Identification of antigen-specific T-cells and protein biomarkers for diagnosis of sarcoidosis

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Abstract

Sarcoidosis is a multisystem granulomatous disease in humans with unknown aetiology and no current definitive diagnostic method. Intradermal inoculation of Kveim reagent (sarcoidosis tissue) was historically used for diagnosis, by causing a granuloma at injection site within 4-6 weeks, with high sensitivity and specificity. The immune mechanisms of this are poorly understood and the antigenic targets of granuloma-associated CD4+ T-cells are unknown. This T-cell infiltrate is oligoclonal, implicating a small number of antigenic targets. Previous work has shown that these are likely proteins.

Aim 1 of this project involved identification of candidate protein antigens within Kveim reagent using proteomics, an established technology for the in depth quantitative differential analysis of complex protein mixtures. These sarcoidosis-specific proteins were analysed using gel electrophoresis with subsequent mass spectrometry and protein database interrogation.

More than 80 proteins were identified as appearing specific to Kveim reagent or of significantly different abundance in sarcoidosis tissue compared to controls. These previously unreported proteins represent novel information that may help in the further understanding of the cellular pathways involved in disease.

Additionally, multiplex cytokine analysis was performed on supernatant from sarcoidosis peripheral blood mononuclear cells incubated with both Kveim reagent and Kveim protein fractions to identify a secreted Th1 signal specific to disease. Information from the proteomics approach combined with this cytokine signal may be beneficial in creating an *ex vivo* immune based assay for diagnosis.

Aim 2 of this project involved the direct analysis of serum and bronchoalveolar lavage (BAL) fluid by multiplex cytokine analysis to investigate signals which could serve as diagnostic biomarkers for the disease. BAL patterns in sarcoidosis were found indistinguishable from tuberculosis but significantly different to healthy volunteers. Significant differences were also found between sarcoidosis and tuberculosis serum, allowing for the creation of a model to reliably distinguish between the two diseases.

For Abi,

You put things on hold and waited for me whilst I did this.

None of this would have been possible without you.

Thank you

Declaration of Originality

This work is by Muhunthan Thillai unless where explicitly referenced in the text.

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Abbreviations

1D	1 Dimensional
2D	2 Dimensional
2-ME	2-Mercaptoethanol
A	Amperes
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ANOVA	Analysis-of-variance
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guérin
^{o}C	Degrees Centigrade
CD	Cluster of differentiation
CFP-10	Culture Filtrate Protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
dH20	Distilled water
DIGE	Difference In Gel Electrophoresis
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
ESAT-6	Early secreted antigenic target
ESI-TOF	Electrospray time of flight
g	gram
G	Gravity
GO	Gene Ontology
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
hrs	hours
IAA	Iodoacetamide
IEF	Isoelectric focussing
IGRA	Interferon Gamma Release Assay
K	Kveim reagent
kDa	kiloDalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
IBD	Inflammatory Bowel Disease
IL	Interleukin
LTBI	Latent tuberculosis infection
m	milli
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
mKatG	Mycobacterial catalase peroxidase
Min	Minutes
mRNA	messenger ribonucleic acid
MS	Mass Spectrometry
MSD	Meso Scale Discovery
m/z	mass-to-charge ratio
NL	Non Linear
p	pico
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pH	potential of Hydrogen
PMT	Photomultiplier
PPD	Purified Protein Derivative
PMF	Peptide Mass Fingerprint
Q-tof	Quadropole time of flight
RA	Rheumatoid Arthritis

RPM	Revolutions Per Minute
RPMI	Royal Park Memorial Institute (culture medium)
S	Sarcoidosis
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	Seconds
SELDI	Surface-enhanced laser desorption/ionization-time-of-flight
SLE	Systemic Lupus Erythematosus
SFC	Spot forming counts
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TB	Tuberculosis
TCR	T-cell receptor
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TIFF	Tagged image file format
TFA	Trifluoroacetic acid
Treg	Regulatory T-cell
Tris	Tris-(hydroxymethyl)-aminomethane
TST	Tuberculin Skin Test
µ	micro
V	Volts
v/v	volume/volume
w/v	weight/volume

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Chapter 1

Background

1.1 Background to sarcoidosis

1.11 Definition and history of disease

Sarcoidosis was first formally described by a British physician named Jonathan Hutchinson who, in 1877, presented to a London Surgical meeting the case of a 58 year old coal miner with unusual lesions on his hands and feet which had appeared over a two year period. He described it as a form of skin disease which had "hitherto escaped special recognition". He later wrote that it was a type of lupus with some similarities but also some differences to tuberculosis and leprosy (Hutchinson, 1898). A year later the Norwegian dermatologist Carl Boeck made illustrations of a patient with the same skin condition and his nephew, also a dermatologist, termed the condition a "multiple benign sarkoid of the skin" as he felt the lesions were a benign form of sarcoma (Boeck, 1899). With growing interest from Physicians into this new disease, the term *sarkoid* subsequently morphed into sarcoid and then finally into sarcoidosis.

Half a century later the disease was described as a "combination of appropriate clinical symptoms and where the clinical manifestations of disease are accompanied by more objective anatomical, radiological, biochemical, immunologic and other laboratory features" (Ricker and Clark, 1949). The joint statement on sarcoidosis by the American Thoracic Society, European Respiratory Society and World Association of Sarcoidosis and Other

Granulomatous Disorders (1999) provide the most comprehensive and up to date definition available:

"Sarcoidosis is a multisystem disorder of unknown cause(s). It commonly affects young and middle-aged adults and frequently presents with bilateral hilar lymphadenopathy, pulmonary infiltration, and ocular and skin lesions. The liver, spleen, lymph nodes, salivary glands, heart, nervous system, muscles, bones, and other organs may also be involved. The diagnosis is established when clinicoradiological findings are supported by histological evidence of noncaseating epithelioid cell granulomas. Granulomas of known causes and local sarcoidosis reactions must be excluded.

Frequently observed immunological features are depression of cutaneous delayed-type hypersensitivity and a heightened helper T cell type 1 (Th1) immune response at sites of disease. Circulating immune complexes, along with signs of B cell hyperactivity, may also be found. The course and prognosis may correlate with the mode of the onset and the extent of the disease. An acute onset with erythema nodosum or asymptomatic bilateral hilar lymphadenopathy usually heralds a self-limiting course, whereas an insidious onset, especially with multiple extrapulmonary lesions, may be followed by relentless, progressive fibrosis of the lungs and other organs."

1.12 EPIDEMIOLOGY

Sarcoidosis affects patients of all ages and races. It is suggested that there is a worldwide prevalence of between 1 and 40 per 100 000 (Rybicki et al., 1997) but difficulties in diagnosis and subsequent misdiagnosis mean that exact figures are difficult to attain (Hennessy et al., 1988). It occurs at different frequencies throughout the world with the highest rates occurring in Denmark, Sweden, Japan and in Americans of black ethnicity. It tends to affect young adults with a peak of presentation in patients between 20 and 29 years old (James, 1994). The

severity and extent of disease varies between ethnicities with black Americans tending to have a more severe course of sarcoidosis than whites (Keller, 1971). Specific forms of extra thoracic manifestation are also present in different ethnic groups e.g. sarcoidosis related erythema nodosum is seen with increased incidence in European patients but is less common in Japan (Pietinalho et al., 1996).

1.13 PATHOLOGY

The hallmark pathology of disease is a non-caseating granuloma and the supporting histopathological features that help with diagnosis (Kitaichi, 1986) can be summarised as follows:

1. Well formed tight granulomas
2. Perilymphatic interstitial distribution of granulomas
3. Lymphocytes and fibrocytes in the outer margins
4. Exclusion of similar diseases using staining methods e.g. tuberculosis and fungal infections

The granulomas are made up of lymphocytes and differentiated mononuclear phagocytes, containing asteroid and Schaumann bodies with a typical example shown in figure 1.1. The granulomas can be discrete or they may be fibrotic in more extensive disease.

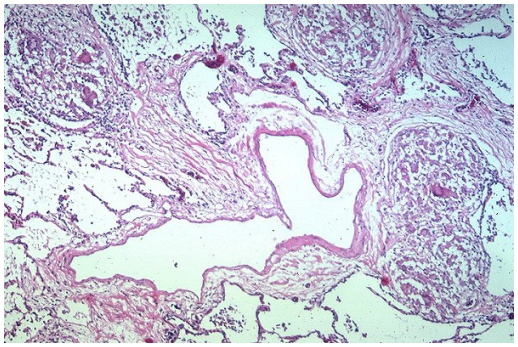


Figure 1.1 Granulomas adjacent to fibrous tissue in the respiratory tract. Image reproduced with permission from Yale Rosen M.D.

1.14 CLINICAL ASPECTS OF DISEASE

Sarcoidosis is a multisystem disease with a large burden of morbidity. The lungs are the primary organ affected in over 90% of cases and pulmonary cases can be staged by chest radiograph depending on the severity of disease (DeRemee, 1983). Other organs which may be involved include the skin, eye, brain, heart, lymphatic system, liver and spleen as shown in table 1.1. Features at presentation depend on the site and extent of organ involvement but in pulmonary disease, patients may present with cough, shortness of breath and chest pain (Hendrick et al., 1976). Löfrgen's disease is the term for a recognised acute form of disease with a good prognosis which classically presents with the triad of pulmonary hilar lymphadenopathy, erythema nodusum and arthritis.

The overall course of disease is highly variable with approximately two thirds of patients resolving spontaneously or with a short course of oral steroid therapy (Siltzbach et al., 1974). A significant proportion of the remainder will have a progressive course of disease before it either stabilises or improves. 10% will have life long sequelue, with the overall mortality varying from 1-5%. Death is usually due to cardiac or neurological involvement or due to progressive respiratory failure (Huang et al., 1981).

Table 1.1 Organ involvement in sarcoidosis

Organ or system involvement	Common disturbances
Respiratory	Lymph node involvement, lung infiltration and fibrosis
Cardiovascular	Cardiac arrhythmias with potential for sudden death
Neurological	Cranial nerve involvement, peripheral neuropathy and symptoms of intracranial lesions
Lymphatic	Lymph node involvement with systemic features including weight loss and low grade pyrexia
Skin	Erythema nodosum, lupus pernio, subcutaneous nodules and plaques
Eye	Uveitis
Liver	Mildly abnormal liver function
Endocrine	Hypercalcaemia, hyperprolactinaemia

1.15 CURRENT METHODS OF DIAGNOSIS

There is no currently available specific or definitive test for sarcoidosis. Diagnosis of disease merely relies on a suggestive clinical and radiological picture supported by compatible histology from tissue samples obtained by invasive biopsy of affected organs.

A compatible clinical story may be supported by radiology such as a chest radiograph, computerised tomography (CT) scan or, less often, a gallium scan showing hot spots of disease. Once the appropriate site for biopsy has been chosen, a histological biopsy is the current 'gold standard' but in some patients with a strong clinical picture and supporting radiology this may not be necessary before making the diagnosis (Hiraga, 1981). In addition, a diagnostic procedure to obtain tissue is not without risk of complications e.g. a pneumothorax post transbronchial lung biopsy. Even once obtained, the histology may not be specific to disease but merely suggestive of sarcoidosis. Furthermore, a histological diagnosis cannot be used to predict disease progression or response to treatment. A number of other relevant investigations are detailed in table 1.2 but although these may be useful in assisting with diagnosis they are usually not enough to make a diagnosis by themselves.

Table 1.2 Investigations that may be warranted in sarcoidosis

Investigation	Associated difficulties
Radiology	Indicates site of disease but does not give definitive diagnosis as the differential diagnosis is extensive
Histology	Invasive procedure with associated risks which cannot predict disease progression or outcome
Spirometry	Lung function testing (e.g. transfer factor) can be used to monitor disease activity but is unhelpful in making the initial diagnosis
Angiotensin Converting Enzyme (ACE) levels in serum	Useful for monitoring disease activity but false positives mean it cannot be used alone for diagnosis e.g. serum ACE is often raised in tuberculosis
Serum and urine calcium	May be elevated due to increased intenstinal calcium absorption which in turn is due to activated vitamin D (1,25-dihydroxyvitamin-D) production from macrophages within sarcoid granulomas. Useful for monitoring disease activity but cannot be used alone for diagnosis.
Raised CD4:CD8 ratio (>3.5) in BAL fluid	Useful for assisting with diagnosis with high sensitivity but requires invasive procedure

Lung function abnormalities are often present when patients are first diagnosed with pulmonary sarcoidosis and the majority of studies prior to 2010 indicate that the predominant pattern is of restriction or normal airways, with a truly obstructive pattern in a minority (Polychronopolous et al., 2009). However, a more recent study of patients presenting to a West London Hospital found that 1 in 4 patients with newly diagnosed pulmonary sarcoidosis presented with a picture of large airways obstruction and an FEV1/FVC of <70% (Thillai et al., 2012).

The mechanisms involved in airway function in sarcoidosis are extensive and range from disease affecting the nasal passages right through to the bronchioles. Processes involved are as diverse as mucosal inflammation, stenosis, extrinsic compression, bronchiectasis and airway hyperreactivity (Polychronopolous et al., 2009). Large airway obstruction in sarcoidosis is a recognised feature of the disease and the higher than expected incidence found in the recent 2012 study may be due to the increasing use of specialised diagnostic methods such as CT-guided biopsy and endobronchial ultrasound sampling for lymph node disease, which may be providing histological confirmation in patients in whom a diagnosis of obstructive airways disease may otherwise have been made.

The differential diagnosis of a patient presenting with pulmonary sarcoidosis is wide and includes infections such as tuberculosis, infiltrative conditions such as pulmonary fibrosis and other diseases including Wegener's granulomatosis, lymphoma and primary lung carcinoma. The differential diagnosis of extra pulmonary sarcoidosis is more extensive and depends on the organ primarily affected e.g. neurosarcoidosis is simply one of a large number of causes of cranial nerve abnormalities. Despite recent advances in molecular immunology there have been no new diagnostic tools for 40 years. Swift and accurate diagnosis with high sensitivity and specificity is paramount as conditions which present with a similar clinical picture (e.g. tuberculosis or lymphoma) require radically different yet equally prompt treatment. A greater understanding of the immune mechanisms and antigenic targets involved in sarcoidosis may help develop more accurate prognostic and diagnostic methods with potentially major and immediate advances for clinical practice.

1.2 AETIOLOGY

1.21 EVIDENCE FOR A TRANSMISSIBLE AGENT

Koch's postulates are a series of rules that can be used to dertemine whether a partiuclar organism is responsible for causing disease (Koch., 1893). They can be summarised using four criteria:

1. The organism should be found in diseased but not in healthy individuals
2. The organism can be isolated from a diseased specimen and successfully cultured
3. The organism cultured from criteria 2 must be able to cause disease when introduced into a new healthy individual
4. The organism from the previously healthy individual in critera 3 must be able to be again isolated and cultured from the new host

The rules were described over 100 years ago and have significant limitations when used for investigating disease aeitiology e.g. an organism may be present in a number of individuals but only cause infection in those who are predisposed to the disease and some organisms such as viruses cannot be simply cultured in the method that Koch envisaged, However, modified versions of the postulates using more advanced molecular biology have been used to investigate aetiology of diseases with a previously unknown cause e.g. the use of 16S rDNA analysis to identify *Tropheryma whippelii* as the cuase of Whipple's disease (Wilson et al., 1991) and the use of complementary DNA cloning to identify hepatitis C virus as the cause of uncharacterised non-A, non-B hepatitis disease (Choo et al., 1989).

Koch's postulates cannot currently be applied to any organism found in sarcoidosis and there is therefore no generally accepted cause of the disease. However, there is some evidence for a

transmissible agent as there have been a number of reports of outbreaks of sarcoidosis within a short period of time. One in particular was reported as a case controlled study from the Isle of Man which found that 40% of 96 cases had prior contact with at least one person who had the disease (Parkes et al., 1985). Later detailed epidemiological work indicated that there may have been a shared infective pathogen amongst many of the cases of disease (Hills et al., 1987). In addition, donor acquired sarcoidosis has been described in patients post lung transplantation (Muller et al., 1996) and after bone marrow transplants (Padilla et al., 2002). At present there are no naturally occurring *in vivo* animal models for sarcoidosis but mouse work indicates that homogenates of sarcoidosis tissue from mice are able to pass along their granuloma causing capabilities into new animals (Mitchell and Rees, 1969).

1.22 MYCOBACTERIA AND SARCOIDOSIS

Tuberculosis infection is caused by transmission of mycobacteria and can result in a wide range of clinical presentations with the lungs as a primary focus of disease. The clinical and histological similarities between sarcoidosis and tuberculosis have led some to believe that the two are related diseases in terms of a directly shared aetiology, similar aetiologies or that sarcoidosis may represent an altered host immune response to a form of mycobacteria. There is much evidence both for and against a relationship as evidenced in table 1.3.

Indeed, in some cases it is clinically difficult to distinguish between the two diseases e.g. a patient presenting with respiratory symptoms and radiology compatible to both diseases. In terms of similarities in radiological presentation, unequivocal bilateral hilar lymphadenopathy accompanying erythema nodosum or febrile arthropathy may be found on routine chest radiographs patients who are otherwise symptom free. However, these findings which appear to be consistent with sarcoidosis do not exclude mycobacterial lymphadenitis (Winterbauer et al., 1973). On biopsy, the histology may be equivocal with epithelioid cell granulomas displaying little central necrosis. Depending on the experience and background of the

investigating histopathologist, these findings could be determined as being of necrosis compatible with tuberculosis or as non-caseating as seen in sarcoidosis (Mitchell and Scadding, 1974).

Table 1.3 Arguments for and against a relationship between sarcoidosis and mycobacteria

Arguments for	Arguments against
Some similarities in clinical presentation and pathology	Differences in systemic involvement between both diseases
Reports of coexisting diseases in the same patient (Scadding, 1960)	Different treatment strategies are needed for the two diseases
Some molecular evidence of mycobacterial DNA in sarcoidosis lymph nodes (Popper et al., 1997)	Some larger studies suggest the true incidence of mycobacteria in sarcoidosis granulomas is low (Bocart et al., 1992)
Some shared familial incidence of both diseases (Wurm et al., 1962)	Worldwide epidemiology of both diseases is not identical (Hosoda et al., 2004)
Mycobacterial catalase peroxidase is a T-cell antigen in sarcoidosis (Chen et al., 2008)	Interferon gamma release assays for tuberculosis are negative in most cases of sarcoidosis (Inui et al., 2008)

Alternative hypotheses for the relationship involve mycobacteriophages which have been found in the sera of some patients with sarcoidosis (Mankiewicz and Liivak, 1967) or atypical forms of mycobacteria which may be present within the granuloma in a form that it is not able to be cultured e.g. a protoplast or L-form, otherwise known as cell wall deficient mycobacteria, which lack the power to produce a characteristic cell wall and persist as an intracellular parasite of mesenchymal cells. The protoplast may only rarely revert to a bacillary form and the infected cells might migrate to initiate foci of disease in other parts of the body. In most cases, the infected cells would be eliminated. The affected individual may also show unusually efficient production of humoral antibody and abnormal cellular immune responses (Moscovic, 1978).

Such mycobacteria have been little studied and the difficulties associated with identification have led some to question their very existence. However, there are reports of findings of cell wall deficient mycobacteria after prolonged culture of sarcoidosis granulomas (Cantwell, 1981). Cell wall deficient forms of mycobacteria have been grown after lengthy blood culture in 19 out of 20 patients with sarcoidosis but were not found in a similar number of controls

(Almenoff et al., 1996). The evidence is by no means clear and other groups found no difference between sarcoidosis and controls in this respect (Brown et al., 2003).

1.23 OTHER SUGGESTED AETIOLOGICAL FACTORS

A number of studies have suggested that *Propionibacterium acnes* could be frequently isolated from patients with sarcoidosis and hypothesised a possible link (Homma et al., 1978). Subsequent studies using PCR found evidence of *P. acnes* or *P. granulosum* rRNA in 15 out of 15 sarcoidosis lymph nodes compared with 2 out of 15 lymph nodes from patients with tuberculosis. Conversely, only 3 out of the sarcoidosis lymph nodes demonstrated evidence of mycobacteria (Ishige et al., 1999). The majority of studies which describe the presence of propionibacteria in sarcoidosis tissue are from Japan and it may be that in this region of the world, propionibacteria do have an aetiological link with some cases of sarcoidosis. However, one study investigating propionibacteria in European sarcoidosis patients also suggests an association (Eishi et al., 2002).

A number of other inciting causative agents for sarcoidosis have been suggested. These include Chlamydophila due to the presence of Chlamydophila-specific antibodies in serum and bronchoalveolar lavage fluid (Gaede et al., 2002), borrelia like organisms (Derler et al., 2009), mycobacteriophages (Mankiewicz and Beland, 1964), *Rickettsia Helvetica* (Nilsson et al., 2002) and *Mycoplasma Orale* (Jansson et al., 1972). Non-organic mechanisms of disease include beryllium (Gentry et al., 1955) and pine pollen (Cummings and Hudgins, 1958).

An investigation into a number of fire department rescue workers who were involved with the World Trade centre attacks in 2001 found that the incidence of sarcoidosis or sarcoidosis-like granulomatous pulmonary disease was significantly increased in this population post 2001 and which was ascribed to the inhaled dust and debris from that area (Izbicki et al., 2007).

1.24 THE ACCESS STUDIES

The ACCESS (A Case Controlled Etiologic Study of Sarcoidosis) collaboration was a large multi-centre study in North America which was initiated by the National Institutes of Health. It represents the largest case controlled study to date to specifically investigate the aetiological causes of sarcoidosis. 706 newly diagnosed patients with sarcoidosis and an equal number of age, sex and geographically placed matched controls were recruited for the study in the late 1990s.

The major hypothesis of the investigation was that sarcoidosis occurs in genetically susceptible individuals through alteration of the immune response following exposure to environmental, occupational or infectious insults. The study investigated these aetiological factors using a detailed questionnaire. In addition, infectious agents in the blood were investigated by Polymerase Chain Reaction of blood looking for a number of specific organisms and culture of blood for cell wall deficient mycobacteria. The study led to a number of publications but no single cause of sarcoidosis was identified.

The familial aggregation of sarcoidosis (ACCESS) publication examined data on disease occurrence in 10,862 first- and 17,047 second- degree relatives of 706 age, sex, race and geographically matched cases of sarcoidosis and controls who participated in the study from 1996 to 1999 (Rybicki et al., 2001). Siblings had the highest relative risk followed by avuncular relationships, grandparents and parents. White relatives had a higher risk when compared with African-Americans. A significantly elevated risk of sarcoidosis was observed amongst first and second degree relatives of sarcoidosis patients when compared with relatives of matched control subjects.

A case control-etiological study of sarcoidosis: environmental and occupational risk factors (ACCESS) investigated the same patients with detailed questionnaires regarding suspected

occupational and non-occupational exposures and the data was assessed by univariable and multivariable analysis (Newman et al., 2004). A history of smoking cigarettes was less frequent amongst patients with sarcoidosis than controls. Positive associations between sarcoidosis and specific occupations were seen e.g. agricultural employment, insecticide exposure or work in environments rich in mould or mildew. No single predominant environmental cause of disease was identified.

Job and industry classifications associated with sarcoidosis (ACCESS) investigated the collated data with reference to Standard Industrial Classification (SIC) and Standard Occupational Classification (SOC) to assess occupational contributions to sarcoidosis risk (Barnard et al., 2005). A number of further detailed exposures were associated with sarcoidosis risk including organic dust exposures, workers for suppliers of building materials, and those in hardware and gardening material work. Jobs associated with metal dust, metal fumes and work with childcare were negatively associated with the risk of sarcoidosis.

1.3 ALTERED IMMUNE RESPONSE

1.31 GRANULOMA FORMATION

Non-caseating granulomas are the hallmark pathology in sarcoidosis. Granulomas have historically been considered part of a host mechanism to seal off harmful foreign material from damaging the body but a recent alternative hypothesis comes from research into tuberculosis indicating that granulomas in that disease may be part of the pathogen's mechanisms for host evasion (Davis and Ramakrishnan, 2009).

The exact aetiology of sarcoidosis remains unknown and it is generally still believed that the granuloma formation seen in disease is a key part of the host immune response and that

disordered immunity in sarcoidosis may lead to the maintenance of granulomas which eventually cause organ dysfunction. The granulomatous inflammation seen in sarcoidosis is typically polarised towards a Th1 response (Muller-Quernheim et al., 1992).

Macrophages are involved as part of granuloma formation and studies have shown them to be of a classical phenotype with production of Th1 associated markers (Muller-Quernheim et al., 1992). Dendritic cells which are involved in antigen presentation have been little studied but there is evidence to show that although circulating numbers may not be low, there may be an altered functionality of circulating cells leading to the decreased delayed type hypersensitivity reactions seen in sarcoidosis (Mathew et al., 2008).

1.32 T-CELL INVOLVEMENT

A number of studies have shown an accumulation of T-cells at the sites of disease in sarcoidosis which can represent as much as 80% of all cells found in the bronchoalveolar lavage from a patient with sarcoidosis (Yeager et al., 1977). These T-cells are predominantly CD4+ in nature (Hunninghake and Crystal, 1981) and spontaneously release Th1 cytokines preferentially (Moller, 1999). These CD4+ T-cells are found scattered throughout the granuloma whereas CD8+ T-cells tend to be only found around the edges (Rosen, 1994). Previous work has also indicated that these T-cells are oligoclonal in nature which implies an antigen driven disorder with limited target antigens.

Regulatory T-cells (Tregs) have been investigated in sarcoidosis and a subset displaying high levels of transcription factor forkhead box P3 (FoxP3) have been found at increased frequency in both the site of disease and the peripheral circulation (Miyara et al., 2006). However, the same researchers showed that the cells were not effective in suppressing granuloma formation *in vitro*.

CD1d-restricted Natural Killer Cells (NKTs) are a subset of T-cells which are thought to protect against disorders with increased CD4-positive Th1 responses in animals. Their presence has been found to be greatly reduced in the peripheral blood and lungs of patients with sarcoidosis and it is thought that this may a mechanism through which there is persistent CD4+ T-cell activity seen in disease (Ho et al., 2005).

1.33 B-CELL ABNORMALITIES

Circulating immune complexes are found at elevated levels in patients with sarcoidosis (Selroos et al., 1980). The B-cells that are present are found to be hyperactive and have a spontaneous production in situ of immunoglobulins and these tend to be IgG. IL-6 (which causes a potent B-cell stimulation) is also found upregulated at the sites of disease in sarcoidosis. A number of antibodies against specific organisms have been discovered but much of this work has not been replicated or corroborated e.g. the increased incidence of anti-chlamydial antibodies in sarcoidosis serum (Puolakkainen et al., 1996) despite further studies showing no evidence of the organism itself in biopsy samples (Blasi et al., 1997).

1.34 CYTOKINES IN DISEASE

Acute sarcoidosis is generally thought of as a polarised Th1 disorder with an accumulation of T-cells secreting interferon gamma (Moller, 1999) and IL-2 (Hunninghake et al., 1983) at sites of disease. Some studies have also shown a co-existing lack of most Th2 cytokines (Hoshino et al., 1995) but IL-13 (a prominent Th2 marker) has been shown to be upregulated in other work (Hauber et al., 2003). The Th1 immunoregulatory cytokines IL-18 (Minshall et al., 1997) and IL-12 (Greene et al., 2000) are also shown to be upregulated at sites of disease. Inflammatory cytokines such as Tumour Necrosis Factor (Baughman et al., 1990), Macrophage Migration Inhibitory Factor and Interleukin-1 (Yamaguchi et al., 1988) have been found upregulated at sites of disease. TH17 cytokines have been investigated

extensively in other lung diseases such as tuberculosis and there is some recent evidence to support their role in sarcoidosis (Facco et al., 2011).

Chronic persistent disease and development of lung fibrosis is accompanied by increased levels of Tumour Necrosis Factor and increased production of macrophage-derived fibrogenic cytokines including platelet derived growth factor, and Insulin like growth factor 1 (Homma et al., 1978). Other pro-granuloma cytokines which play a role in chronic disease include RANTES, MIP-1α and IL-16 (Kunkel et al., 1996).

1.4 KVEIM REAGENT

1.41 DEFINITION AND HISTORICAL DEVELOPMENT OF KVEIM REAGENT

Kveim reagent is material most often derived from sarcoidosis spleen or lymph node tissue but can be successfully prepared using most diseased tissue specimens. The reagent was historically used to diagnose disease in a method analogous to use of the tuberculin skin test in patients with tuberculosis. Intradermal inoculation of Kveim reagent caused a pathognomonic granuloma within 4-6 weeks at the site of injection as seen in figure 1.2. Kveim reagents were prepared in batches of tissue; each batch coded from a single patient. These would usually be tested for sensitivity and specificity using patients with biopsy proven pulmonary sarcoidosis as a gold standard. An American study of 750 patients with biopsy proven disease (or who had a strong clinical picture in keeping with sarcoidosis) showed that Kveim reagents varied in their ability to diagnose disease but that the best Kveim reagents had a sensitivity of 80% and a specificity of greater than 98% (Siltzbach, 1961).

The Kveim reagent was named after the Norwegian pathologist Morten Ansgar Kveim who first reported that the injection of a heated suspension of sarcoidosis lymph node tissue caused a skin nodule to develop at the site of injection in 12 out of 13 patients with the disease (Kveim, 1948). The nodule occurred between nine days and four weeks after injection and subsequent biopsy revealed histological changes consistent with typical sarcoidosis granulomas from the lungs of patients with the disease. The reaction was not seen in an unspecified number of control patients with tuberculosis, lupus vulgaris and syphilis. Further validation work by the American physician Louis Siltzbach in the 1950s led to preparation of specific Kveim reagents for diagnostic use in sarcoidosis worldwide. The reagent was withdrawn from use in the UK in 1988 due to the lack of availability of standardised bioactive *in vivo* diagnostic material as well as the potential for the suspension to transmit prion diseases such as new variant Creutzfeldt-Jakob disease.

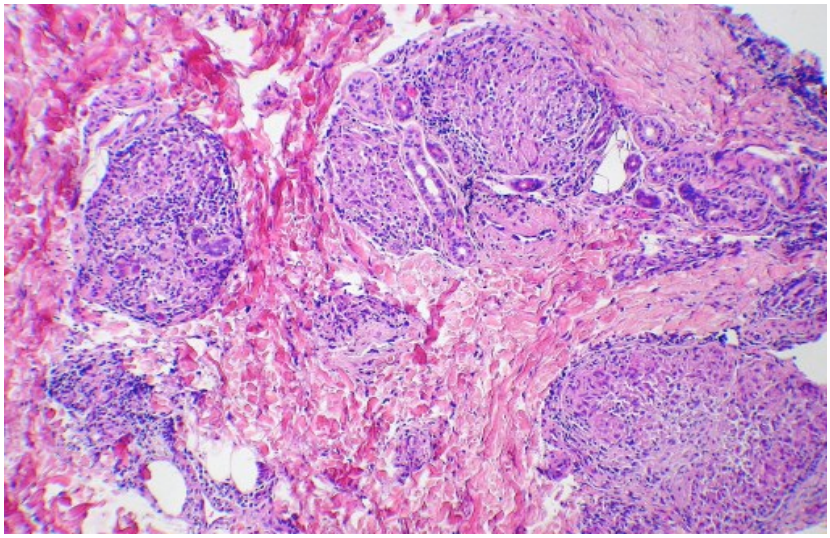


Figure 1.2 Granulomas seen in the skin after biopsy of Kveim test injection site in patient with sarcoidosis. Image reproduced with permission from Yale Rosen M.D.

After injection of the reagent a simple punch biopsy was necessary from the site of the Kveim test for final confirmation of diagnosis. Most patients reacted to Kveim reagent within two years of the onset of sarcoidosis but the proportion of responders fell with length of disease. Of those with pulmonary hilar node involvement about 90% responded whilst only 50% of

those with pulmonary infiltration only displayed a positive Kveim reaction (Siltzbach, 1961). It was also reported that ethnic factors could influence the size of the developing papules with black patients developing larger reactions than whites (Siltzbach, 1964).

The Kveim reaction has been investigated intensively in the past and it was found that a number of different sarcoidosis tissue specimens could be used for preparation including spleen, lymph node and tonsil but that individual reagents varied greatly in their ability to produce the diagnostic reaction and that this ability was independent to the effects of immunospuression on the donors or the length of time since diagnosis (Danbolt, 1951a). Detailed histological and immunological investigations of the reaction have provided few answers (Klein et al., 1995).

1.42 PREPARATION OF KVEIM REAGENT AND ATTEMPTS TO ISOLATE THE ACTIVE COMPONENT

The majority of Kveim reagent was derived from spleen (due to the large amount of tissue available) and lymph node which was thought to have had a higher potential for the reaction despite the smaller amount of available material. Approximately 20% of sarcoidosis spleens were suitable for use as an *in vivo* diagnostic test due problems with weak reactivity or low specificity of the other batches (Mitchell et al., 1976). The level of reactivity was not found to be related to the extent of granulomatous infiltration in the tissue.

Kveim reagent is prepared using a specific protocol (Chase, 1961) and although the method has been modified in a number of subtle ways for use in different institutions, the underlying principle was that Kveim reagent remained a relatively crude suspension of human sarcoidosis tissues which were heated and irradiated to destroy any co-existing viable microorganisms. A number of research groups have tried to both increase the potency of

Kveim reagent as well as to identify the active component. The conclusions of this work are varied and contradictory but one group felt that the biologically active material within Kveim may be particulate and not water soluble and that it did not pass a Berkefeld or Seitz filter (Danbolt, 1951b). Whilst the opalescent supernatant after centrifugation at 2,500rpm retained activity, the clear supernatant after ultra centrifugation at 30,000rpm became inactive (Rogers and Haserick, 1954). Some work has shown that the particles sedimenting from spleen suspension in 15 minutes at centrifugation at 5,500g appeared to have the highest activity (Chase, 1961).

Removal of lipids by extraction in the cold with ether or with chloroform-methanol did not reduce activity and ether extraction was actually thought to have increased activity (Lyons et al., 1992). Repeated extraction with a series of hot organic solvents destroyed activity and one theory was that this was perhaps due to the disruption of lipoprotein linkages. Boiling in aqueous suspension seemed to reduce but did not destroy potency. Removal of nucleoprotein by 2M sodium chloride did not reducing potency and the discarded nucleoprotein fraction was not active.

Exposure to sodium hydroxide at low concentrations appeared to somewhat reduce activity. Proteolytic enzymes, nucleases, hyaluronidase and neuraminidase appeared to have little effect in diminishing the granuloma causing capacity of the reagent. Preparation of different cellular compartments has been used to argue that the active component was located in the cell membrane (Izumi et al., 1973).

Some Kveim reagents are known to retain activity after prolonged storage e.g. several years at temperatures at around 4^0C (Siltzbach and Ehrlich, 1954) whereas other batches have lost activity after just a few weeks of storage in similar conditions (Nelson and Schwimmer, 1957) but the reasons for these differences are unknown.

1.43 THE KVEIM REACTION IN OTHER DISEASES

Reactions of patients with diseases other than sarcoidosis to Kveim reagent may provide a clue as to the immune mechanisms for the response. A number of Kveim suspensions were reported to cause granulomatous reactions in a higher proportion of tuberculosis patients than expected. One study specifically looking at this phenomenon reported a significant difference in this respect between suspensions prepared at different times from the cervical lymph nodes of the same patient with sarcoidosis (Israel et al., 1958).

A number of studies have also investigated the effect of prior Bacille Calmette Guerin (BCG) vaccination on Kveim responses. The largest study to date involved 13 598 BCG vaccinated individuals (Hart et al., 1964) of whom 12 failed to convert despite repeated BCG vaccination. 10 of these 12 were found to have a positive Kveim reaction with no medical history or clinical evidence suggesting previous sarcoidosis. Explanations of this effect include the hypothesis that serial BCG vaccinations are responsible for priming the adaptive immune system to respond to Kveim reagent with the possibility that there is a cross-reactive antigen present in both BCG and Kveim.

Some studies have shown a higher incidence of positive Kveim reactions in sub groups of patients from with leprosy from Japan and China but the majority of studies elsewhere contradict these findings (Pearson et al., 1969). There is no previous research on the reactivity of patients with sarcoidosis to lepromatin which can be used as part of the diagnostic Mitsuda reaction in leprosy.

Kveim reactions in Crohn's disease has been investigated with the attempt to find a causal link between the two granulomatous diseases. One study showed that Kveim reactions were histologically positive in 38 of 74 (51%) patients with diagnosed Crohn's disease (Mitchell et al., 1970). However, this phenomenon has not been replicated with different batches of

Kveim reagent. A study of Kveim reagent injection into 32 patients with chronic brucellocis showed a positive reactivity in seven (Williams, 1974). Despite the potential for liver granulomas in patients with brucellocis, none of the study patients had any clinical or radiological evidence of sarcoidosis.

1.5 PROTEOMIC ANALYSIS OF SARCOIDOSIS TISSUES

1.51 PROTEOMIC ANALYSIS

Proteomics involves the in depth analysis of proteins including deducing their identities, looking for patterns in disease and analysis of structure, function and protein-protein interactions. A number of different techniques are currently in use and include gel based separation of proteins by size and charge, chip based approaches e.g. surface-enhanced laser desorption/ionization (SELDI) and shotgun proteomics involving the fractionation and samples with high performance liquid chromatography and direct analysis with Mass Spectrometers.

Comparative proteomics involves the direct comparison of multiple samples e.g. to look for protein identities or patterns that can be used in diagnosis and prognosis of disease. The diagnostic potential of proteomics has been confirmed in a range of conditions including non-small-cell lung cancer (Yanagisawa et al., 2003) and tuberculosis (Agranoff et al., 2006). The incorporation of specific discovered proteins into simpler diagnostic immunoassays may allow for the development of novel biomarkers to accurately and swiftly diagnose disease.

1.52 PROTEOMICS OF KVEIM REAGENT

There appears to be no previous published work directly analysing Kveim reagent for identities of potential candidate protein antigens. However, immunoblotting techniques have been used to attempt identification. One such investigation used a model of sarcoidosis where rabbits were sensitized with soluble Kveim reagent. A small number of potential anti-Kveim antibodies were subsequently found through immunoelectrophoresis but these were never identified (Stahl and Veien, 1981). A later investigation demonstrated a monoclonal IgM antibody (termed IHY-1) which was found to react more strongly to sarcoidosis granulomas than to controls with tuberculosis. Despite much evidence for the existence of the antibody, the potential protein antigenic target remains unknown (Ishioka et al., 1990). The antibody is not commercially available and the last published use was in 1999 in studies by the same team who developed two antibodies with slight variations (IHY-2 and IHY-3) and were able to replicate their work from 1990 in a limited number of sarcoid granulomas (Ishioka et al., 1999). If the antibodies are still available from this group, proteomics capture studies e.g. using MALDI or SELDI (see 1.51) could potentially be used to attempt to determine the identity of the Kveim antigen targets.

1.53 MYCOBACTERIUM CATALASE PEROXIDASE

In arguably the most important study to date to investigate sarcoidosis solid tissue for protein antigens, sarcoidosis lymph node extracts were treated with neutral detergent and proteases which is consistent with the known physical properties of the granuloma causing extracts within Kveim reagent. Tissue antigens were detected with immunoglobulin (Ig)G or F(ab')(2) fragments from pooled sarcoidosis sera in 9 out of 12 (75%) sarcoidosis tissues but only 3 out of 22 (14%) control tissues (Song et al., 2005).

One of these sarcoidosis tissue antigens was subsequently identified as mKatG using Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The identity of mKatG as a tissue antigen was further confirmed in a number of sarcoidosis tissues with immunoblotting using anti-mKatG monoclonal antibodies. IgG antibodies to recombinant mKatG were also detected in a number of sera from patients with sarcoidosis (Song et al., 2005).

1.54 PROTEOMIC ANALYSIS OF SERUM AND BRONCHOALVELOAR LAVAGE FLUID

Two dimensional gel electrophoresis has been used to create a proteomic profile map of proteins found in sarcoidosis bronchoalveolar lavage (BAL) fluid and compare it to a proteomic profile of control samples. A detailed comparative analysis showed that the number of proteins in the pH range 5.5–6.7 were increased in sarcoidosis compared to controls (472 vs. 384) and were decreased in the pH range 4.5–5.5 (399 vs. 518). A large number of the identified proteins were those involved with general inflammation and as such were not deemed useful as biomarkers (Sabounchi-Schutt et al., 2003).

A number of smaller studies have used high throughput methods to investigate protein profiles in sarcoidosis patients. One study using surface-enhanced laser desorption/ionization-time-of-flight mass spectroscopy (SELDI-TOF-MS) looked at BAL fluid in 65 patients with sarcoidosis and 23 healthy controls. 40 proteins were detected as being significantly differentially expressed between the groups and some of these were associated with specific chest radiograph stages. Human serum albumin, alpha1-antitrypsin and protocadherin-2 precursor were found upregulated in sarcoidosis patients and although not specific to disease, these proteins were postulated as potential biomarkers (Kriegova et al., 2006).

SELDI-TOF-MS has also been used to investigate serum profiles in sarcoidosis. One such study compared the Mass Spectrometric profiles of 35 patients with sarcoidosis to the same number of healthy controls. A peak with a mass-to-charge *(m/z)* value of 11,955 was able to distinguish between the sarcoidosis and healthy sample sets with an 86% sensitivity and 63% specificity but this analyte was unable to be identified and may have simply represented an acute phase protein (Bons et al., 2007). Mass spectrometry has also been used to directly identify proteins from BAL fluid in patients with sarcoidosis using MALDI MS (see section 1.66).

In addition to the limited studies investigating protein profiles in sarcoidosis, high throughput multiplex cytokine analysis has been used to identify cytokine profiles specific to the serum of patients with sarcoidosis. An investigation using multiplex bead array technology of patients with sarcoidosis, systemic sclerosis and healthy controls revealed no single markers of distinction between the groups. However, a combination of 17 innate and adaptive cytokines could correctly classify 90% of patients into the three groups (Beirne et al., 2009). The immune processes behind this particular pattern are unclear and further work is needed to both replicate the work in different cohorts and investigate the underlying immune processes associated with the pattern specific to sarcoidosis.

It would also be interesting to investigate cytokine profiles at the site of disease in sarcoidosis e.g. using lung tissue biopsies or bronchoalveolar lavage. Comparison of these cytokine profiles with those from the relevant site of disease in other granulomatous diseases of known aeitiology (e.g. tuberculosis, leprosy, and schistosomiasis) may reveal clues as to the both the aetiology and immune processes involved in development of sarcoidosis.

1.6 EX-VIVO T-CELL RESPONSES TO PROTEIN ANTIGENS IN SARCOIDOSIS

A greater understanding of the immune mechanisms and potential antigenic targets involved in sarcoidosis disease pathogenesis may help to develop more accurate diagnostic methods with potentially major and immediate advances for clinical practice. To this end, Interferon Gamma Release Assays (IGRAs) have been investigated in sarcoidosis for *ex vivo* responses to specific antigens. The implied association between sarcoidosis and tuberculosis has resulted in much recent research focusing on T-cell responses to mycobacterial antigens in patients with proven sarcoidosis but results of studies to date remain far from conclusive.

1.61 IGRAS ARE CURRENTLY USED FOR DIAGNOSIS OF TUBERCULOSIS INFECTION

The low infective burden in latent tuberculosis infection results in *Mycobacterium tuberculosis* remaining hidden from view through direct observation methods. The weak humoral response makes antibody testing unhelpful but infection does result in a strong T-cell response with T helper 1 (Th1)-type CD4+ T-cells secreting interferon gamma (IFNγ) in response to infection. T-cell responses to Early secreted antigenic target (ESAT-6) or Culture Filtrate Protein (CFP-10) (antigens specific to *Mycobacterium tuberculosis* and which are deleted from most environmental mycobacteria and all forms of BCG (Behr et al., 1999)) are used in current diagnostic tests for tuberculosis infection. Two methods for identification of antigen-specific IFNγ- secreting T-cell responses are used for diagnosis and they and detailed in figure 1.3. T-cells from people infected with *M tuberculosis* which have become sensitised to ESAT-6 or CFP-10 during infection release IFNγ (amongst other cytokines) when they are re-exposed to these antigens in the laboratory.

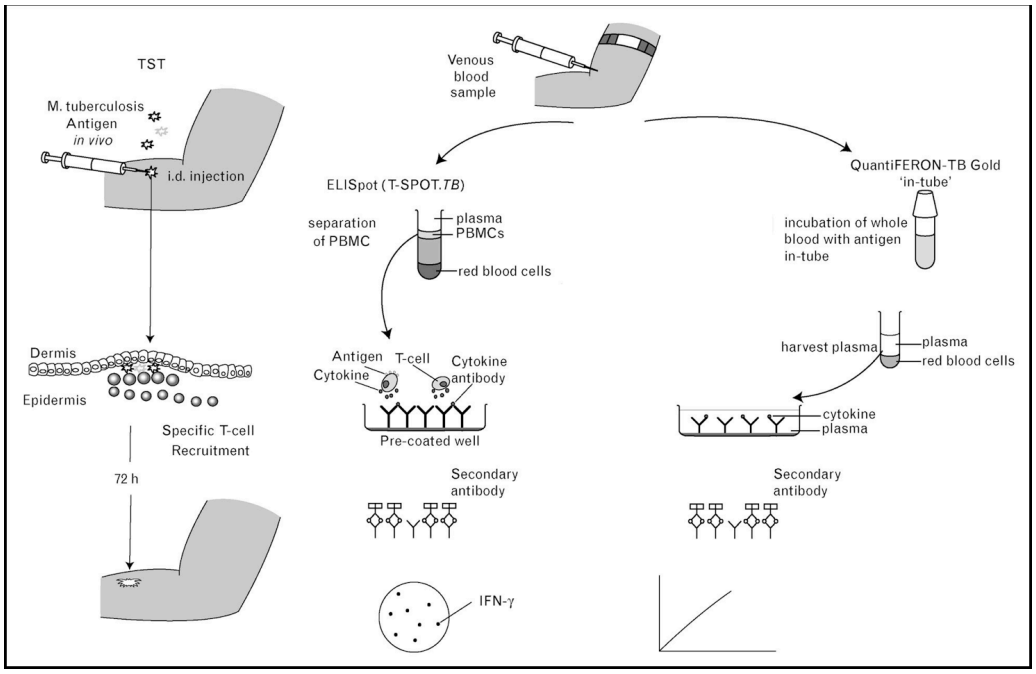


Figure 1.3 Diagrammatic representation of the Tuberculin Skin Test (TST), Enzyme-linked immunosorbent spot (ELISpot) and Enzyme-linked immunosorbent assay (ELISA) for diagnosing *M tuberculosis* infection. **TST** *(left)* is performed when purified protein derivative is intradermally injected into the volar surface of the forearm. The delayed-type hypersensitivity response (induration) is measured 72 h later. **ELISpot** *(centre)* uses peripheral blood mononuclear cells which are separated from the blood sample by density centrifugation and then incubated with ESAT-6 and CFP-10 in a standard 96-well microtitre plate for 16–20 h. If the patient is infected with *M tuberculosis*, T-cells will recognize the antigens and secrete IFNγ. **ELISA** *(right)* uses whole blood from the patient is incubated with ESAT-6, CFP-10 and TB7.7 in the blood collection tube for 16–24 h. If the patient is infected with *M tuberculosis*, T-cells will recognize the antigens and secrete IFNγ. Image reproduced from BREATHE (Lalvani & Thillai, 2009).

1.62 RATIONALE FOR EXPECTING ANTIGEN SPECIFIC T-CELL RESPONSES IN SARCOIDOSIS

The immune mechanisms of the Kveim reaction are poorly understood and the antigenic targets of the granuloma-associated CD4+ T-cells that predate the immunopathology of disease are unknown. However, previous work has implicated an oligoclonal CD4+ T-cell infiltrate which precedes formation of the diagnostic local epithelioid intradermal granulomas. The study also showed that the TCR V beta repertoire from Kveim reaction sites (using a PCR technique) demonstrated an oligoclonal pattern of V beta gene expression with limited junctional variability in the hypervariable section of the TCR (Klein et al., 1995). This

implicates a small number of Kveim reagent derived targets which invoke host T-cell responses. It is also important to note that the removal of non-proteinaceous material from Kveim did not alter the efficacy of the resultant suspension retained the ability to induce a Kveim reaction. This led to the conclusion that the causative agent was most likely a protein (Lyons et al., 1992).

1.63 INVESTIGATION OF T-CELL RESPONSES TO MYCOBACTERIAL ANTIGENS

Based on the hypothesis that there exists a limited number of antigens within both Kveim reagent and sarcoidosis granulomas targeted by granuloma-associated T-cells, and that these antigens may be mycobacterial in origin, a number of research groups have investigated the use of the commercial tuberculosis interferon gamma release assays in patients with sarcoidosis. The majority have found no increase in response in this patient group e.g. an investigation into a Japanese cohort of patients at low risk of LTBI found that only 3 of 90 tested with the IFNγ ELISA assay used for diagnosis of tuberculosis had a positive response (Inui et al., 2008). However, other mycobacterial antigens have been investigated in the belief that the antigens involved are not necessarily from viable mycobacterial organisms. Rather, that they are responsible for initiating sarcoidosis in some patients through a mechanism that may be different to that from a standard tuberculosis infection.

Mycobacterium tuberculosis mycolyl transferase (Antigen 85A) is one of three secreted subunit proteins in *M tuberculosis* that together form the Antigen 85 complex. IFNγ ELISpot assays have been used to assess for recognition of this antigen by Peripheral Blood Mononuclear Cells (PBMCs) from patients with sarcoidosis and controls. Reactivity to Ag85A whole protein was observed in 15 of 25 (60%) sarcoidosis subjects compared to 2 of 22 (9%) PPD negative subjects ($p=0.0006$) and to 14 of 16 (88%) Purified Protein Derivative (PPD) positive subjects. Peptide-mapping studies identified four immunogenic Ag85A

peptides, which induced Th-1 immune responses in individual subjects, suggesting that multiple epitopes from this protein may have a role in be involved in antigen-specific T-cell recognition in some patients with sarcoidosis (Hajizadeh et al., 2007).

ESAT-6 is one of the components of the IGRAs for diagnosis of TB infection but has also been investigated alone in patients with sarcoidosis. One study showed a significant difference within the sarcoidosis group with 12 of 30 (40%) patients responding to the antigen compared to one of 26 (4%) PPD- volunteers ($p=0.0014$). The same study further investigated BAL fluid and found that one patient who had no recognition of ESAT-6 by PBMC demonstrated strong BAL cell Th1 immune responses to ESAT-6 (Drake et al., 2007). A more detailed study by the same research group found that when combined with the mycobacterial antigen mKatG, BAL T-cells from 32 out of 44 sarcoidosis patients displayed antigen-specific recognition to either one of the whole proteins compared with one of 27 controls with other lung diseases ($p<0.0001$). The primary cells responding to the antigens were shown to be CD4+ T-cells although CD8+ T-cell responses were observed in the BAL of some of the sarcoidosis patients. Blocking of Toll-like receptor 2 reduced the strength of the observed immune response (Oswald-Richter et al., 2009).

In addition to mycobacterial expressed proteins, the virulence factor Superoxide dismutase A (SodA) which is responsible for generating host cellular immune responses in infected hosts has also been investigated. The protein sequence has been identified from sarcoidosis tissue using Polymerase Chain Reaction (PCR) (Drake et al., 2002) and in one study SodA amplicons were detected in 12 of 17 (71%) sarcoidosis specimens, compared to 2 of 16 (13%) controls ($p = 0.001$) and in 3 of 3 (100%) tuberculosis specimens and the sequences were shown to be identical to that found in *M. tuberculosis*. ELISpot analysis found that 6 of 12 (50%) sarcoidosis subjects recognised the sodA peptides, compared to 1 of 26 (4%) PPD negative controls ($p = 0.002$) and six out of 11 (55%) of PPD positive subjects (Allen et al., 2008).

A follow up to this work has been the development of a mouse model of pulmonary sarcoidosis. Mice were sensitized by subcutaneous injection of SodA and then challenged with intravenous injection SodA or control. Subsequent histological analysis revealed hilar lymphadenopathy and noncaseating granulomas in the lungs of sodA treated mice. BAL cells from these mice demonstrated CD4+ T cell responses against sodA peptide and this was present in the SodA sensitized mice only (Swaisgood et al., 2010).

1.64 MYCOBACTERIAL CATALASE PEROXIDASE (MKATG)

Although T-cell responses to a number of mycobacterial antigens have been investigated in sarcoidosis, the majority of these have been chosen for study based on their potential for responses in patients infected with mycobacteria. Aside from the initial work on SodA, there has so far been limited effort to investigate sarcoidosis material itself for potential T-cell antigens. A major exception is that of mycobacterial catalase peroxidase (mKatG), a mycobacterial protein which is clinically relevant as it is involved in the conversion of isoniazid from the prodrug to the active form. The protein has been isolated from sarcoidosis tissues (see 1.53) and T-cell responses to mKatG have since been investigated with higher frequencies of mKatG-reactive, IFN-gamma-expressing T-cells in the peripheral blood of sarcoidosis patients compared with healthy controls in phenotypically diverse patient groups from North America and Sweden. Sarcoidosis patients who displayed T-cell responses to mKatG showed preferential accumulation of mKatG-reactive CD4+ Th1 cells in the lung, indicating a compartmentalised response (Chen et al., 2008). In addition, circulating mKatG-reactive T-cells were found in patients with active sarcoidosis only. The absence of these cells in patients with inactive disease may indicate that they play a role in causality of acute disease only.

Further experimental work conducted to identify the cells responsible for immune responses to mkatG peptides have indicated that CD4+ T cells may be the cell type primarily responsible for these systemic responses. Recognition of mkatG is inhibited using monoclonal antibody against HLA-DR and HLA-DQ, but not HLA-DP. mkatG presented by antigen-presenting cells expressing DRB1*1101-induces Th-1 (IFN-γ and IL-2) responses from sarcoidosis T-cells. This demonstrates the likely immunological relevance of the association of HLA DRB1*1101 with sarcoidosis (Oswald-Richter et al., 2010). To date mKatG and sodA are the only tissue antigens identified using an immunological based proteomic approach. Analysis of sarcoidosis spleen and lymph node tissue using more diverse techniques such as comparative proteomics of sarcoidosis and control tissues may yield still further proteins of interest.

1.65 LTBI AS A CONFOUNDING FACTOR IN PATIENTS WITH SARCOIDOSIS

The hypothesis that mycobacteria have a causal role to play in disease pathogenesis is not thought of as being universally applicable to all cases of sarcoidosis. IGRAs used for diagnosis of LTBI have been investigated in patients with sarcoidosis and it has been generally found that there is no increased response above the expected in this group. One point of contention with papers investigating T-cell responses to individual specific mycobacterial antigens is the somewhat unexplored possibility of patients being incidentally co-infected with latent tuberculosis infection which is unrelated to their sarcoidosis. Such a possibility would lead to false positive responses in these individuals. Given the relative low incidence of such occurrences stringent study groups are difficult to define. For example the selection of a PPD negative (e.g. assessed by a negative TST) control group may bias towards an increased incidence of Latent tuberculosis infection (LTBI) in the sarcoidosis group. Some of the published studies are from regions of high incidence of background LTBI and where the sarcoidosis patients did not have TST results. Indeed, when studies have examined IGRAs

in patients with sarcoidosis in areas of low levels of LTBI, the findings are generally negative (Inui et al 2008).

An ideal study would involve sarcoidosis patients who are shown to have no evidence of latent tuberculosis infection. It could then be hypothesised that any resulting T-cell responses to mycobacterial antigens other than those seen in Region of Difference 1 (RD1) would be due to sarcoidosis, rather than any latent tuberculosis infection. The inherent difficulty with this scenario is that by removing all patients with LTBI from the sarcoidosis study group, the investigators may be selecting out cases of sarcoidosis that are actually caused in some way by mycobacteria, leaving only a cohort of patients with disease caused by other stimuli. This 'catch-22' situation is hard to resolve and will remain the case until an actual molecular mechanism for disease pathogenesis by mycobacterial proteins can be proven in at least some cases of sarcoidosis.

1.66 INVESTIGATION OF T-CELL RESPONSES TO SELF PROTEINS

One study to investigate T-cell responses to non-mycobacterial proteins involved the sequencing of peptides eluted from HLA-DR molecules of bronchoalveolar lavage fluid to identify self-antigens in disease. This study showed sarcoidosis patients expressing the HLA-DR allele DRB1*0301 are characterized by large accumulations in the lungs of CD4+ T cells expressing the TCR AV2S3 gene segment (Wahlstrom et al., 2007). Analysis of these antigenic peptides by reversed-phase HPLC and liquid chromatography–mass spectrometry resulted in the identification of 78 amino acid sequences from self-proteins including vimentin and ATP synthase. Of these 78 proteins, only one has been investigated for antigenic T-cell activity. The cytoskeletal protein vimentin has since been used in ELISpots with PBMCs of patients with sarcoidosis and also of controls. 6 out of 11 (55%) patients with the disease displayed strong responses to vimentin peptides compared to 2 out of 16 (13%) healthy controls (Wahlstrom et al., 2009). T-cell responses to citrullinated vimentin peptides

have also been found in rheumatoid arthritis (Feitsma et al., 2010b). Characterisation of blood and lung T-cell autoimmune responses to these self-antigens may help in our understanding of the persistent pulmonary inflammation seen in sarcoidosis.

1.67 FUTURE T-CELL DIAGNOSTIC WORK

The difficulty in investigating T-cell responses in peripheral blood and bronchoalveolar lavage fluid to mycobacterial antigens lies with our limited understanding of the T-cell infiltrate associated with the Kveim reagent. Highly validated Kveim reagent has almost total specificity i.e. it does not produce a reaction in patients with other disorders including those with tuberculosis. However, if the active component within Kveim is a mycobacterial protein, it would be expected to cause a skin response in most patients with tuberculosis which is not the case. One explanation for this apparent disparity is that the antigens that have been investigated (including mKatG) may be more antigenic in patients with sarcoidosis than in those with tuberculosis and there is some evidence to support this theory (Chen et al., 2008).

An alternative explanation is that if sarcoidosis is caused by a number of different aetiological factors, a sub set of patients may have increased responses to mycobacterial antigens compared to controls. The difficulty here lies again with ensuring that the sarcoidosis patients chosen for the study do not have latent tuberculosis instead as a confounding factor causing false positive T-cell responses. A number of areas of interest need to be further explored including T-cell responses to self antigens such as the HLA-bound peptides which are presented *in vivo* during pulmonary inflammation as well as the investigation of T-cell responses to other antigens in select cohorts e.g. responses to propionibacteria in patients from Japan. A thorough analysis of these responses in large patient cohorts is needed before we can make any solid conclusions about T-cell responses to specific aetiological agents.

1.7 HYPOTHESES, AIMS AND METHODS OF INVESTIGATION

1.71 HYPOTHESES AND AIMS

Hypothesis 1

I hypothesise that there is a limited set of T-cell protein antigens contained within Kveim reagent and sarcoidosis tissue that cause the diagnostic Kveim reaction seen in sarcoidosis patients. Identification of these proteins may allow for further investigation of T-cell responses to these antigen(s) and detection *ex vivo* may result in the improved diagnostic sensitivity and specificity of the Kveim test itself.

Hypothesis 2

I hypothesise that although there are no pathognomonic features of sarcoidosis granulomas, there are signatures or fingerprints of immune mediated inflammation that are specific both to sarcoidosis and to stage of disease.

Aim 1

This relates to hypothesis 1. I will use proteomic techniques to investigate validated Kveim reagent and whole sarcoidosis spleen tissue for proteins that are specific to sarcoidosis and are not found in control tissue. Future immune studies may be able to investigate the *ex vivo* T-cell antigenicity towards these identified proteins.

Aim 2

This relates to hypothesis 2. I will use multiplex protein analysis methods to investigate profiles in sarcoidosis and related controls to see if there are cytokine signatures that are specific to either sarcoidosis or to stage of disease.

1.72 METHODS OF INVESTIGATION USED TO ACHIEVE THE AIMS

Aim 1 is primarily achieved using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) to identify proteins of interest that are specific to Kveim reagent and DIGE (Difference In Gel Electrophoresis) to quantify proteins that are of increased abundance in sarcoidosis spleen tissue. SDS-PAGE with immobilised pH gradients allows for an accurate separation of up to several thousand proteins on a polyacrylamide gel by mass and charge (pI) (Gorg et al., 2000). The method allows for detailed protein separation (Gorg et al., 1988), high loading of protein samples (Hanash et al., 1991) onto the gel and reproducible results (Corbett et al., 1994). The imaging, spot digestion and Mass Spectrometric analysis of peptides allows for rapid and reliable identification of the separated proteins (Wilm et al., 1996).

DIGE takes the identification process further by allowing for accurate quantitative analysis of differences between sarcoidosis and control spleens. The technique involves labelling of each spleen extract using spectrally resolvable, size and charge-matched fluorescent dyes (Marouga et al., 2005a) and has produced novel results in a number of diseases including glioblastmoa multiforme (Collet et al., 2011), coronary artery disease (de la Cuesta et al., 2009) and schizophrenia (English et al., 2009).

In addition to protein identification, MSD (Meso Scale Discovery) is used to investigate cytokine responses from sarcoidosis peripheral blood mononuclear cells towards Kveim reagent by multiplex analysis of the supernatant produced after overnight stimulation. MSD has been shown to be a rapid and reproducible method for multiple cytokine analysis in a number of diseases including Alzheimer's disease (Rajendran et al., 2006), diabetes (Fosgerau et al., 2010) and asthma (Ayanoglu et al., 2011). MSD is also applied directly to sarcoidosis bronchoalveolar lavage and serum samples to look for patterns of disease as part of Aim 2. The methods used to achieve both aims are summarised in figure 1.4.

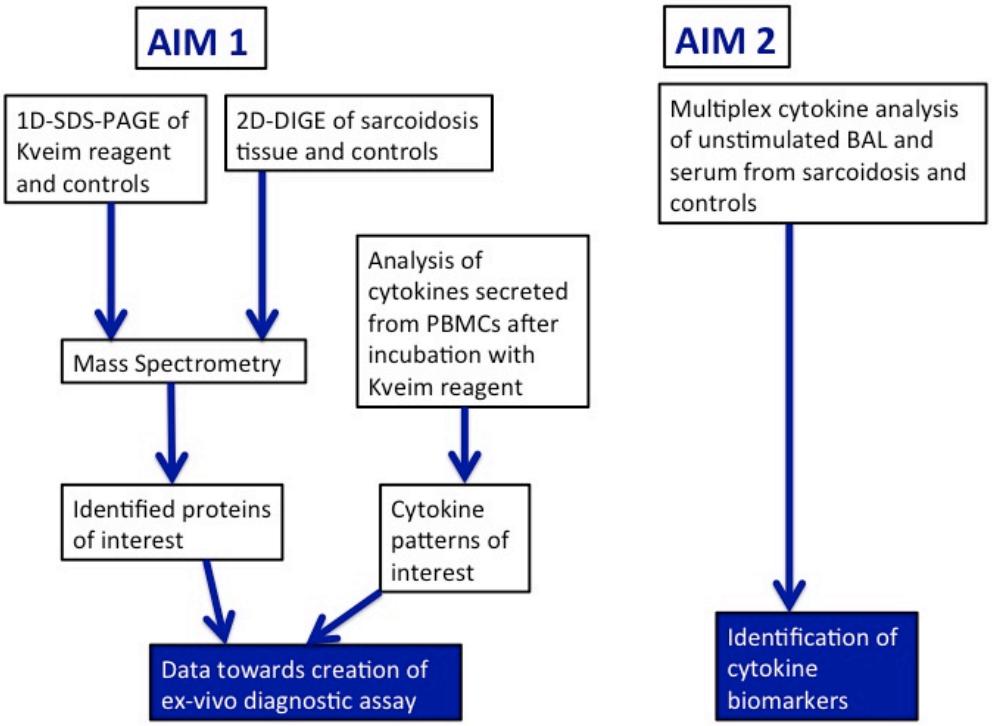


Figure 1.4 Flow chart of methods used to achieve the aims of this work. Aim 1 is achieved using 1D-SDS-PAGE to identify proteins specific to Kveim reagent. Comparative proteomics with 2D-DIGE is additionally used to discover proteins present in sarcoidosis and control tissues but found upregulated in sarcoidosis. Kveim regant is incubated with sarcoidosis PBMCs and the secreted cytokine profile is investiagetd. Informstion from both secreted cytokines and identified proteins may be used for the future creation of an *ex vivo* assay for diagnosis. Aim2 is achieved by use of MSD to look for BAL and serum cytokine patterns specific to sarcoidosis.

Chapter 2

Materials and methods

2.1 Clinical aspects of the work

2.11 Ethical approval

All human tissue collected for the purposes of this work has been under the ethical approval of a Research Tissue Bank which is based within the Tuberculosis Research Unit at the Department of Respiratory Medicine, Imperial College London. Ethical approval for the tissue bank was granted by the West London St. Mary's research ethics committee (reference 07/H0712/85).

2.12 Sample resources and storage conditions

Kveim reagent which had been deemed suitable for diagnosis of patients with sarcoidosis prior to 1996 was provided as a gift expressly for the purposes of this project by Dr. Donald Mitchell (Royal Brompton Hospital London and Porton Down Research Institute). The reagents have been coded (designated with 'K' numbers e.g. K3, K12, K51) and each prepared from a different sarcoidosis spleen. There is little remaining recorded information as to the exact source of each individual Kveim reagent. They have been validated *in vivo* for diagnosis of sarcoidosis with over 80% sensitivity and 95% specificity and were created for use over a number of years. This sample resource possibly represents the largest collection of Kveim reagents worldwide.

Fresh splenic tissue, lymph node tissue, bronchoalveolar lavage (BAL) fluid and blood specimens were acquired from consenting donors within the Imperial College NHS healthcare trust, primarily at St. Mary's and the Royal Brompton hospitals.

Additional spleen tissue samples have been purchased for this project from ethically sourced donors (AMS Biotechnology, Oxford and Cambridge Biosciences, Cambridge) or have been donated as part of a collaboration with the National Disease Research Interchange (Philadelphia, United States). The majority of spleens used for this work (see table 3.3) were from anonymous donors where limited clinical information is available. Some of these donors may have had immunospuressive treatment for their sarcoidosis (but this is unknown) and it is unknown what effect this may have had on the spleen tissue. However, the efficacy of historic Kveim reagent for *in vivo* diagnosis is known to be independent to the effects of immunospressive therapy on the donor or the length of time since diagnosis (as discussed in section 1.41).

All samples were stored under appropriate conditions to ensure high quality retrieval for downstream experiments e.g. Peripheral Blood Mononuclear Cells (PBMCs) were stored within liquid nitrogen tanks whereas serum samples were stored at -80 ^{o}C. All samples were stored under the permissible ethical approval of the Research Tissue Bank.

2.13 PARTICIPANTS AND PROCEDURES

Aside from spleen samples already mentioned, patients were recruited from hospitals within Imperial College Healthcare Trust for donation of blood samples for use in this work. Bronchoalveolar lavage (BAL) fluid from sarcoidosis patients was obtained from consenting patients at St. Mary's hospital London and BAL fluid from patients with other conditions (for use as controls) were obtained as apart of a collaboration from other ethically approved studies within Imperial College Healthcare Trust.

2.14 STATISTICAL ANALYSIS

The experimental data has been analysed using the appropriate statistical methods and these have been detailed where used throughout this work. Graphpad Prism version 5.04 for Macintosh (La Jolla, California, USA) was used to perform this analysis and create graphs.

2.2 PROTEOMIC METHODS

2.201 PREPARATION OF NEW (*DE NOVO*) KVEIM REAGENT FOR PROTEOMIC ANALYSIS

This method follows an original protocol describing creation of Kveim reagent (Chase, 1961). Previously frozen sarcoidosis spleen tissue (having been donated peri-operatively or post-mortem and confirmed to contain the characteristic sarcoidosis granulomas) was thawed from frozen and rinsed free of blood with Phosphate Buffered Saline (PBS) (Sigma-Aldrich, St. Louis, USA). 100 µg of spleen was processed at a time by disintegration using a sample grinding kit (GE Healthcare, Piscataway, NJ, USA) into 250 µl of PBS which had been pre-stored at 4°C. The resulting suspension was diluted up to a total volume of 500 µl with further cold PBS and washed 4 times by spinning at 5,500g for 20 min at 4°C (using a Hettich Table Top Shaker) with successive removal of supernatant and suspension of the pellet in 500 µl of cold PBS in between each spin. The final resulting resuspension was poured through a sterile stainless steel mesh (425 µm aperture) and the volume was adjusted to 625 µl total volume using additional cold PBS. This suspension was then heated for 2 hours to 56°C in a water bath on two consecutive days with storage at -20°C in between heating steps. The final material was stored at -80°C.

2.202 AMMONIUM ACETATE/METHANOL PRECIPITATION FOR PHENOL REMOVAL FROM HISTORIC *IN VIVO* VALIDATED KVEIM REAGENT

A number of Kveim reagents (e.g. K51) were stored in phenol which needed to be removed prior to 1D-SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as phenol interfered with protein visualisation in some instances. The protocol used originates from a method of phenol extraction of plant tissues (Faurobert et al., 2007) This method has been shown to be a viable alternative to TCA-acetone extraction (which was also used to remove phenol from Kveim reagents but with less success in terms of protein visualisation). 200 µL of Kveim reagent was incubated overnight at -20°C in 1.5 ml 0.1M ammonium acetate in methanol (which was pre-stored at -20°C) prior to centrifuging at 10,000g for 5 min at 4°C. The pellet was washed in 1.5 ml of -20°C methanol, vortexed for 30 sec and centrifuged at 10,000g for 5 min at 4°C. The supernatant was discarded and 1.5 ml of 80% acetone 20% water was added to the pellet. After a further centrifuging at 10,000g for 5 min at 4°C the supernatant was again discarded and the resultant pellet was solubilised at 37°C into Laemmli buffer (appendix 1) prior to 1D-SDS-PAGE.

2.203 SAMPLE PREPARATION STEPS

In addition to phenol extraction from Kveim reagents by ammonium acetate/methanol precipitation, a number of sample preparation steps were evaluated prior to determining optimal conditions for SDS-PAGE. These steps were necessary as rule out methods but due to their inability to improve SDS-PAGE resolution when used alone, the final protocol for 1D-SDS-PAGE (as described in section 2.206) used a combination of some of the steps and did not rely on one single method alone. However, all methods evaluated are detailed in brief in table 2.1.

Table 2.1 Summary of sample preparation steps evaluated prior to SDS-PAGE.

PREPARATION STEP	METHOD (IN BRIEF)
Sample dilution	Kveim reagent was analysed by two dimensional gel electrophoresis in a pure state or in dilutions of 1:2, 1:10 and 1:50
Sonication	Kveim reagent was placed in a Sonication bath for 10, 30 or 60 sec at 37°C
Microconcentration and desalting	Kveim reagent underwent microconcentration and desalting with Microcon centrifuge filter units (Millipore, Massachusetts, USA)
Boiling with SDS	Kveim reagent was boiled in SDS reagent at 95 ˚C for 10, 20 and 30 min
Detergent addition	Zwitterioninc detergent, R3 reagent and R3 reagent without urea (all from Sigma-Aldrich) were added to Kveim reagent prior to solubilisation into buffer
Sample grinding	Kveim reagent and fresh sarcoidosis tissue underwent sample grinding using a commercially available kit (GE Healthcare)
Protein extraction	Kveim reagent and fresh sarcoidosis tissue underwent a protein extraction process using a cocktail of protease inhibitors and commercially available kits to separate proteins into total protein, membrane proteins and nuclear and cytoplasm proteins (Fermentas Life Sciences, Oxford, UK)
2D clean up kit	Kveim reagent and fresh sarcoidosis tissue underwent a two stage cleanup process using a commercially available kit (GE Healthcare)
Destreaking reagents	Destreak reagent (GE Healthcare) was added to Kveim reagent and fresh sarcoidosis tissue prior to solubilisation in traditional solutions

2.204 PROTEIN QUANTIFICATION

All protein concentrations were determined using a Nanodrop 2000c Spectrophotometer (Thermo fisher Scientific, Massachusetts, USA). Concentrations were measured after a 1:20 dilution with Bradford assay buffer (Sigma-Aldrich). The optical density of each sample was read in duplicate at 595 nm and the initial protein concentration was calculated by plotting this density against a pre-created standard curve made up in the same assay buffer (see appendix 2).

2.205 ANALYSIS OF KVEIM REAGENT AND SARCOIDOSIS SPLEEN EXTRACTED PROTEINS BY MEANS OF PHAST GEL ELECTROPHORESIS

1D-SDS-PAGE phast gels (GE Healthcare) were used to separate protein samples by adding 1:1 v:v ratio of protein (50 μg) and SDS phastgel buffer (GE Healthcare) and then heated to 80°C in a heating block for 5 min. 2 μl of this solution was taken up with a sample applicator (GE Healthcare) and was then run on the Phastgel system (GE Healthcare) using SDS buffer

strips and phastgels with continuous acrylamide gradients 10-15% (GE Healthcare) and a standard protocol run at 15 ^{o}C for 20 minutes as detailed in table 2.2. This summary of optimal run parameters was determined by makers of the Phastgel system (GE Healthcare) and provided by the company as a technical protocol for proteomic analysis of solid tissues. Gels were immediately removed upon completion of the run and stained with instantblu (Expedeon, Cambridge, UK) or Colloidal Coomasie (appendix 1) as per section 2.210.

Table 2.2 Summary of optimal run parameters for 1D-SDS-PAGE phastgel

Step	Voltage (V)	Current (mA)	Time
1	2000	2.5	3 min 45 sec
2	200	2.5	7 min 30 sec
3	2000	2.5	20 min

2.206 Analysis of Kveim reagent and sarcoidosis spleen extracted proteins by means of 1D-SDS-PAGE

1D-SDS-PAGE was attepted prior to 2D-DIGE (2.209) for one main purpose. It permitted the running of individual Kveim reagents in individual lanes as detailed in Chapter 4. This allowed for the identification of proteins which were specific to Kveim reagent only and not found in any control tissues. Conversely, 2D-DIGE analysis allowed for identification of proteins present in both sarcoid and control tissues but that were of increased abundance in sarcoidosis. These two approaches therefore allowed for identification of Kveim-specific proteins and those of increased abundance in disease. As detailed figure 5.7 there is some overlap in terms of protein identity between these two approaches but a number of proteins were found through a single methodology only. The 1D-SDS-PAGE phast gels (2.205) allowed for rapid protein separation (30 minutes) but the protein staining and visualisation was less intense than the 1D-SDS-PAGE gels used here. These non-phast gels took longer to run (1-1.5 hours until the gel front had moved to within 5 mm of the bottom of the gels) but

their superior attributes of protein staining and visualisation resulted in these gels being used (rather than the Phast gels) for the protein identification detailed in chapter 4.

For 1D-SDS-PAGE analysis, a total of 50 µl Kveim reagent was centrifuged at 10,000g for 20 min at 4°C. The pellet was discarded and the supernatant removed and added to 50 µl 2x Laemmli buffer (appendix 1). This was then vortexed for 30 sec and sonicated in a water bath for 15 min at 37°C to disperse any remaining solid material into a suspension. The sample was heated at 100°C for 5 min in a water bath prior to centrifugation at 10,000g for 1 min at 4°C. On cooling, 100 µg of protein as measured by spectrophometer (appendix 2) was loaded onto a 4–20% precast polyacrylamide gel (Bio-Rad Laboratories, Hercules, California, USA). One lane was kept free for the addition of 5 µl molecular weight marker (20–120kDa, Fermentas Life Sciences). The gels were run in a mini protean tera cell gel tank (Bio-Rad Laboratories) at a fixed voltage of 150V for 5 minutes (entry phase) and subsequently at 50V until the gel front had moved to within 5 mm of the bottom of the gels. The gels were then opened and added to Colloidal Coomasie solution (appendix 1).

2.207 Analysis of Kveim reagent and sarcoidosis spleen extracted proteins by means of 2D-SDS-PAGE: 1ST dimension

All steps were carried out in a dedicated clean room within the proteomics facility at Imperial College London. 100 µg batches of spleen were lysed with a mortar and pestle (pre-stored at -80°C) into 1 ml lysis buffer (appendix 1) and protein concentrations were adjusted to the concentration required. Immobiline dry strips (GE Healthcare) containing pre-formed pH gradients immobilised onto gel were rehydrated with rehydration buffer (GE Healthcare) with 1% w/v DTT (Sigma-Aldrich) and 2% v/v IPG Buffer of pI appropriate to the strip (GE Healthcare) and placed in a drystrip reswelling tray for 16 hr (460µl buffer for 24 cm strips and 340µl buffer for 18cm strips). Air bubbles present below the strips were removed with a needle prior to the rehydration period. The strips were then rinsed in distilled water and

blotted using 3mm Whatman paper (Sigma-Aldrich) before being transferred to an Ettan IPGPhor 3 machine (GE Healthcare). Filter wick pads (GE Healthcare) pre-soaked in 150 µl distilled water and blotted on Whatman 3mm paper were added to the anodic and cathodic ends of the strip and electrodes were inserted and placed on the filter wicks. The protein samples (containing 150 µg of protein for analytical loading and 450 µg of protein for preparative gels) were cup loaded at the anodic strip ends and paraffin oil used to cover the strips the strips thus preventing crystallisation of urea. Once the isoelectric focusing was complete the strips were either stored at -80˚C (for up to 4 weeks) or run immediately in the 2nd Dimension. Table 2.3 details the optimal conditions for 24 cm strips of different pIs all run in the first dimension at 20˚C with a maximum current of 50 mA per strip (see appendix 3 for IPGPhor run graph).

Table 2.3 Summary of optimal IPGphor voltage steps for sarcoidosis spleen tissue protein extracts (150 µg analytical phase, 450 µg preparative phase) run in 1st dimension on 24 cm strips (pI 3-7, NL and pI 7-11, NL)

Step	Voltage type	Voltage (V)	Time (hours)
1	Step and hold	500	1
2	Step and hold	1000	4
3	Gradient	4000	4
4	Gradient	6000	4
5	Step and hold	4000	4

2.208 2D-SDS-PAGE: 2ND DIMENSION

All steps were carried out in a dedicated clean room within the proteomics facility at Imperial College London. 50 ml of SDS equilibration buffer (appendix 1) was thawed and 1% w/v DTT (Sigma-Aldrich) was added (reduction phase). The strips were then shaken using a benchtop shaker at 100rpm at for 15 min to allow the gels to equilibrate. The strips were then shaken again in 4.8% w/v iodoacetamide in SDS equilibration buffer for a further 15 minutes (alkylation phase). Strips of 24 cm in length were placed on top of precast SDS-PAGE gels at

12% bis acrylamide (GE Healthcare) and bonded with 1 ml high quality agarose (GE Healthcare) with trace amounts of bromphenol blue. These were then run in an Ettan DALT 12 tank with dedicated power supply, buffer circulation pump and Peltier cooling device for up to 12 x 24cm precast gels (GE Healthcare). The gels were run in SDS running buffer (GE Healthcare) and run until the gel edge was within 5 mm of the bottom of the gel. The following protocol (table 2.4) was found optimal for 24 cm strips run in the second dimension at 20˚C. Times are given as approximate as step 2 was shortened or lengthened (up to 2 hour either way) so that the gel edge reached the bottom of the gel but did not over run.

Table 2.4 Summary of optimal voltage steps for sarcoidosis tissue run in 2nd dimension using 24 cm strips (pI 3-7 and 7-10, NL).

Step	Voltage (V)	Time (approx.)
1	1V per gel	2 hrs
2	4V per gel	10-16 hrs

2.209 DETECTION OF DIFFERENTIAL PROTEINS BY MEANS OF DIFFERENCE IN GEL ELECTROPHORESIS (DIGE)

Differential protein pools were labelled with fluorescent dyes (N-Hydroxysuccinimidyl (NHS) Cy2, Cy3 and Cy5- all from GE Healthcare) as follows: 100 mg of spleen tissue was extracted with DIGE lysis buffer (appendix 1) and the concentration was initially adjusted to 10mg/ml of protein using a spectrophotometer (appendix 2). The dyes were diluted by adding 25 µl high grade dimethylformamide (Sigma-Aldrich) to of 25 µl dye. 1 µl of this dye solution (containing 400 pM of pre-diluted CyDye 2 (Cy2)) was added to 50 µg of each protein sample to constitute the internal standard and all internal standards were pooled. Cy3 and Cy5 dyes were used for reciprocal labelling (as shown in table 2.5) with 400 pM of dye added to each 50 µg of protein sample. These were individually mixed and briefly centrifuged and then left on ice for 30 minutes in the dark. 1 µl of 10 mmol/L lysine was added to stop the labelling reaction and the samples were mixed and briefly centrifuged and then left on ice for

10 minutes in the dark. Prior to 1st dimension electrophoresis, an equivalent volume per sample was added of 2x DIGE lysis buffer containing an additional 4% (w/v) DTT (Sigma-Aldrich) and 2% (v/v) IPG buffer at a pI range matching that of the immobiline strip (GE Healthcare). Samples were combined with the internal standard, one disease and one control sample per combination. The typical layout of a 6 gel DIGE experiment is detailed in table 2.5. The IPGphor strips were cup loaded and run in the first and second dimensions as described in sections 2.207 and 2.208 with the use of DIGE low fluorescent plates and DIGE running buffer (both from GE Healthcare) in the second dimension. Preparative gels for spot excision were run under identical conditions but with 450 µg of protein per each gel.

Table 2.5 Typical layout of a DIGE experiment comparing sarcoidosis and normal spleen.
S= sarcoidosis spleen, N=normal spleen. Cy3 and Cy5 dyes were used to reciprocally label protein samples to ensure there was no bias due to a particular dye fluorescence.

Gel	Internal standard cy2	Sample cy3	µl vol. to achieve 50 µg total protein	Sample cy5	µl vol. to achieve 50 µg total protein	IPG strip no	Gradient/cm	µl 2x lysis buffer
1	20 µl	S1	18	N4	25	76035	3-7 pI. 24cm	63
2	20 µl	S2	25	N6	19	76036	3-7 pI. 24cm	64
3	20 µl	S3	18	N8	18	76038	3-7 pI. 24cm	56
4	20 µl	N11	16	S4	25	76037	3-7 pI. 24cm	61
5	20 µl	N12	19	S5	18	75984	3-7 pI. 24cm	57
6	20 µl	N13	25	S6	18	76039	3-7 pI. 24cm	63

2.210 VISUALISATION OF PROTEINS FROM 1D- AND 2D-SDS-PAGE GELS

1D-SDS-PAGE gels were stained with either Instantblu (Expedion) or Colloidal Coomasie (appendix 1). 2D-SDS-PAGE gels were stained with Colloidal Coomasie only. Instantblu was applied for 1-2 hours with gels in a tray on a benchtop shaker at enough volume to cover the gel when placed in a shallow container of area slightly larger than the gel. Gels stained with Colloidal Coomasie (amount similar to instantblu) were left on a benchtop shaker overnight at 100rpm and some required destaining with repeated changes of distilled water (up to four changes needed over a one hour period at the end of overnight staining). In some cases, gels

which appeared heavily stained were destained with 1% (v/v) acetic acid instead of distilled water.

2.211 DETECTION OF PROTEINS FROM 1D- AND 2D-SDS-PAGE GELS

Proteins analysed using 1D-SDS-PAGE phast gels were imaged using a Diversity 3 Imager (Syngene, Cambridge, UK) in the white light mode with all default settings from start up of machine maintained. 1D-SDS-PAGE precast gels were imaged using an Epson Perfection V750 Pro Flatbed Scanner in the gel scanning mode with all default settings from start up of machine maintained. 24 cm 2D-SDS-PAGE gels with prior DIGE-labelled proteins were imaged using a Typhoon 9400 laser scanner (GE Healthcare) as follows: fluorescent labelled proteins were visualised by exciting the Cy dyes at particular excitation wavelengths (optimal excitation/emission wavelengths used for detection were: Cy2= 488/520 nm, Cy3 = 532/580 nm, Cy5 = 633/680 nm). PMT voltage settings were adjusted during preliminary low resolution scans such that signal intensities measured across a specified area of each gel were within 0-5% of each other. All high resolution scans were subsequently performed using these predetermined optimal scan settings. Images from all three scanning devices were saved as TIFF files for further analysis.

2.212 ANALYSIS OF DIFFERENTIAL PROTEIN SPOTS FROM DIGE 2D-SDS-PAGE

Images from DIGE experiments were analysed using Progenesis SameSpots version 3.0 (Nonlinear Dynamics) software to allow qualitative and quantitative comparison between sarcoidosis and normal spleen proteins. Gel images were superimposed on each other and compared to a reference image (pre-selected as the gel which appeared to contain the best spot pattern) which contained all protein spots from both sarcoidosis and control spleens. Automatic alignment was chosen for all images analysed. All other settings from start up of the software were left in the default mode for comprehensive unbiased spot analysis. The

software used Principle Component Analysis to identify spots that were significantly up or down regulated or that appeared specific to sarcoidosis tissue only. A statistically ranked list of spots was then produced to allow accurate evaluation of spots of interest which included sarcoidosis protein spots present in one or more disease samples (and which appeared absent from controls) or sarcoidosis spots which after normalisation had a significantly increased or decreased spot volume at least 1.5 fold ($p<0.05$ on ANOVA analysis). The spots of interest were subsequently excised from the gels using clean scalpel blades (one per spot) and underwent tryspin digestion and Mass Spectrometric identification.

2.213 IN GEL TRYPSIN DIGESTION OF PROTEIN BANDS/SPOTS OF INTEREST

This method is adapted from that used by the Proteomics, Metabolism and Bioimaging Unit at the Veterinary Laboratories Agency, UK. All chemicals used are from Sigma-Aldrich unless otherwise mentioned. Excised Colloidal Coomassie gel slices and/or 2D gel spots were placed in 500 µl eppendorf low protein bind microfuge tubes and washed and destained three times in 30% methanol / 7% acetic acid solution total 100 µl (or of a higher volume which was sufficient to be double the spot or band volume). At each wash step, the tubes were shaken briefly on a rotary mixer. The wash was discarded between each step. The gel pieces/spots were then covered in 50% acetonitrile solution (ACN) i.e. 50 - 100 µl and incubated at room temperature for 10 min. The solution was removed and this step was repeated once more. The gel pieces were dried at 50°C in heat block for 10 min. The disulphide bonds of proteins were reduced by the addition of 50 - 100 µl of 10 mM DTT in 10 mM ammonium hydrogen carbonate solution (pH 8). The tubes were incubated for 45 min at 50°C in a heat block. The DTT solution was removed and alkylation of protein cysteine residues promoted by the addition of 50 - 100 µl 50mM iodoacetamide (IAA) in 10 mM ammonium hydrogen (pH 8) carbonate solution. Tubes were incubated for 60 min at 50°C in the dark. The IAA solution was removed and gel pieces washed twice in 50% ACN i.e. 100

µl. The tubes were incubated for 45 min at 50°C in a heat block. Trypsin Gold Grade (Promega, Wisconsin, USA) was reconstituted in 50 mM acetic acid to a final concentration of 1 µg/ µl. 50 mM of acetic acid was prepared by the addition of 143 µl glacial acetic acid to 50 ml distilled H_2O and a stock solution of 30 ng/µl modified porcine trypsin was prepared in 10mM ammonium hydrogen carbonate. 50 ng of trypsin solution was added per gel spot (100 ng per slice) and tubes were incubated at room temperature for 10 min. Each gel spot/slice was covered with 30-50 µl of 10 mM ammonium bicarbonate solution (enough to cover the gel). The tubes were incubated at 37°C for a period of 12 – 16 h on a flat shaker running at 50 RPM. The tubes were then centrifuged at 10,000g for 2 min. 10 µl of 50% ACN / 5% Trifluoroacetic acid (TFA) was added to each tube and gently agitated for 5 min on the horizontal shaker. Supernatants were transferred to fresh tubes and 10 µl of 50% of ACN / 5% TFA was added twice more to the gel pieces with subsequent removals to ensure efficient extraction of all peptides. The peptides were then analysed immediately or stored at -80°C prior to MS analysis.

2.214 Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-ToF)

Proteins bands from 1D phastgels were excised with clean scalpel blades and underwent trypsin digestion as detailed in section 2.213. 1µl of trypsin digested peptides (taken from the supernatant formed immediately after an overnight trypsinisation of protein bands) was added to 1 µl of Matrix solution (appendix 1) and spotted onto wells on a MALDI plate (Micormass, Manchester, UK). Additionally, 1 µl lock solution (10 pmol/µl ACTH - 18-39 fragment peptides, (Sigma-Aldrich) in 0.1% TFA) was mixed with equal volume of matrix solution and spotted in the centre of each 4 spot cluster of wells to be used for internal calibration at 2469.155 Da. The spotted plate was then dried. In addition to sample analysis, the MALDI-Tof Mass Spectrometer (MALDI micro MX, Waters, Millford, Massachusetts, USA) was

calibrated using calibration mix (Polyethylene glycol 1000, 2000 and 3000 from Sigma-Aldrich). The machine was used once the vacuum was on or less than 10^{-7} in reflectron mode. Twenty spectra of peptides in the mass range between 0 and 3500 were collected from each sample with three spectra of mass lock. Raw data was then processed using MassLynx software (Waters). The backgrounds were subtracted, peaks were smooth and centroided and data was then searched using Protein Lynx Global Server version 2.2 (Waters) against an updated SwisProt database (version 04092010) firstly against homo sapiens and then against an all organisms database. The searches were repeated using MASCOT Daemon search mechanism version 2.2.1 (Matrix Science, Boston, MA, USA) using the following parameters: One miscleavage was tolerated, fixed modification of carbamidomethylation, variable modifications of methionine oxidation and carboxymethylation. Peptide mass tolerence was set to 100 ppm with no restrictions made on the protein M_r and the pI. Trypsin was set as the digesting enzyme. The resulting protein identification was supported with MASCOT data for each identification showing the Mowse search score, the number of peptides found and the percentage of the amino acid sequence covered. The theoretical mass was also compared to the position on the gel for further validation, taking into account that isoforms, protein degradation products and post-translational modifications would result in differences between the theoretical mass and mass of the protein product found. Identified proteins were analysed for function using the NCBI database (version 04092010).

2.215 QUADROPOLE TIME OF FLIGHT (Q-TOF)

Proteins bands (1D-SDS-PAGE) and spots (2D-DIGE) were excised with clean scalpel blades and underwent trypsin digestion as detailed in section 2.213. The low protein bind Eppendorf tubes containing digested peptides were taken to dryness in a SpeedVac prior to re-suspension in 8.5 µl of 0.1% v/v formic acid (Sigma-Aldrich). The liquid in the tubes was carefully pipetted up and down to ensure total resuspension of the dried peptides. The peptide solutions were decanted into lined HPLC sample vials (Agilent Technologies) then 7.0 µl per sample

was immediately analysed by electrospray Q-TOF LC/MS with a chip cube interface (model 6520, Agilent Technologies, Santa Clara, California, USA). Machine settings were applied by Dr. Mark Weeks, VLA. The chip type used was ProtID-Chip-150 II 300A C18 150mm column. HPLC settings applied were: capillary pump (buffer A 100%), flow rate 4 µl / min (micro flow), nanopump: (buffer A, buffer B), 0.55 µl per min, gradient setting: time 0 (buffer B 4%), 11 min (75%), 12.5 min (90%), 14 min (90%), 15.5 min (4%), 16 min (4%). The mass spectra were recorded with following settings: drying gas 3.0 l /min, maximum 5 precursors per cycle, mass range 250-3000 m/z at acquisition rate of 3 spectra per sec (MS) and 50-2000 m/z at acquisition rate of 5 spectra sec (MS/MS). Reference mass correction was done by internal standard of 922.009798 *m/z* (Agilent Technologies). Capillary pump buffers used were made in HPLC H_20 (Thermo Fisher Scientific), and Nanopump buffers used were made in LCMS grade H_20 (Thermo Fisher Scientific) (appendix 1). Resulting MS/MS data was analysed using MassHunter Mass Profiler software version B.02.01 (Agilent Technologies) and searched against Spectrum Mill version A.03.03 (Agilent Technologies) and MASCOT version 2.2.1 (Matrix Science) databases. Resulting data was searched firstly against an updated Swisprot homo sapiens database and then against an all organisms database (version 04092010). The following parameters were used: Two miss-cleavages were tolerated, fixed modification of carbamidomethylation, variable modifications of methionine oxidation and carboxymethylation. A precursor mass tolerence of +- 30 ppm and product mass tolerence of +-50 ppm was set. Trypsin was set as the digesting enzyme. The resulting protein identification was supported with Spectrum Mill data (with additional MASCOT data to support the protein identification from Spectrum Mill) for each identification showing the MS/MS search score, the number of peptides found and the percentage of the amino acid sequence covered. The theoretical mass was also compared to the position on the gel for further validation, taking into account that isoforms, protein degradation products and post-translational modifications would result in differences between the theoretical mass and mass of the protein product found. Identified proteins were subsequently analysed for function using the NCBI database (version 04092010).

2.216 OFFGEL PROTEIN FRACTIONATION

Proteins extracted from spleen tissue into lysis buffer (as described in section 2.207) were fractionated by pI using the Agilent 3000 Offgel fractionator (Agilent Technologies). Distilled water was added to extracted proteins to a final volume 720 µl , to which was added 2.88 ml 1.25x Offgel Stock Solution (Agilent Technologies). 24 cm IPG (3-10 Linear gradient) strips were placed in the tray where a 24 well plastic well strip was position over the gel such that each well was sealed against the gel surface. 40 µl of Offgel rehydration solution (Agilent Technologies) was applied per well to the 24 wells used. Electrode pads were wetted with rehydration solution and placed on the ends of the strips. The strip gels were allowing to swell for 15 min at room temperature, prior to the addition of 150 µl of previously prepared sample solution (containing 600 µg of extracted spleen proteins) to each well. A cover seal was placed over the wells and 10 µl of dH_20 was applied to the electrode pads at the end of each gel. Cover oil (GE Healthcare) was placed over the strips to ensure adequate covering and the cathode and anode electrodes were placed at either end. A current was then applied over a 100 hr period (max 50 µA) to separate the proteins by virtue of their isoelectric point. During the run, cover oil was added to the electrodes at regular intervals (24 hrs) to prevent evaporation. 100 µl of sample was recovered per well and transferred to 24 individual low bind tubes. Pools 1 and 24 were discarded (as they were deemed to contain the most impurities and least protein content) and the remaining 22 pools were combined into 11 protein pools each containing 2 pools of adjacent pI e.g. 2+3, 4+5, 6+7 etc. Each of the 11 pools then underwent an acetone protein clean up step as detailed in materials and methods section 2.218 and the resulting protein pellet was dissolved in RPMI-1640 media (Sigma-Aldrich) with the final concentration adjusted by nanodrop to 10 µg/ml (see appendix 4 for Offgel run graphs of sarcoidosis and control spleen extracts).

2.217 PROTEIN ELUTION FROM SDS-PAGE

This method of elution of intact proteins from SDS-PAGE gels follows an original protocol (Hager and Burgess, 1980). Slices of gel (approximately 2 mm x 2 mm) were added to 100 µl of Elution Buffer (appendix 1) in individual tubes (1 ml low bind). The tubes were sonicated in a water bath for 15 min at 37˚C and then placed in a heat block for 1 hr at 25˚C with gentle agitation every 15 min. The tubes were then spun at 10,000g for 15 min at 4˚C. The supernatant was taken off for protein quantification (appendix 2) and use in downstream applications.

2.218 ACETONE PRECIPITATION OF SPLEEN PROTEIN EXTRACTS

Kveim reagent derived proteins were cleaned up prior to use in ELISpot and MSD assays using an acetone precipitation in order to remove contaminants such as SDS and DTT which may have proved toxic to cells as follows: 1 volume of protein solution was added to 4 volumes of -20ºC acetone. This was mixed and kept –80ºC for 12 hrs. The tubes were then centrifuged at 10,000g for 10 min at 4ºC. The supernatant was removed and the pellet retained. The pellet was dried in air for 5 min and resuspended in buffer appropriate for downstream applications.

2.219 SERUM PROCESSING FOR PROTEOMIC ANALYSIS

The entire process from taking blood to storage at -80˚C was complete within 90 min. 5-8 ml blood was collected in 10ml glass vacutainer tubes (Sigma-Aldrich). The tubes were placed in an upright position and the blood was allowed to clot for 30 min (up to 60 min maximum if blood did not clot within the 30 min time period). The tubes were centrifuged at 1000g for 10 min at 4ºC and the resulting serum was stored in 500 µl aliquots at -80˚C.

2.220 BRONCHOALVEOLAR LAVAGE FLUID PROCESSING FOR PROTEOMIC ANALYSIS

The entire process from taking BAL fluid to storage at -80˚C was complete within 90 min. BAL fluid was passed through a 425 μm aperture mesh and the resulting filtrate was stored in 500 μl aliquots at -80˚C.

2.3 IMMUNOLOGICAL METHODS

2.31 COLLECTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PMBCS)

A total of 40 ml of blood was collected in heparinised 50 ml falcon tubes (Sigma-Aldrich). This was diluted 1:1 with RPMI-1640 media (Sigma-Aldrich) and then two volumes of diluted blood were gently layered over one volume of Ficoll Paque PLUS (GE Healthcare). After centrifugation at 900g for 20 min at 21°C the resulting layer of PBMCs was transferred to a 10 ml polypropylene tube. RPMI-1640 was added to wash the cells and the resultant solution underwent centrifugation at 650g for 7 min at 21°C. The supernatant was discarded and the resulting cell pellet was washed with 5 ml RPMI-1640. The cell suspension underwent centrifugation at 650g for 7 min at 21°C. The supernatant was discarded and the resulting cell pellet was gently suspended in AIM-V media (Sigma-Aldrich). The cell suspension was diluted 1:2 in trypan blue for counting and assessment of viability with haemocytometer (Invitrogen Countess automated cell counter). Cells were prepared for storage in liquid nitrogen as detailed in section 2.32 or used immediately in downstream applications.

2.32 FREEZING AND THAWING OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

PBMCs were stored for future use by aliquoting $10x10^6$ cells into 1 ml freezing mix (appendix 1) and then placed in Mr. Frosty containers (Thermo-Scientific) and stored at -80˚C for 24-96 hours before being transferred to permanent storage in liquid nitrogen.

Frozen cells were thawed for 1-2 minutes in a 37˚C water bath before being placed one drop at a time into 10 ml of pre-warmed R10 solution (appendix 1). Cells were then centrifuged 3 x at 650g for 5 minutes at 21˚C with resuspension of the pellet in 5ml RPMI in between centrifugation steps. The cell pellet was resuspended in 5 ml AIM-V for cell counting and viability prior to downstream applications.

2.33 PREPARATION OF KVEIM TEST ANTIGENS FOR DOWNSTREAM APPLICATIONS

Whole Kveim suspension (historical validated *in vivo* Kveim or newly created *de novo* Kveim) was put through a commercial 2D precipitation kit (GE Healthcare) which involved the use of precipitant and co-precipitant proprietary reagents to quantitatively precipitate proteins. 100 µl of Kveim reagent was added to 300 µl precipitant and mixed by vortexing for 30 sec. It was then incubated on ice for 15 min. 300 µl of co-precipitant was added and again mixed by vortexing for 30 sec. The tubes were centrifuged at 12 000g for 5 min at 4°C. The supernatant was removed and the tubes spun again briefly to remove further supernatant.

40 µl of co-precipitant was placed on top of each pellet and the tubes were incubated on ice for 5 min before being centrifuged at 12 000g for 5 min at 4°C. The supernatant was again removed and 25 µl of dH20 was placed on top of each pellet. The tubes were vortxed for 30 sec and 1 ml of wash buffer (pre-chilled for at -20 ^{o}C) and 5 µl wash additive were added. The tubes were vortexed and the resultant suspension was incubated at -20 °C for 30 min. Tubes were vortexed for 20–30 sec every 10 min during this time. The tubes were then removed and centrifuged at 12 000g for 5 min at 4°C. The supernatant was discarded and the

resulting pellet was dissolved in 600 µl RMPI-1640 (Sigma-Aldrich) and then underwent sonication in a water bath for 45 minutes to disperse the solid material into a suspension. The protein concentration was determined using a nanodrop spectrophotometer and adjusted to 10µg/ml prior to storage at -80˚C.

2.34 INTERFERON GAMMA AND INTERLEUKIN-2 ELISPOT

Enzyme-linked immunosorbent spot assays (ELISpot assays) were performed using in-house protocols developed within the TB Research Unit at Imperial College London (Millington et al., 2007b). In terms of the Interferon gamma (IFNγ) ELISpot, precoated IFNγ plates (Mabtech, Stockholm, Sweden) were seeded with 2.5 x 10^5 PBMCs per well. Wells contained no peptide (negative control), 5 µg/ml PHA (positive control; MP Biomedicals, Solon, Ohio, USA) or antigen e.g. 20 µg/ml tuberculin PPD (United States Serum Institute), ESAT-6 or CFP-10 peptide pools at 10 µg/ml (Louisiana State University Health Science Center Core Laboratories, New Orleans, LA). Whole Kveim antigens were prepared as detailed in section 2.33. Plates were incubated at 37°C 5% CO2 for 16 to 20 hours. The plates were then washed 4 times with PBS (200 µL/well) and developed with an alkaline phosphatase-conjugated IFNγ detection antibody (Mabtech) followed by the chromogenic substrate BCIP/NBT Plus (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Moss Substrates, Maryland, USA). Spot-forming cells (SFCs) were counted using an automated ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). Settings for the intensity and size of a counted spot were predefined and the same settings were used throughout.

The Interleukin-2 (IL-2) ELISpot was performed in the same manner with the following changes: the membrane of a MAIPS4510 plate (Millipore, Billercia, Massachusetts, USA) was prewetted with 50 µl of 70% ethanol per well for 2 min at room temperature. Wells were washed five times with 200 ml/well sterile water and incubated overnight at 4°C with 10

μg/ml coating IL-2 antibody (Mabtech). Wells were washed five times with 200 ml/well sterile PBS (Sigma-Aldrich) and blocked for 30 min at room temperature with R10 (appendix 1). After incubation with appropriate stimulus (e.g. Kveim reagent antigen) for 16-20 h, plates were developed with 1 μg/ml biotin-conjugated IL-2 detection Ab (Mabtech) for 2 h at room temperature and then streptavidin-ALP (Mabtech) diluted 1/1000 in PBS for 1 h at room temperature followed by the chromogenic substrate BCIP/NBT Plus (Moss). For both IFNγ and IL-2 ELISpots, all wells were measured in duplicate and mean SFC (spot forming counts) were recorded as per 10^6 PBMCs minus the value in the negative well. Positive results were defined as a SFC of at least twice the negative well and at least 5 spots greater than seen in the negative well.

2.35 CELL INCUBATION WITH PROTEIN ANTIGENS

2.5×10^5 PBMCs in a total volume of 100 μl of AIM V media (Sigma-Aldrich) were placed per well in 96 well flat bottom plates (Thermo-Fisher) with 20 μl of Kveim antigen (as detailed in section 2.33) (or other antigen e.g. ESAT-6 as detailed in section 2.34) at a concentration of 10 μg/ml. The plates were incubated for 20 hrs at 37˚C and 5% CO_2. The plates were then spun at 500g for 1 min at 21˚C and the supernatant was aliquoted off for immediate cytokine analysis or storage at -80˚C for later analysis.

2.36 MULTIPLEX CYTOKINE ANALYSIS

Multiplex cytokine analysis was carried out using 96 well precoated plates and proprietary reagents (Meso Scale Discovery – MSD, Maryland, USA). 25 μL of Diluent 2 was dispersed into each well. The plate was sealed with an adhesive plate seal and incubated for 30 min with vigorous shaking (300–1000rpm) at room temperature. 25 μL of the sample (e.g. serum or

BAL fluid) was then added per well and all samples were measured in duplicate. A calibrator for the 8 point standard curve was also aliquoted in duplicate on each plate. The plate was sealed with an adhesive plate seal and incubated for 2 hours with vigorous shaking (1000 rpm) at room temperature. The plate was then washed 3 times with 0.05% Tween 20 (Abcam, Cambridge, UK) in PBS (Sigma-Aldrich). 25 μL of the 1x detection antibody solution (specific to the cytokines being measured) was then placed into each well of the MSD plate. The plate was sealed and incubated for 2 hours with vigorous shaking (1000 rpm) at room temperature. The plate was again washed 3 times with 0.05% Tween 20 in PBS. 150 μL of 2X Read Buffer T was added to each well of the MSD plate which was then analysed using the MSD SECTOR Imager. The majority of cytokine readings were found above the lower detection limits as defined by the standard curve created for each experiment and from supplied company literature of between 0.07 and 0.5 pg/ml depending on the cytokine measured. For the purposes of data display, the intravariability of the platform has meant that accuracy of the readings has been limited to 2 significant figures after the decimal point (Meso Scale Discovery, 2009). Any concentrations recorded lower were kept as absolute values rather than normalised up or down. All results of 0 were normalised to 0.01pg/ml for purposes of accurately displaying all results on logarithmic curves.

Chapter 3

Identification of kveim reagent-specific Th1-cells in sarcoidosis

INTRODUCTION

Antigen-specific T-cell responses in the peripheral blood and BAL fluid towards a number of proteins have been previously investigated in sarcoidosis and research has primarily focused on proteins of mycobacterial origin (Hajizadeh et al., 2007, Drake et al., 2007). Perhaps the best characterised protein investigated to date is mycobacterial catalase peroxidase (mKatG), a mycobacterial enzymatic virulence factor which allows for prolonged survival of the organism inside host macrophages and was directly identified from sarcoidosis spleen tissues (Moller, 2007). Antibodies and T-cells directed towards mKatG have been identified in two patient cohorts representing the diverse environments of North America and Northern Europe (Chen et al., 2008). There has been no previous attempt to identify proteins from Kveim reagent itself but prior to such identification, it must be shown that Kveim reagent has the capacity to elicit specific cellular responses from patients with sarcoidosis.

Most Kveim reagents can be stored for long periods of time without loss of *in vivo* granuloma forming activity (Mitchell et al., 1976, Siltzbach and Ehrlich, 1954, Nelson and Schwimmer, 1957) and although such Kveim reagents have been extensively used *in vivo*, there are no published reports of Kveim reagent being employed *ex vivo* with this aim of eliciting specific cellular responses. However, a major concern of the validated Kveim reagent with respect to proteomic analysis, is that despite the ability to cause an *in vivo* granulomatous response and perhaps an *ex vivo* response in a cellular assay, it is probable that the long term storage at

$+4^0$C will have led to the proteolytic degradation of whole proteins into peptides (Cleland et al., 1993).

Whilst the peptides may well retain the ability to induce an antigen-specific cellular response, the degradation of the original proteins may result in difficulties in identification through proteomic methods. Such methods often require intact proteins which are digested to produce protein-specific peptides. These peptides constitute a 'peptide mass fingerprint' which is searched against updated databases for accurate identifications. Proteolytic processes which have degraded Kveim reagent will mean that individual proteins cannot be identified from single peptides without resorting to 'shotgun proteomics' (section 1.51) which requires intensive data analysis to identify the original proteins. To overcome this potential difficulty in protein identification, it must be shown that new Kveim material can be made from fresh sarcoidosis spleen (hereafter designated *de novo* Kveim reagent) and that it is also capable of causing an *ex vivo* cellular response specific in patients with sarcoidosis. The protein constituents of Kveim reagent may be fractionated by size (mass) and/or charge (pI) to identify specific fractions that engender the same cellular responses as whole Kveim reagent. This is important as work on validated Kveim reagent has shown it to be relatively resistant to chemical and physical stress but such stresses could in fact be used to concentrate the active component (Lyons et al., 1992). The process of physicochemical stress when fractionating the proteins into separate groups may help towards this concentration process.

Most published sarcoidosis *ex vivo* T-cell studies to date have involved the use of a single method i.e. INFγ ELISpot assays (Drake and Newman, 2006) with one study demonstrating IL-2 specific T-cell responses towards mKatG (Oswald-Richter et al., 2010). In order to better understand the cellular response towards Kveim-derived proteins it is important to investigate a number of different cytokines. Infections such as tuberculosis evoke a strong

Th1 response from T-cells activated by specific antigens of interest (Taha et al., 1997) whilst other pulmonary diseases such as atopic asthma display a stronger Th2 response (Robinson et al., 1992). As the only antigen-specific T-cell cytokines investigated in sarcoidosis to date are INFγ and IL-2, it is important to investigate a wider range of Th1 and Th2 cytokines.

A confounding factor in the investigation of host responses to mycobacterial proteins is the possibility of co-existing latent mycobacterial infection which may need to be excluded in patients with a diagnosis of sarcoidosis prior to investigations with Kveim reagent. Some studies in patients resident in environments at lower risk of mycobacterial infection have shown they have low or non-existent T-cell responses to the immunodominant mycobacterial protein antigens early secreted antigenic target protein (ESAT-6) and culture filtrate protein (CFP-10) (Inui et al., 2008, Horster et al., 2009). Thus, by pre-selecting sarcoidosis patients at presumed low clinical risk of mycobacterial infection and investigating T-cell responses to mycobacterial proteins with standard clinically validated interferon-gamma release assays (IGRAs), it may be possible to exclude patients with co-infection. This clinically distinct phenotype of patients with sarcoidosis only can then be used in *ex vivo* investigations with Kveim reagent.

The selection of a distinct clinical phenotype of sarcoidosis patients without tuberculosis infection (which may have otherwise confounded any cellular Kveim reagent response) and the investigation of multiple cytokine-secreting T-cell responses in the peripheral blood towards Kveim reagent, provides the first step in validating the premise of an *ex vivo* Kveim reagent based antigen-specific T-cell assay. These investigations were therefore completed prior to a proteomic analysis of Kveim reagent itself.

RESULTS

3.1 PRECISE CLINICAL PHENOTYPING OF SARCOIDOSIS PATIENTS WITHOUT TUBERCULOSIS INFECTION

The demographic and clinical characteristics of the patients with sarcoidosis (n=14) and controls with tuberculosis (n=9) investigated by ELISpot are detailed in table 3.1. The patients with sarcoidosis represented a broad time period from first presentation. In all, 4 patients were investigated less than one month following diagnosis and the remaining 10 more than one month from time of diagnosis. The 4 patients who were within one month of diagnosis all had blood sample taken at time of bronchoscopy prior to starting any treatment for sarcoidosis. The remaining ten patients who were more than one month from diagnosis were all on immunosuppressive therapy (<10mg prednisolone daily) but no other immunosupressants. All sarcoidosis patients had diagnosis supported by tissue biopsy with radiological evidence of pulmonary disease and 4 patients had additional clinical or radiological evidence of extra-pulmonary involvement. A significant limitation of this experiment is that power calculations were not performed prior to data analysis and so a larger data set may be needed to exclude the possibility that further differences are found between the sarcoidosis and tuberculosis groups (e.g. figure 3.1b). All sarcoidosis patients studied were designated as having a low clinical risk of latent tuberculosis infection. All were either born in the United Kingdom or if born abroad had arrived in the UK in early childhood. None had a family or contact history of tuberculosis and none had the classically described risk factors for tuberculosis e.g. HIV infection, alcohol or substance misuse. Of the 14 patients, 10 had definite evidence of prior BCG vaccination (as evidenced by skin scar) with vaccination status unclear in the remaining four.

Of the 14 patients with sarcoidosis, 8 (57%) were female and the median age was 42 yrs. (range 30-64). Seven were classed as Caucasian, two as Asian (Indian-sub continent)

ethnicity and five were black. Of the patients with tuberculosis 3 (33%) were female, the median age was 36 yrs. (range 29-77) and 5 were classed as Caucasian, 2 as Asian (Indian-sub continent) ethnicity and 2 were black. All control group patients had definite evidence of tuberculosis infection by standard diagnostic methods including tuberculin skin tests, chest radiograph findings, IGRAs and sputum/bronchoalveolar lavage culture; 4 were classified as having active tuberculosis infection with the remaining 5 as having latent infection. Frequencies of PPD-, ESAT-6- and CFP-10-specific IFNγ-secreting T-cells were enumerated by ELISpot assay and results shown in figure 3.1. There was a statistically significant difference ($p<0.0001$) in the median spot forming cell counts (SFCs) per 10^6 PBMCs of PPD-specific IFNγ-secreting T-cells between the two groups as shown in figure 3.1a (sarcoidosis median 0, IQR 0-34; tuberculosis median 288, IQR 206-561). A statistically significant difference was also seen in the median SCFs per 10^6 PBMCs of CFP-10-specific IFN-γ-secreting T-cells (sarcoidosis median 0; tuberculosis median 20, IQR 0-71) as seen in figure 3.1c. ($p=0.01$) The statistical value of difference between the median SFCs per 10^6 PBMCs of ESAT-6-specific IFNγ-secreting T-cells as shown in figure 3.1b is $p=0.06$ (sarcoidosis median 0, IQR 0-6; tuberculosis median 42, IQR 0-156). None of the 14 patients with sarcoidosis exhibited an ELISpot response to CFP-10 whilst 3 showed a positive response to ESAT-6 but 2 of these 3 are below the median SFC value seen in the patients with tuberculosis.

Table 3.1 Demographic and clinical characteristics of investigated patients with sarcoidosis (n=14) and tuberculosis (n = 9)

	Sarcoidosis n=14	**Tuberculosis n=9**
Median age (range)	42 (30-64)	36 (29-77)
Female gender (%)	8 (57)	3 (33)
Ethnic origin: Caucasian/Indian subcontinent/Black	7/2/5	5/2/2
Site of disease: **Sarcoidosis:** pulmonary/extrapulmonary/both **Tuberculosis:** pulmonary/extrapulmonary/both/not applicable(LTBI)	10/0/4	2/1/1/5
Stage of disease: **Sarcoidosis:** Acute (<1 month)/chronic (>1 month) **Tuberculosis:** Active/Latent	4/10	4/5

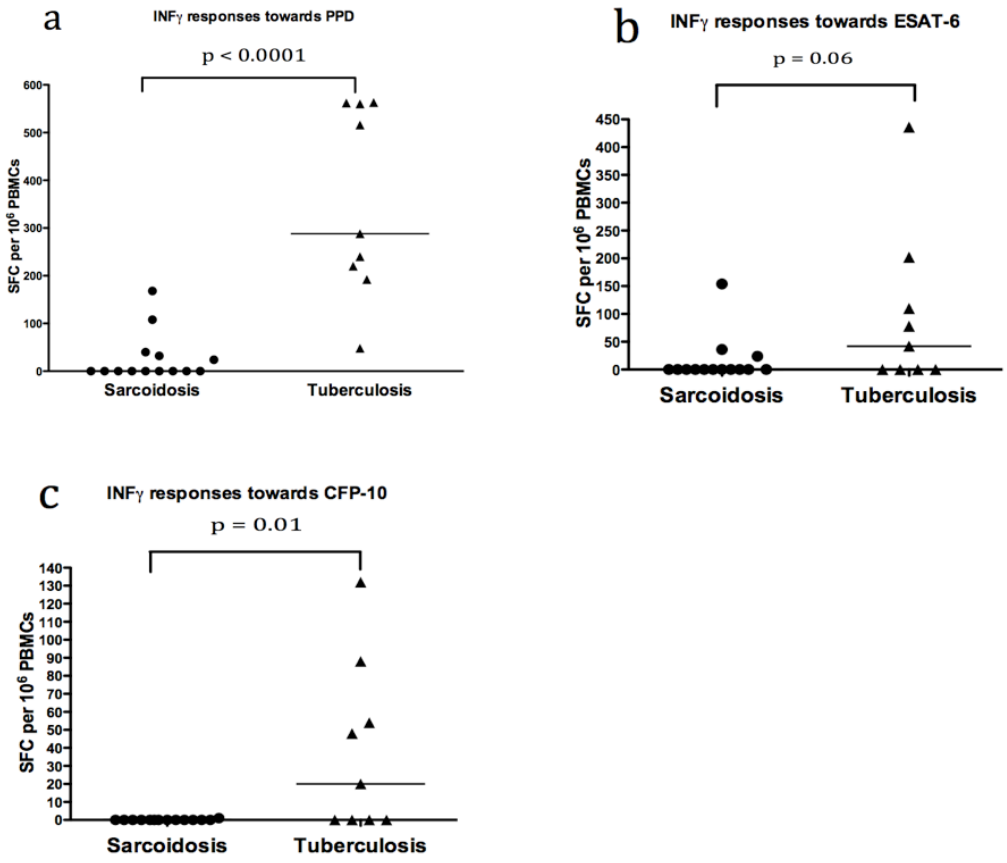


Figure 3.1 Frequencies of PPD-, ESAT-6- and CFP-10-specific IFNγ- secreting T-cells enumerated by *ex vivo* ELISpot assay in patients with sarcoidosis and tuberculosis. Dot plots represent mean number (from two wells assayed per experimental condition) of IFN-γ- secreting T-cells in antigen well minus mean negative control wells per 10^6 PBMC fro m individual patients. Significant differences in median value of sarcoidosis and tuberculosis groups (which were determined using Mann-Whitney test) were found with (a) PPD and (c) CFP-10. A p value just above the traditionally accepted value for statistical significance (p=0.06) was found with (b) ESAT-6. Display using logarthmic scales was attempted but the data here is not as disparate as that shown in figure 3.8 and the decision was made to use traditional linear scales to display the SFC values. The ratio of tuberculosis to sarcoidosis results (rather than median values) can be attempted but is not perhaps the traditionally accepted form of diplaying ELISpot data (Millington et al. 2007a).

Interestingly, only 5/14 (36%) of patients with sarcoidosis displayed a positive response to PPD despite at least 10/14 (72%) patients having evidence of prior BCG vaccination (there is significant antigenic cross-reactivity between PPD and BCG). A further analysis of these low

PPD responses in the 10 definite BCG vaccinated sarcoidosis patients was compared to responses from 14 BCG vaccinated healthy volunteers (figure 3.2), 6 of whom were female, 10 were Caucasian and 4 Asian, with a median age of 37.6 years (range 21-37). The data again show a significantly reduced response towards PPD in the sarcoidosis group.

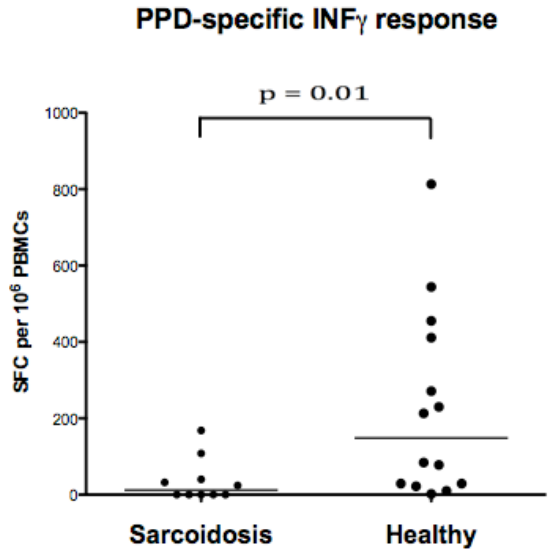


Figure 3.2 Frequencies of PPD-specific IFNγ- secreting T-cells enumerated by *ex vivo* ELISpot assay in BCG vaccinated patients with sarcoidosis and healthy volunteers. Dot plots represent mean number (from two wells assayed per experimental condition) of IFN-γ- secreting T-cells in antigen well minus mean negative control wells per 10^6 PBMC from individual patients. Median results from each group were compared by Mann-Whitney testing. Healthy volunteers demographics: 6 were female, 10 were Caucasian and 4 Asian, with a median age of 37.6 years (range 21-37). Healthy data provided by Dr. Damien Montamat-Sicotte, Tuberculosis Research Unit, Imperial College London.

3.2 IFNγ- AND IL-2-SECRETING T-CELL RESPONSES TOWARDS PROTEINS EXTRACTED FROM VALIDATED KVEIM REAGENT ARE FOUND IN THE PERIPHERAL BLOOD OF PATIENTS WITH PULMONARY SARCOIDOSIS

Proteins were extracted from 4 *in vivo* validated Kveim reagents (K3, K12, K51 and K54) (section 2.33). The patient samples were the same as used for results section 3.1 but only 11/14 sarcoidosis patients were used-all of whom had negative responses to both CFP-10 and ESAT-6, making it unlikely that they had co-existing tuberculosis infection. The limiting number of PBMCs from patients resulted in only some samples being exposed to all Kveim

protein antigens tested but all data generated from these experiments are shown. A total of 6 sarcoidosis patients were exposed to all 4 Kveim reagents. Frequencies of K3-, K12-, K51- and K53-specific IFN-γ-secreting T-cells enumerated by *ex vivo* ELISpot assay are shown in figure 3.3. The p value is significant in 2 out of the 4 validated Kveim reagents investigated as detailed in table 3.2. In total, 9 of 11 (82%) of patients with sarcoidosis produced a positive (as defined in 2.34) IFNγ-ELISpot response to at least 1 of the 4 Kveim reagents compared to 1 of 9 (11%) patients with tuberculosis. However, limited availability of PBMCs from each patient meant that not all patients with sarcoidosis were exposed to all 4 Kveim reagents extracts so the true percentage of sarcoidosis patients responding may be higher.

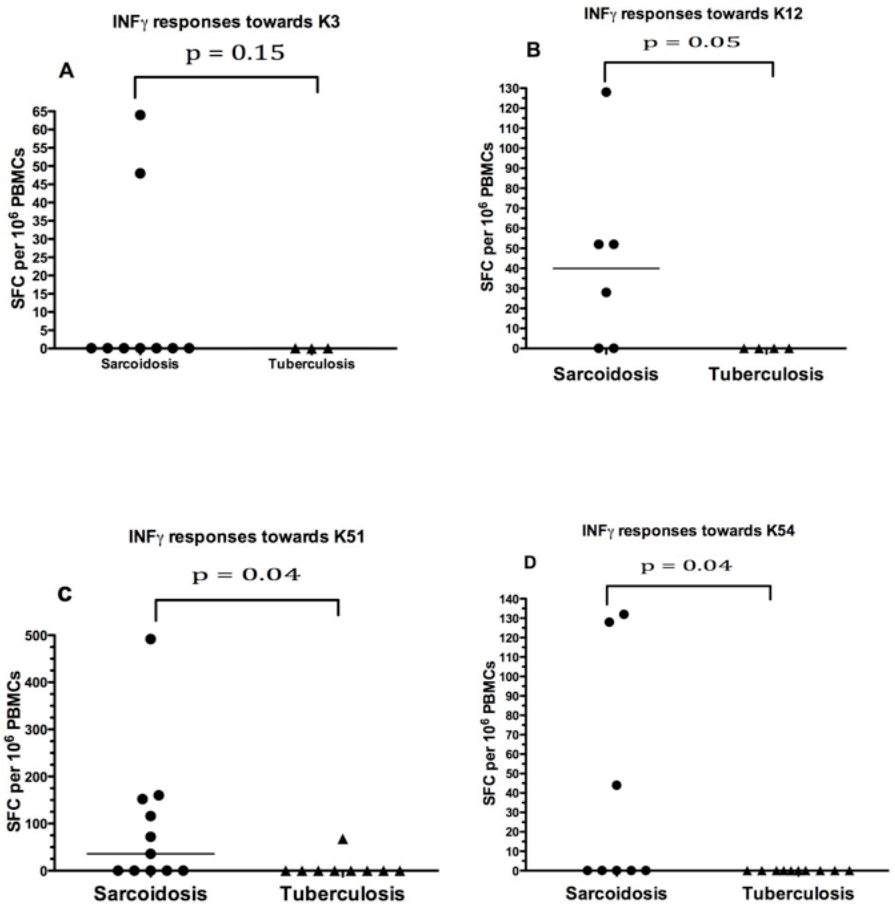


Figure 3.3 Frequencies of validated Kveim protein antigen-specific IFNγ-secreting T-cells enumerated by ELISpot assay in patients with sarcoidosis and tuberculosis. Dot plots represent mean number (from two wells assayed per experimental condition) of IFNγ-secreting T-cells in antigen well minus mean negative control well per 10^6 PBMC from individual patients. A number of different Kveim antigens (K3, K12, K51, K54) were investigated and results (of differences in median value of sarcoidosis and tuberculosis groups as determined using Mann-Whitney testing) are shown above (A-D).

Table 3.2 Total SFCs per 10^6 PBMCs from INFγ ELISpot measurements of sarcoidosis and control PBMCs incubated with Kveim reagent extracted proteins.

Kveim reagent	Number of sarcoidosis patients responding	Number of control patients responding	Sarcoidosis median (IQR) SFCs	Control median (IQR) SFCs	P value of difference between sarcoidosis and control medians
K3	2/9 (22%)	0/3	0 (0-24)	0	0.15
K12	4/6 (67%)	0/4	40 (0-71)	0	0.05
K51	6/11 (55%)	1/9 (11%)	36 (0-152)	0 (0-0)	0.04
K54	3/8 (38%)	0/10	0 (0-107)	0	0.04

Taken overall, 6 patients with sarcoidosis were investigated by INFγ ELISpot towards all 4 Kveim protein extracts. All 6 patients responded to at least one Kveim reagent as shown in figure 3.4 with 1 patient (S46) responding to 3 Kveim reagents (K12, K51 and K54) and 3 patients responding to 2 Kveim reagents. S46 was a newly diagnosed (known duration of less than one month) patient with pulmonary sarcoidosis and who had no unusual clinical features.

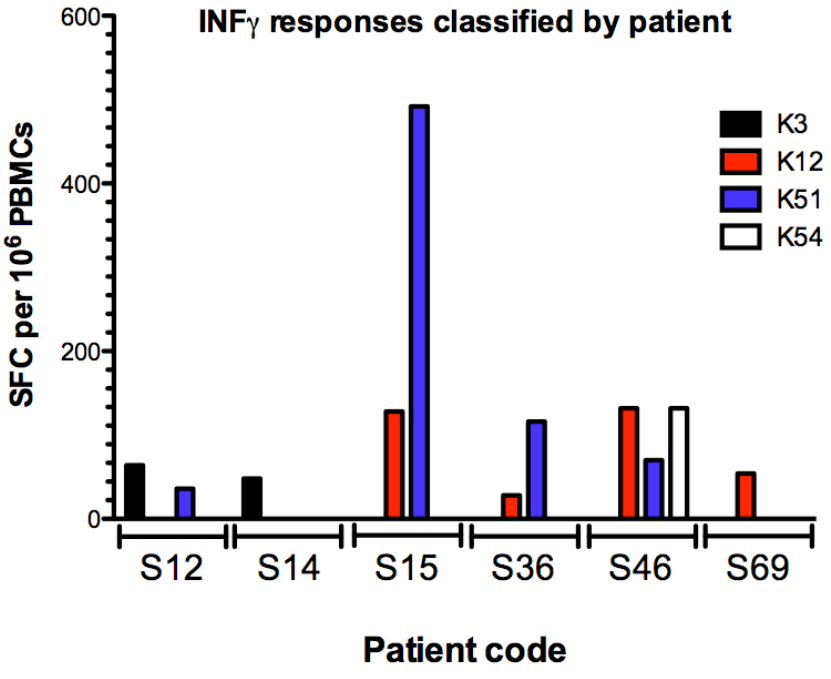


Figure 3.4 Frequencies of validated Kveim protein antigen-specific IFNγ-secreting T-cells enumerated by ELISpot assay in the 6 patients with sarcoidosis who were tested against all 4 validated Kveim reagents. Bars represent mean number of IFNγ-secreting T cells in antigen well minus mean negative control well per 10^6 PBMC.

Interestingly, S46 was initially presumed to have pulmonary tuberculosis at the first clinical visit but the diagnosis was only refuted following bronchoscopy and lung biopsy which showed changes consistent with sarcoidosis. The majority of SFCs were less than 200 but the highest response was displayed by patient S15 who had a SCF per 10^6 PBMCs of 492 in response to proteins extracted from K51. S15 was a patient with newly diagnosed pulmonary sarcoidosis with no other unusual clinical features other than that she was 3 months pregnant at the time of diagnosis and blood donation (the possible implications of pregnancy in sarcoidosis are discussed in section 7.13)

All 4 Kveim reagent-extracted proteins produced an INFγ ELISpot response in at least one of the 6 patients as shown in figure 3.5. K51 and K12 both exhibited a response from 4/6 (67%) of the sarcoidosis patients in this group who were tested against all 4 Kveim reagent protein extracts.

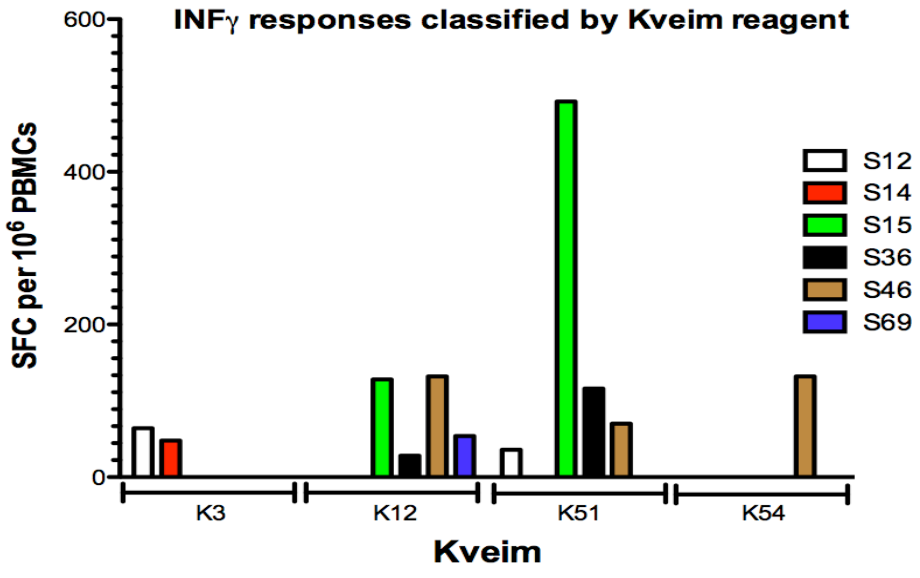


Figure 3.5 Interpretation of the data from figure 3.4 displaying the results for each of the four validated Kveim reagents. Bars represent mean number of IFNγ-secreting T cells in antigen well minus mean negative control well per 10^6 PBMC.

PBMC availability allowed for 4 of the patients with sarcoidosis to have a further investigation of Kveim reagent-specific IL-2-secretion from T-cells by means of an IL-2 ELISpot and the data were compared to that found using an INFγ ELISpot. The data for one sarcoidosis patient (S36) shown in figure 3.6 shows that there were no INFγ or IL-2 positive responses seen to ESAT-6 or CFP-10. However, IL-2-secreting T-cells were detected in response to PPD. Both K12 and K51-specific INFγ and IL2-secreting cells were detected by ELISpot but higher INFγ SFC responses were found in the case of K51. K54-specific IL2-secreting T-cells were found alone with no evidence of K54-INFγ secreting T-cells. A further 3 sarcoidosis patients were investigated with an IL-2 ELISpot but none of the three displayed positive responses to these antigens (data not shown). All 4 patients investigated by IL-2 ELISpot had pulmonary sarcoidosis diagnosed more than one month prior to donation of PBMCs and S36 had pulmonary sarcoidosis diagnosed 5 years previously and was still requiring long term immunosuppressive treatment with oral steroids.

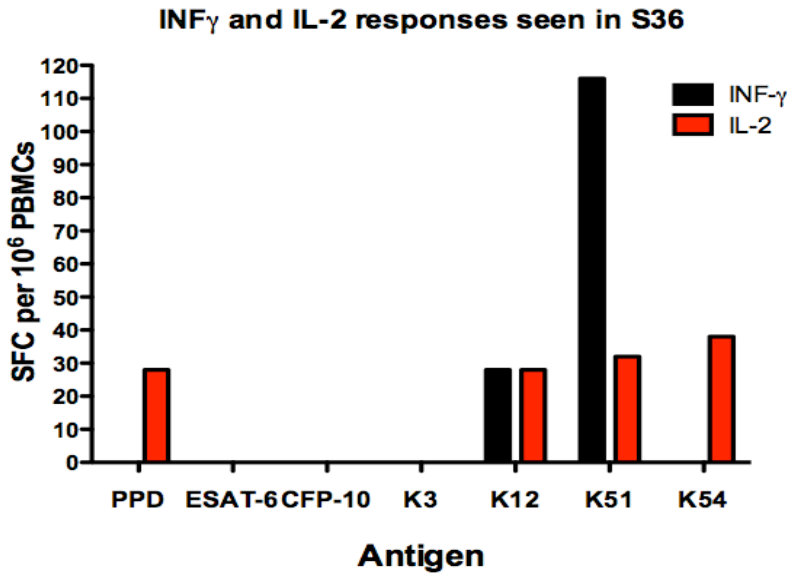


Figure 3.6 Frequencies of *in vivo* validated Kveim reagent antigen-specific IL-2- secreting T-cells enumerated by ELISpot assay in one patient with sarcoidosis. Bars represent mean number of IL-2-secreting T cells in antigen well minus mean negative control well per 10^6 PBMC. A further 3 patients with sarcoidosis were investigated with IL-2 ELISpot but none had positive responses to any of the antigens tested.

3.3 IFNγ-SECRETING T-CELL RESPONSES TO PROTEINS EXTRACTED FROM *DE NOVO* KVEIM REAGENT IN THE PERIPHERAL BLOOD OF PATIENTS WITH PULMONARY SARCOIDOSIS

Fresh sarcoidosis spleen was used to create *de novo* Kveim (materials and methods section 2.201). A sarcoidosis spleen coded S1 was taken from a 45 year old black female with gross extrapulmonary sarcoidosis and histological evidence of extensive granulomatous infiltration into her spleen. The organ was removed due it's size as it had been causing extensive abdominal pain for the patient. *De novo* Kveim proteins were extracted as detailed in materials and methods 2.33. The patient samples were the same as used for results section 3.2. The limiting number of PBMCs from patients resulted in only some samples being exposed to all Kveim protein antigens tested but all data generated from these experiments are shown with 6 sarcoidosis patients and 6 controls with tuberculosis investigated by means of INFγ ELISpot.

As seen in the figure 3.7, although the median sarcoidosis SFC value was significantly higher than that seen in the tuberculosis group, all 6 tuberculosis patients displayed a T-cell INFγ response by ELISpot method to proteins extracted from *de novo* Kveim reagent. Furthermore, on analysis of the sarcoidosis group, there was no clinical difference found (e.g. higher SFCs in patients with acute onset of disease) that corresponded with SFC values.

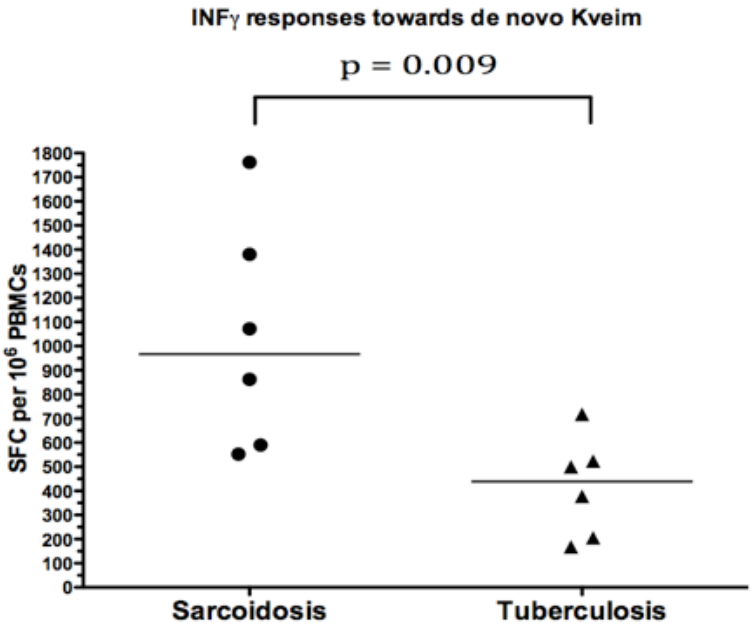


Figure 3.7 Responses towards de novo Kveim reagent show that there is a statistically significant (p = 0.0087, Mann Whitney analysis) difference in the median spot forming cell counts (SFCs) per 10^6 PBMCs of *de novo* Kveim protein extract-specific IFNγ-secreting T-cells in the two groups (sarcoidosis median 966, IQR 582-1476; tuberculosis median 440, IQR 195-573). Dot plots represent mean number of IFNγ-secreting T-cells in antigen well minus mean negative control well per 10^6 PBMC from individual patients.

3.4 Th1 TYPE T-CELL RESPONSES TO FRACTIONATED PROTEINS EXTRACTED FROM *DE NOVO* KVEIM REAGENT IN THE PERIPHERAL BLOOD OF PATIENTS WITH PULMONARY SARCOIDOSIS

Proteins were extracted from 6 *de novo* Kveim reagents and fractionated by pI as detailed in materials and methods section 2.16 and combined together to form one combined pool of extracted sarcoidosis spleen proteins. The same method was applied to 6 control spleens form one combined pool of extracted control spleen proteins. The demographic patient data for the 12 spleens used is detailed in table 3.3.

Table 3.3 Demographic and clinical characteristics of sarcoidosis spleens (n=6) and control spleens (n = 6) used in protein fractionation. All sarcoidosis spleens are from patients with evidence of splenic disease at surgery or autopsy. Key: C= Caucasian, A= Asian (Indian sub-continent), B= Black, M= Male, F= Female. Full clinical information on all spleen samples used for this project is given in appendix 6.

Category	Donor code	Age / Ethnicity / Sex
Control	N4	83 / C / M
Control	N6	70 / C / M
Control	N8	49 / C / F
Control	N11	72 / C / F
Control	N12	53 / C / M
Control	N13	68 / C / F
Sarcoidosis	S1	45 / B / F
Sarcoidosis	S2	48 / C / M
Sarcoidosis	S3	68 / C / M
Sarcoidosis	S4	50 / B / M
Sarcoidosis	S5	75 / C / M
Sarcoidosis	S6	43 / C / F

Eleven of the twelve spleens detailed in table 3.3 were donated for the purposes of this study from the National Disease Research Interchange (NDRI) as detailed in section 2.12. These spleens were removed at post mortem from patients classified as 'normal controls' or from those with sarcoidosis who had biopsy proven evidence of splenic disease. They were ethically donated to the NDRI. They were either frozen directly at -80^{0}C or 'snap frozen' in liquid nitrogen at the post mortem site. Samples were then transorted by air (in dry ice containers) to Imperial College London for the express purposes of use in this project. Samples arrived in individual batches (1 per spleen) of 15-30g at time of shipping and were imemdiatley transferred to -80^{0}C on arrival.

These 11 spleen samples were taken from anonymous donors and hence limited clinical information is available. However, it is known that the 6 patients who donated control spleens for this experiment had a number of different conditions, many of them age related e.g. Alzheimer's disease, history of cardiac bypass surgery, hypertension, hyperlipidaemia. A number of the sarcoidosis patients also had, in addition to biopsy proven sarcoidosis, other co-morbidities (some of which may have been related to their sarcoidosis) including renal

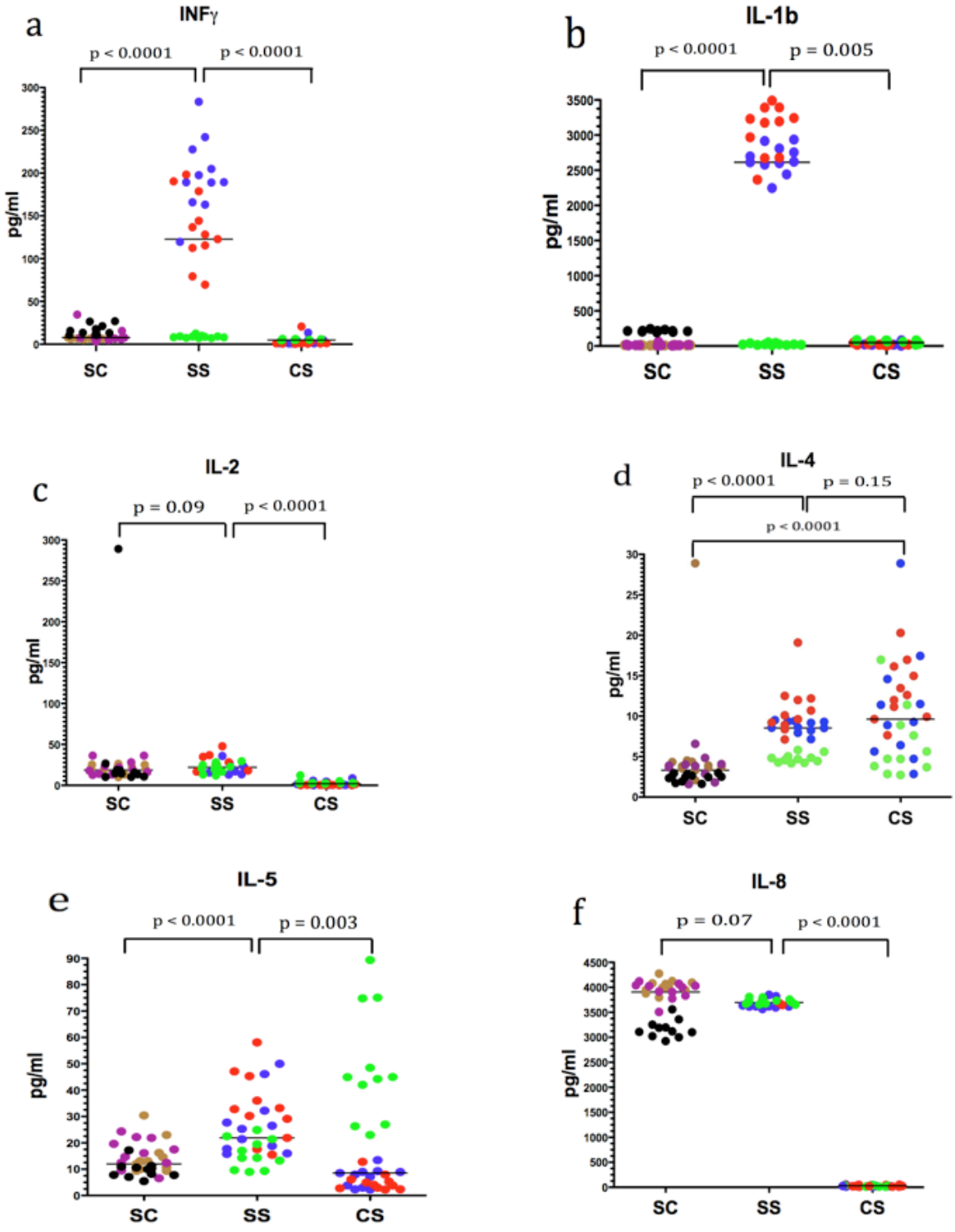
failure and chronic obstructive airways disease. The remaining one spleen samples (S1) was taken from a consenting patient within Imperial College London hospitals. This was a spleen removed fully for purposes of relieving abdomional pressure in a patient with gross spleneomegaly due to sarcoid infiltrates. The spleen was taken fresh from the operation and dissected and frozen at -80^{0}C within 2 hours of harvest. All clinical information where available for all 12 spleens (plus an additional spleen used in Chapter 4 table 4.1) are given fully in Appendix 6. The clinical data in appendix 6 relates to all known clinical information about these samples.

PBMCs from 6 patients (3 with sarcoidosis and 3 controls as detailed with in table 3.4) were then incubated with individual fractions as described in materials and methods section 2.35 and supernatant was taken off and levels of measured by multiplex cytokine assay (2.36). The full clinical information for all 6 patients is given in Appendix 7.

Table 3.4 Demographic and clinical characteristics of sarcoidosis PBMCs (n=3) and control PBMCs (n = 3). Key: C= Caucasian, A= Asian (Indian sub-continent), B= Black, M= Male, F= Female. See Appendix 7 for ful clinical details.

Category	Diagnosis	Donor code	Age/Ethnicity/Sex
Sarcoidosis	Pulmonary Sarcoidosis (new presentation)	SA69	56/A/F
Sarcoidosis	Pulmonary Sarcoidosis (new presentation)	SA92	51/C/M
Sarcoidosis	Pulmonary Sarcoidosis (new presentation)	SA143	41/B/M
Control	Healthy Control	KP	33/C/F
Control	Healthy Control	CE	34/C/M
Control	Pulmonary Hypersensitivity Pneumonitis (new presentation)	H2	29/B/F

The results from this experiment are detailed in figure 3.8 (a-j) which continues on the next page. Prior to this experiment a proof of principle study confirmed that the incubation of acetone cleaned up protein pellets did not adversely affect the cell viability of the incubated PBMCs (see appendix 5). Multiplex assay was also repeated to show that the protein fractions themselves did not contain differences in measureable cytokine level (data not shown).


a
INFγ
p < 0.0001
p < 0.0001
pg/ml
SC
SS
CS
b
IL-1b
p < 0.0001
p = 0.005
pg/ml
SC
SS
CS
c
IL-2
p = 0.09
p < 0.0001
pg/ml
SC
SS
CS
d
IL-4
p < 0.0001
p = 0.15
p < 0.0001
pg/ml
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IL-8
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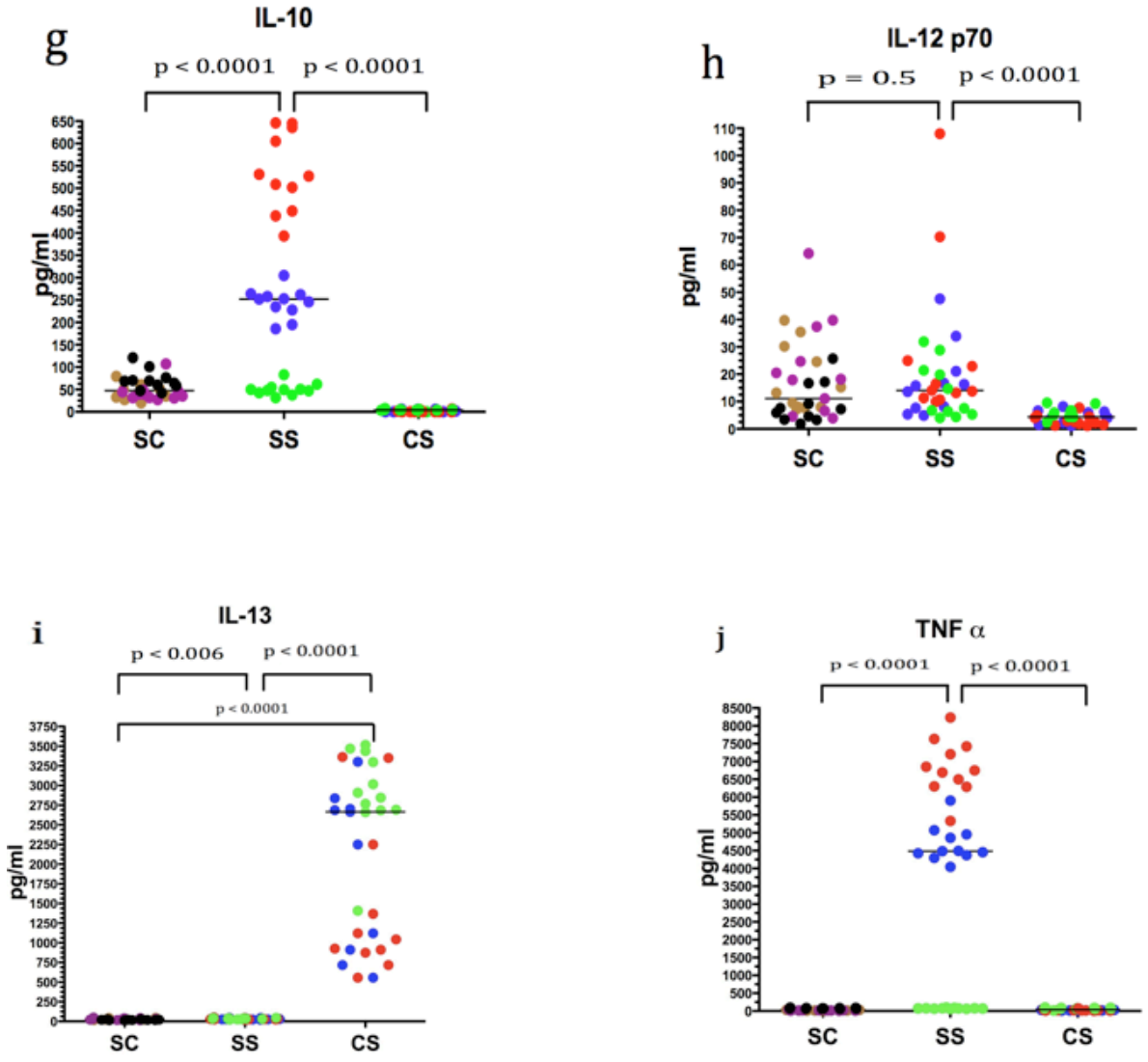


Figure 3.8 Concentration of cytokine measured in supernatant after 20 hr incubation of PBMCs with protein fractions. All measurements made using multiplex cytokine methods (Meso Scale Discovery). Each data point represents one protein fraction incubated with the corresponding PBMCs. Incubation scenarios are as follows: SC= Sarcoidosis spleen fractions + Control PBMCs, SS = Sarcoidosis spleen fractions + Sarcoidosis PBMCs. CS = Control spleen fractions + Sarcoidosis PBMCs. PBMC samples are colour coded as follows: Controls; KP = brown, CE = purple, H2 = black. Sarcoidosis: SA69 = blue, SA92 = red, SA143 = green. A significant difference is found between SC and SS (analysed using Mann-Whitney test for non-parametric data) when comparing median cytokine levels of INFγ, IL-1β, IL-4, IL-5, IL-10, IL-13 and TNFα.

The results from figure 3.8 have been interpreted as each data point (representing a single incubation of PBMCs with one fraction) being a single independent value. This results in 33

data points per scenario (SC, SS or CS) which represents PBMCs from 3 patients in that scenario incubated with 11 fractions of protein per patient. The SS condition is the one of interest in this experiment i.e. sarcoidosis spleen fractions + sarcoidosis PBMCs. The SC and CS conditions are both control for this SS group.

A total of 7 of the measured cytokine responses (INFγ, IL-1β, IL-4, IL-5, IL-10, IL-13 and TNFα) from the supernatant were found to have a significantly elevated median concentration in the SS (sarcoidosis spleen + sarcoidosis PBMCs) condition compared to the SC condition (sarcoidosis spleen + control PBMCs). The details of these differences (plus those of the three cytokines where differences did not reach statistical significance) are detailed in table 3.5.

Table 3.5 Concentration of cytokine measured in supernatant after 20 hr incubation of Peripheral Blood Mononuclear Cells (PBMCs) with protein fractions. All measurements made using multiplex cytokine methods (Meso Scale Discovery). Incubation scenarios are as follows: SC= Sarcoidosis spleen fractions + Control PBMCs, SS = Sarcoidosis spleen fractions + Sarcoidosis PBMCs. CS = Control spleen fractions + Sarcoidosis PBMCs. Analysis by Mann-Whitney testing and bonferroni correction for multiple comparisons (by multiplying each p value by 10).

Cytokine	SC median (IQR) pg/ml	SS median (IQR) pg/ml	CS median (IQR) pg/ml	P value of difference between SC and SS medians
INFγ	7.77 (6.63-14.52)	122.7 (9.16-189.3)	4.90 (1.05-5.38)	<0.0001
IL-1β	15.5 (10.5-207)	2613 (31.75-2953)	47.66 (22.13-75.05)	<0.0001
IL-2	18.7 (13.8-24.5)	22.1 (17.45-26.45)	1.98 (0.59-3.20)	0.09
IL-4	3.32 (2.48-3.95)	8.54 (4.95-9.46)	9.64 (5.179-14.03)	<0.0001
IL-5	12 (9.46-16.7)	21.9 (15.9-32.5)	8.54 (3.83-34.48)	<0.0001
IL-8	3905 (3327-4035)	3697 (3651-3734)	29.72 (16.15-39.51)	0.07
IL-10	47.3 (39.05-66.46)	252 (52.5-475)	3.91 (0.26-4.90)	<0.0001
IL-12 p70	11.1 (6.88-24.65)	14.1 (7.59-22.15)	4.39 (2.35-6.11)	0.5
IL-13	24.4 (19.45-31.6)	31.1 (26.05-40.9)	2666 (985-2963)	0.006
TNFα	26.82 (15.86-67.22)	4483 (78.9-6401)	36.82 (15.86-67.22)	<0.0001

With the exception of IL-8, the median level of all cytokines were higher in the SS condition compared to the SC condition. With the exception of IL-4 and IL-13, median levels were higher in the SS condition than the CS.

The cytokine concentration values exhibited in figure 3.8 and table 3.5 indicate that in some cases, cytokine responses are significantly elevated in the SS condition (which is supernatant taken from sarcoidosis PBMCs incubated with fractionated sarcoidosis spleen) compared to the two control conditions of SC (fractionated sarcoidosis spleen with control PBMCs) and CS (fractionated control spleen with sarcoidosis PBMCs). This data suggests that T-cells from the peripheral blood of sarcoidosis patients are specifically responding to the fractionated sarcoidosis spleen and producing multiple cytokines in response to this recognition; a response which is generally not seen in the two control conditions.

The cytokine response in the SS condition is directed towards a Th1 and inflammatory response as seen with significantly elevated levels of cytokines such as INFγ (SS median 122.7, IQR: 9.16-189.3 ; SC median 7.77, IQR: 6.63-14.52 ; CS median 4.90, IQR: 1.05-5.38) and TNFα (SS median 4483, IQR: 78.9-6401 ; SC median 26.82, IQR: 15.86-67.22 ; CS median 36.82, IQR: 15.86-67.22). The median level of both INFγ (figure 3.7a) and TNFα (figure 3.7j) is significantly higher in the SS condition than either the SC or the CS ($p < 0.0001$ for all scenarios).

The Th2 response seen in the data is less dominant e.g. IL-4 and IL-5 may be significantly higher in the SS conditions than the SC condition (figure 3.8d and 3.8e) but unlike INFγ and TNFα, this median level is only mildly elevated: IL4 (SS median 8.54, IQR: 4.94-9.46 ; SC median 3.32, IQR: 2.48-3.95 ; CS median 9.637, IQR: 5.18-14.03) and IL-5 (SS median 21.9, IQR: 15.9-32.5 ; SC median 12, IQR: 9.46-16.7). In the case of IL-4 the median level in the control condition CS is actually higher (but without significance) than in the condition SS (9.64 vs. 8.54).

IL-13 is a potent Th2 cytokine and although the level in the SS condition is significantly ($p = 0.006$) mildly elevated over the SC condition (SS median 31.1, IQR: 26.05-40.9 ; SC

condition median 24.4, IQR: 19.45-31.6), it is interesting to note that in the CS condition the levels are massively and significantly elevated ($p = <0.0001$ for both for CS vs. SS and CS vs. SC) above both conditions (CS median 2666, IQR 985-2963). In fact the lowest IL-13 concentration found in the CS condition is 556 pg/ml (SA69 with protein fraction 8) which is higher than all data points seen in conditions SS or SC (figure 3.8i).

On detailed analysis of the data it appears that a number of cytokines in the SS scenario are grouped showing two sarcoidosis patients (SA69 and SA92) who respond with high levels of cytokine and one patient who does not (SA143). For example, the median INFγ and TNFα levels are significantly higher in the SC conditions than the SS or CS but within the SS condition the three patients respond differently to the sarcoidosis spleen protein fractions as shown in figure 3.9a and 3.9b (SA69 INFγ median 189.4, IQR: 165.9-227.7 ; SA92 INFγ median 128.1, IQR: 112.6-178.8; SA143 INFγ median 8.565, IQR: 7.39-9.24. SA69 TNFα median 4483, IQR: 4370-4955 ; SA92 median 6751, IQR: 6303-7423, SA143 median 74.1, IQR 66.2-79.4). The data shows that INFγ levels are significantly higher in SA69 than in SA92 but vice versa with regards to levels of TNFα.

This differential cytokine production amongst the three sarcoidosis patients appears mainly limited to the high Th1 and inflammatory responses seen. It is found to some extent in the Th2 responses of IL-4 and IL-5 but the median levels of all Th2 cytokines show less variation between SS and SC conditions (data not shown). In some of the other cytokines measured there is no difference in median levels between the three patients as seen with IL-12p70 and IL-13 shown in figure 3.9c and 3.9d. There were no clinically relevant differences between the 3 sarcoidosis patients i.e. all 3 patients had biopsy proven pulmonary sarcoidosis with no extra-pulmonary involvement. All donated blood samples at time of bronchoscopy, prior any treatment for disease and all had presented to hospital with pulmonary symptoms no longer than 3 weeks prior to blood donation (see appendix 6 for full clinical details).

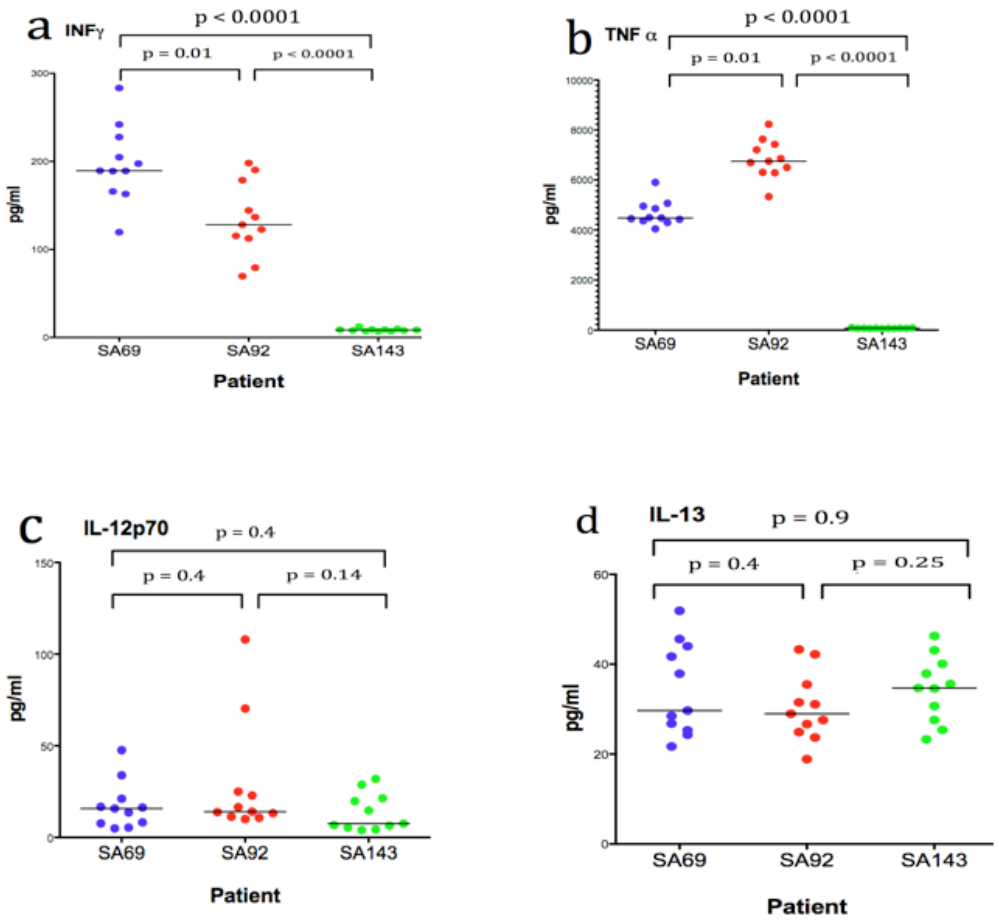


Figure 3.9 Concentration of cytokine measured in supernatant after 20 hour incubation of Peripheral Blood Mononuclear Cells (PBMCs) from three patients with sarcoidosis incubated with protein fractions. All measurements made using multiplex cytokine methods (Meso Scale Discovery). Each data point represents one protein fraction incubated with the corresponding PBMCs. Four cytokines are detailed: (a) IFNγ, (b) TNFα, (c) IL-12p70 and (d) IL-13. Mann Whitney testing used for data analysis.

The summation of all cytokine responses for a particular fraction in scenario SS can be used to see whether a particular fraction evokes a stronger T-cell response than other fractions. This data for INFγ and TNFα is displayed in figures 3.10a and 3.10b. The data indicates that no one single fraction was able to evoke a stronger cellular response than another fraction.

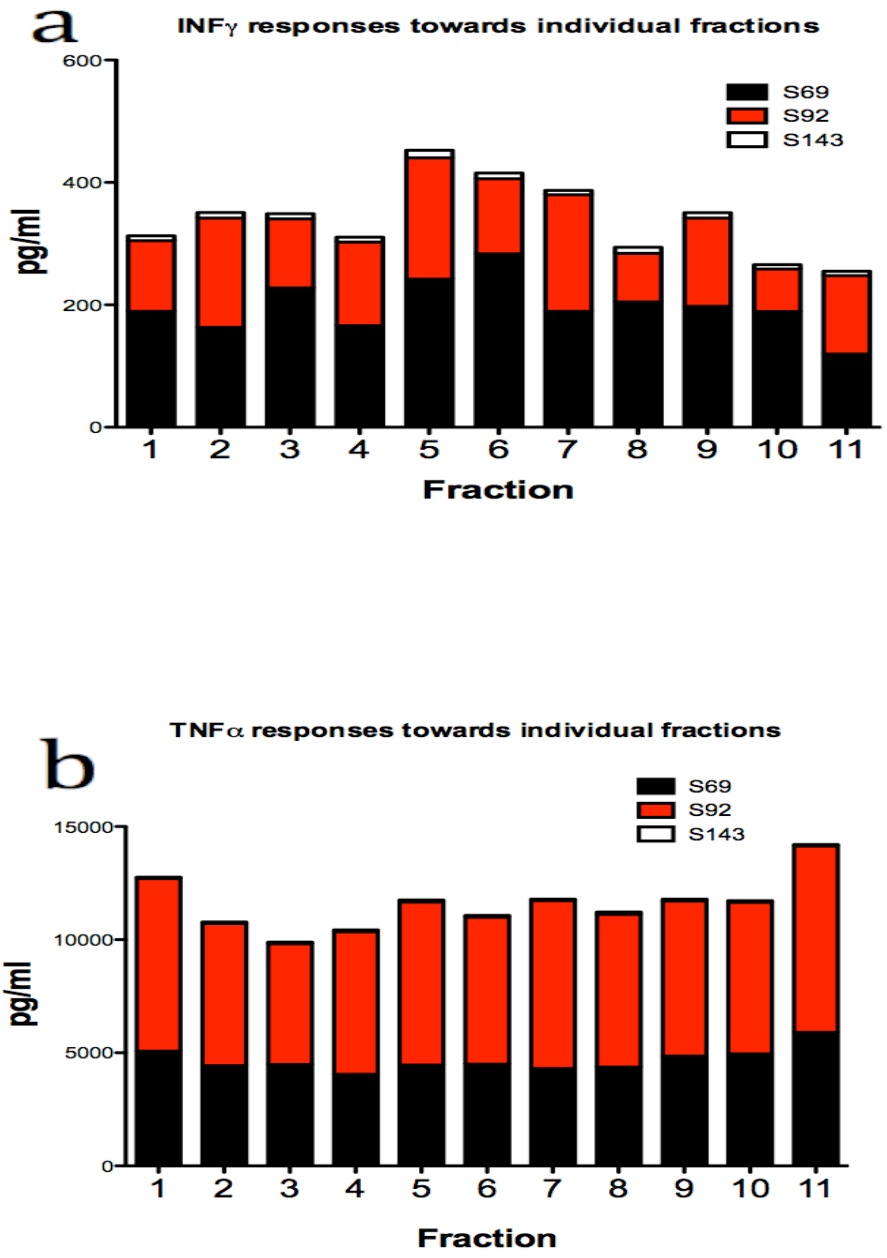


Figure 3.10 Total cytokine concentrations for the three sarcoidosis patients (SS condition) as sub-divided by protein fraction showing total (a) INFγ and total (b) TNFα.

3.5 PROTEIN ELUTION FROM SDS-PAGE IS UNABLE TO RESOLVE PROTEINS WHICH ELICIT PROTEIN-SPECIFIC T-CELL CYTOKINE SECRETION IN SARCOIDOSIS PBMCS

Proteins from a number of 1D and 2D-SDS-PAGE gels from results shown in Chapters 4 and 5 have been fractionated by cutting up the gel pieces into equal sections, eluting the proteins and subjecting them to acetone clean up (as described in materials and methods section 2.33) and then incubating the proteins pools with sarcoidosis and control PBMCs as detailed in section 3.4. These methods did not yield protein pools which capable of inducing specific T-cell cytokine secretion similar to the results seen in section 3.4 (data not shown).

CONCLUSIONS

The data show that patients with a distinct clinical phenotype of pulmonary sarcoidosis who do not have co-existing tuberculosis infection can be selected through clinical risk analysis and IGRA testing (see section 7.1 for full discussion of these results). Interestingly, despite at least 10 patients with sarcoidosis displaying evidence of prior BCG vaccination, an INFγ-secreting T-cell response was only found in 5 patients. A further analysis of these 10 sarcoidosis patients with definite BCG vaccination reveal that they show responses that are significantly lower when compared to healthy BCG vaccinated volunteers. It is recognised that patients with sarcoidosis who are BCG vaccinated show a decreased response to the tuberculin skin test (which uses PPD injected intracutaneously to produce a skin response) used in diagnosis of tuberculosis infection (Hartston, 1964) but the ELISpot data here show for the first time that the reason for this decreased response may be due to a reduction in the number of PPD-specific INFγ-secreting T-cells compared to BCG vaccinated healthy volunteers. Depressed immune response towards skin recall antigens has been investigated

before in sarcoidosis (Mathew et al., 2008) but this is the first data specifically examining the phenomenon of a possible decrease in *ex vivo* T-cell response towards PPD.

The data show for the first time that INFγ- and IL-2-secreting peripheral blood T-cell responses can be measured by ELISpot towards proteins extracted from *in vivo* validated Kveim reagent that has been stored in sub-optimal conditions for long periods of time. The data show that different patients with sarcoidosis respond to different Kveim reagents and that individual reagents themselves have differing abilities to evoke a cytokine-specific T-cell response. It also shows that the level of response (in this limited data set) is not related to the type of sarcoidosis e.g. patients with pulmonary sarcoidosis do not have larger responses than those with extra-pulmonary disease. These findings concur with previous work using Kveim as an *in vivo* skin test for diagnosis of disease (Mitchell et al., 1976).

Finally, the data shows that new Kveim reagent can be created from fresh sarcoidosis spleen and that INFγ responses as measured by ELISpot are higher to *de novo* Kveim reagent than to historic *in vivo* Kveim reagent. The analysis of multiple cytokines from the supernatant of *de novo* Kveim reagent protein fractions incubated with PBMCs from patients with acute pulmonary sarcoidosis shows high levels of the Th1 cytokines INFγ and TNFα whereas levels of the Th2 cytokines IL-4, IL-5 and IL-13 are not significantly raised when compared to the control scenarios investigated. This data showing a biased Th1 response (after incubation) is to be expected and concurs with previous work investigating acute pulmonary sarcoidosis (Moller, 1999) but this is the first time that it has been reported in response to sarcoidosis spleen proteins. The inability to resolve particular antigenic fractions through OFFgel fractionation may indeed provide clues as to the identity of these proteins (see 7.1 for full discussion).

Importantly, the fractionation data (figure 3.8) support the hypothesis that the cytokine response seen in sarcoidosis towards Kveim reagent is specific to Kveim itself, and not due to a general disorder of heightened Th1 cytokine production (as shown by higher Th1 responses in the SS compared to CS conditions). These findings further strengthen the argument towards creation of a protein-based *ex vivo* antigen-specific assay for diagnosis of sarcoidosis, and the INFγ and TNFα responses found here will be key once candidate protein antigens are identified from Kveim reagent and sarcoidosis spleen.

Chapter 4

Identification of Kveim reagent specific proteins through 1D-SDS-PAGE

INTRODUCTION

It is hypothesised that investigation of multiple Kveim reagents for proteins of commonality would lead to the discovery of candidate T-cell antigens responsible for the Kveim reaction itself. Although storage conditions have varied, the majority of reagents are likely to contain the active component as shown by *in vivo* diagnostic testing several years after storage at $+4^{O}C$ (Mitchell et al., 1976). However, whilst this reagent is still able to elicit a specific T-cell response it is likely that candidate antigen(s) have undergone significant proteolytic degradation over time resulting in numerous peptide fragments. Such fragments cannot be easily resolved through standard proteomic methodologies. In order to overcome this limitation, a complementary approach was adopted with the preparation of *de novo* Kveim reagent (from fresh sarcoidosis spleens) to the same exacting methods as validated Kveim reagent (section 2.201).

The main disadvantage with the analysis of fresh sarcoidosis spleens is that not every sarcoidosis spleen was historically suitable for making highly specific Kveim reagents. The newly made *de novo* Kveim reagents cannot be tested for activity in patients with sarcoidosis due to the possibility of transmission of diseases such as new variant CJD. However, the analysis of different sarcoidosis spleens from a number of patients may help overcome these limitations. The production of controls using fresh control spleens (prepared in the same manner as Kveim reagents) can be used as a direct comparison to discover proteins specific to sarcoidosis Kveim reagent only.

1D-SDS-PAGE can be used to accurately and reproducibly separate proteins by molecular weight on polyacrylamide gels. Sodium Dodecyl Sulfate (SDS) is used to denature proteins and combines with them at a constant weight ratio giving them a negative charge. The proteins can then be separated by this level of charge which acts as a substitute for their mass. Once the gels are run, the proteins are visualised with colloidal Coomassie stain, excised and subjected to in-gel trypsin digestion before Mass Spectrometry to identify proteins found in Kveim reagent only, some of which may represent the candidate antigen(s).

RESULTS

4.1 PRECIPITATION OF PROTEINS ALLOWS FOR IDENTIFICATION OF 1D-SDS-PAGE BANDS FROM KVEIM REAGENT

Protein extraction and 1D-SDS-PAGE of Kveim reagent was initially unsuccessful with no protein bands isolated. It was then hypothesised that the phenol (which had been used to preserve the Kveim reagent in a number of cases) may have interfered with the SDS-PAGE process. A number of subsequent trials resulted in the acetone/methanol precipitation (which produced bands from Kveim reagent), examples of which are shown in figures 4.1 and 4.2. Examples of proteins bands visualised through this approach (see materials and methods sections 2.202 & 2.205) are shown circled and MS MASCOT data is given in appendix 6 as evidence of this approach being successful in visualising and identifying proteins from Kveim reagent (denoted with the prefix ‘K’ or ‘S’) that is in some cases more than 10 years old.

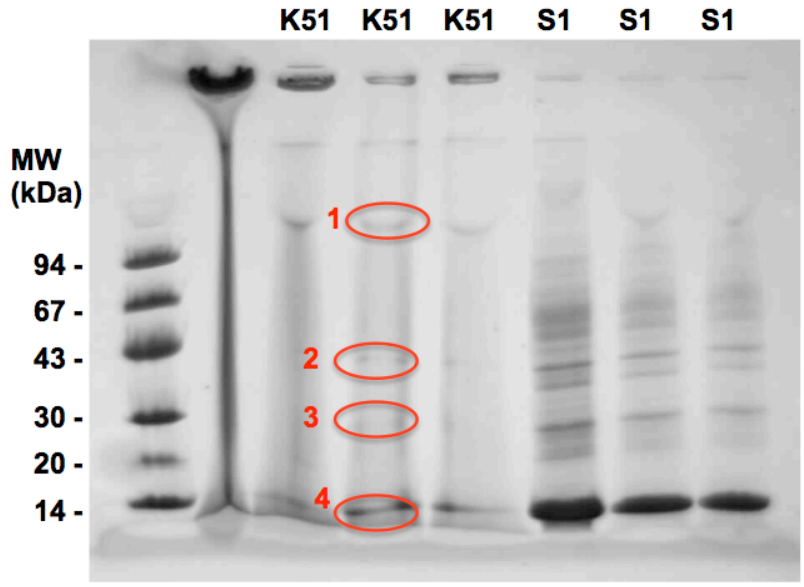


Figure 4.1 1D-SDS-PAGE Phastgel of Kveim reagent (K51) after ammonium acetate / methanol protein precipitation (colloidal Coomassie stain). Circled protein bands were identified through MS (appendix 6).

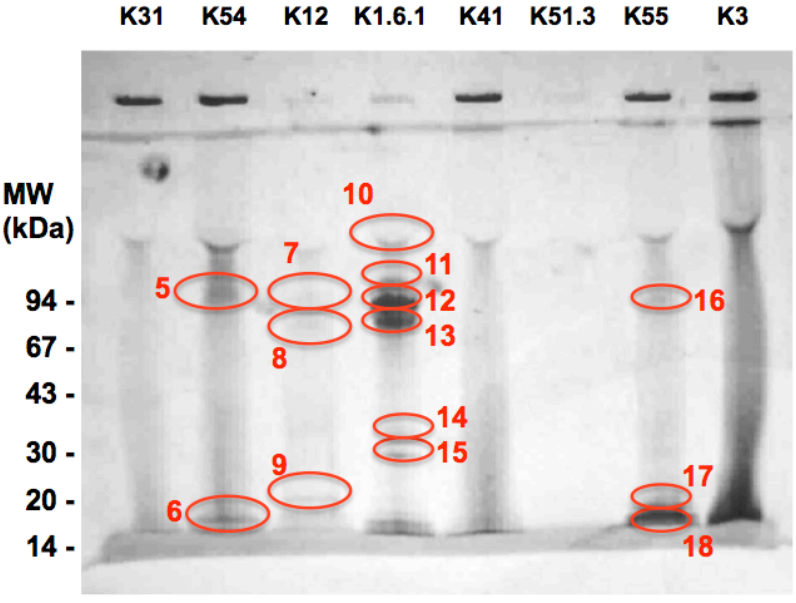


Figure 4.2 1D-SDS-PAGE Phastgel of multiple Kveim reagents after ammonium acetate / methanol protein precipitation (colloidal coomassie stain). Circled protein bands were identified through MS (appendix 8).

4.2 1D-SDS-PAGE AND ESI-TOF-MS IDENTIFIES PROTEINS SPECIFIC TO KVEIM REAGENT

The methods optimised for phastgel analysis were applied to 1D-SDS-PAGE (for more accurate 1D analysis) of validated *in vivo* Kveim (figure 4.3), *de novo* Kveim (figure 4.4) and normal spleens prepared as Kveim reagent (figure 4.5). Full methods including running conditions and protein loading concentrations are detailed in section 2.206. The clinical details for the sarcoidosis spleens to produce figures 4.4 and 4.5 are detailed in table 3.3.and the accompanying text. The same 12 spleen samples were used for this experiment as in chapter 3 with the single change being N13 substituted here for N5. The clinical information for N5 (as well as full clinical information on all spleen samples used for this project) is given in appendix 6.

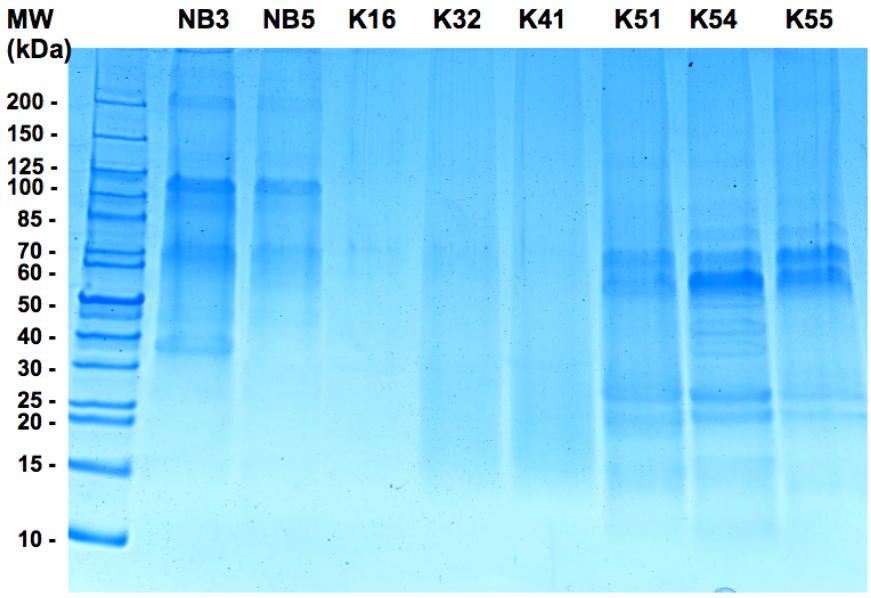


Figure 4.3 1D-SDS-PAGE of validated *in vivo* Kveim reagent. There is no remaining available clinical information for these historic Kveim samples (as discussed in 2.12) other than that they are fully validated Kveim reagents prepared in the UK over a 30 year period ending in 1990.

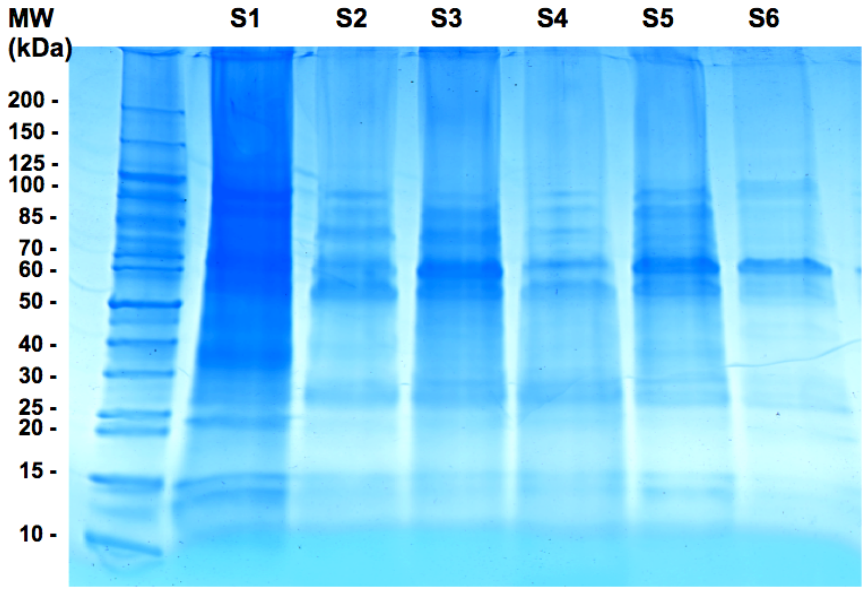


Figure 4.4 1D-SDS-PAGE of *de novo* Kveim reagent

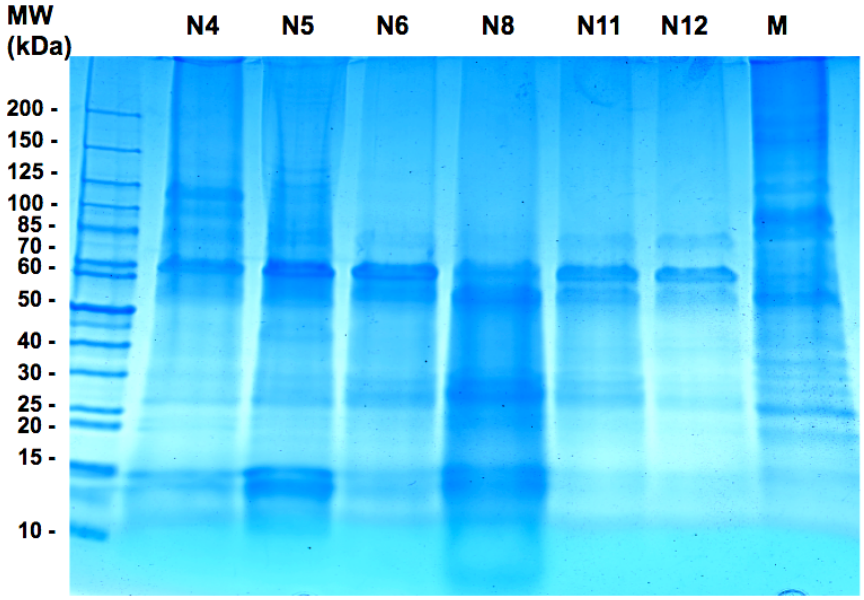


Figure 4.5 1D-SDS-PAGE of normal spleen prepared in the same way as Kveim reagent. M=Massey (an old control spleen validated *in vivo* as non-diagnostic for sarcoidosis with no remaining clinical information regarding the donor spleen.)

Each Kveim reagent was subject to protein precipitation (materials and methods section 2.206), and 2x individual replicates were separated by 1D-SDS-PAGE and stained with colloidal Coomassie to visualise protein bands as shown in shown in figures 4.3-4.5. Gels were sliced into 10 equal pieces and subject to in-gel digestion and MS/MS analysis (materials and methods section 2.215). Data generated were searched and validated (materials and methods section 2.215) A total of 482 proteins were identified, 63 of which were specific to Kveim reagent seen in figures 4.3 & 4.4 (i.e. not found in any of the 7 control spleens prepared in the same manner as Kveim reagent as seen in figure 4.5). Proteins identities are displayed in table 4.1 and full Spectrum Mill data is given in appendix 9.

Table 4.1 Identifications of proteins found specific to validated *in vivo* and newly created *de novo* Kveim reagents (out of a maximum 14 different Kveim reagents investigated) and which were not detected in any of the 7 control spleen tissues prepared in the same manner as Kveim reagent. The gels were run in duplicate but the identification of a protein from a single Kveim reagent was taken as a positive identification for that Kveim reagent. Proteins are identified from sections cut out of each lane which were then summed together for entire lane searches. Where proteins are found in multiple Kveim reagents, the identification with the highest number of peptides is shown. All identifications made with Spectrum Mill (see appendix 9 for full data) and confirmed with MASCOT (data not shown).

Protein	**Identified in n (out of 14) Kveim reagents**	**MS/MS score**	**Peptide matches**	**% Amino acid coverage**
Superoxide dismutase	9	155.76	10	45
Dermcidin	8	40.85	3	25
Serotransferrin	7	157	12	18
Membrane primary amine oxidase	6	95.29	10	10
Beta-actin-like protein 2	6	79.63	5	11
Fibrillin-1	5	171.74	17	25
HLA class II histocompatibility antigen, DRB1	5	42.42	4	12
Neutrophil gelatinase-associated lipocalin	5	91.57	6	28
Alpha-actinin-4	4	106.47	7	8
Catalase	4	148.54	13	25
Vimentin	4	165.09	12	15
Basement membrane-specific heparan sulfate proteoglycan core protein	4	104.81	9	1
Fibronectin	4	44.23	5	2
Histone H2B	4	39.22	3	19
Dipeptidyl peptidase 4	4	59.84	5	5
Neurofilament heavy polypeptide	4	28.24	1	2
Neutrophil elastase	4	45.2	3	9
Peroxiredoxin-1	4	98.99	8	34

Table 4.2 continued

Protein	Identified in n (out of 14) Kveim reagents	MS/MS score	Peptide matches	% Amino acid coverage
Ig lambda chain C regions	3	67.43	4	49
Ig kappa chain C regions	3	62.14	4	59
Thioredoxin-dependent peroxide reductase, mitochondrial OS	3	33.95	3	12
Transforming growth factor-beta	3	35.87	3	4
Complement C3	2	41.36	5	2
Peroxiredoxin-2	2	27.74	2	10
Annexin A2	2	34.87	3	9
Cathepsin G	2	75.49	5	19
Glial fibrillary acidic protein	2	36.94	3	4
Myeloblastin	2	47.08	3	11
Sulfide:quinone oxidoreductase	2	56.94	4	9
Thioredoxin	2	30.79	2	20
Voltage-dependent anion-selective channel protein 1	2	23.43	2	5
78 kDa glucose-regulated protein	2	24.17	2	3
Proteasome subunit alpha type-4	2	21.35	2	6
Alpha-amylase	2	39.01	4	6
Nucleophosmin	2	25.04	2	5
Peroxiredoxin-4	1	42.83	3	7
30S ribosomal protein S2 (Pseudeomnas)	1	25.11	2	13
Annexin A6	1	38.31	3	5
Annexin A5	1	95.41	7	20
Cathelicidin antimicrobial peptide	1	14.09	2	9
Complement C5	1	14.26	2	2
Cytochrome C	1	16.66	2	20
Desmin	1	20.53	2	3
Desmoplakin	1	20.98	2	0
Elongation factor Tu	1	61.92	4	14
EMILIN-1	1	66.31	4	5
Lamin-A/C	1	32.69	3	3
Glutathione reductase	1	27.33	3	5
Transaldolase	1	23.45	3	7
Non-secretory ribonuclease	1	30.15	2	9
Erythrocyte band 7 integral membrane	1	71.44	6	26
Peptidyl-prolyl cis-trans isomerase B	1	34.09	3	13
Proteoglycan-3	1	26.05	2	7
Tenascin	1	19.16	2	0
Nicastrin	1	61.97	6	8
Integrin alpha-M	1	55.15	4	3
Acid ceramidase	1	42.8	3	8
Endoplasmin OS	1	42.05	4	4
Scavenger receptor cysteine-rich type 1 protein M130	1	38.67	3	1
Ceruloplasmin	1	32.63	3	2
Glyceraldehyde-3-phosphate dehydrogenase	1	28	2	8
Beta-glucuronidase	1	24.47	2	1
Cystatin-S	1	32.03	2	13

Proteins were searched against human and all organisms databases but almost all the proteins found were identified as *Homo sapiens* in origin with the exception of two proteins: 30S ribosomal protein S2 (*Pseudomonas*) and Elongation factor Tu (identified as being from either *Pseudomonas, Psychrobacter, Shwanella* or *Acinetobacter*.).

4.3 SUBCELLULAR CHARACTERISATION OF IDENTIFIED PROTEINS

Figure 4.6 details the primary sub-cellular locations of the 63 Kveim reagent specific proteins as determined by Uniprot (Universal Protein Resource, version 2011_05) database search. The majority of proteins identified are either cytoplasmic or secreted. Proteins assigned to the 'other' category include proteins associated with the peroxisome, lysosome and ribosome.

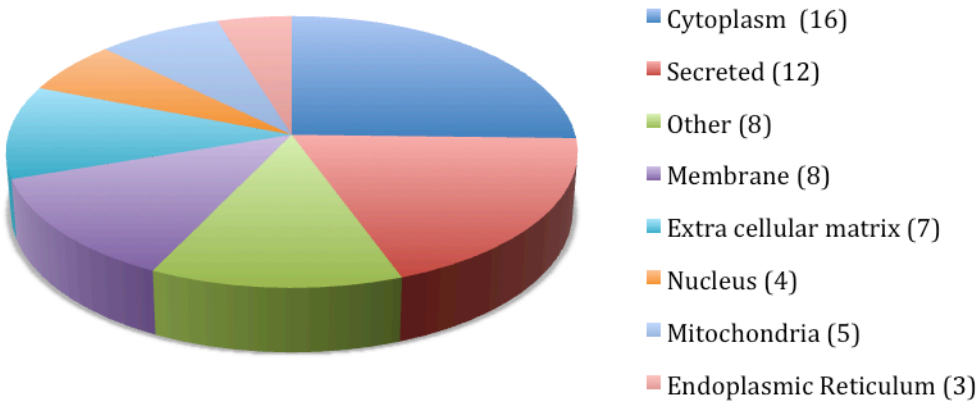


Figure 4.6 Subcellular location of proteins identified as specific to Kveim reagent.

4.4 FUNCTIONAL CLASSIFICATION OF IDENTIFIED PROTEINS

Each protein found specific to a Kveim reagent can fulfil multiple functions as detailed by the Gene Ontology (GO) (AMIGO version 1.8) database but the protein's main biological function as determined by Uniprot database search (version 2011_05) is given in table 4.2.

Table 4.2 Primary biological function of proteins specific to Kveim reagent as determined by Uniprot database searches

Protein	Biological function
Superoxide dismutase	Destroys radicals which are normally produced within the cells and which are toxic to biological systems.
Dermcidin	DCD-1 displays antimicrobial activity thereby limiting skin infection by potential pathogens in the first few hours after bacterial colonization. Highly effective against E.coli, E.faecalis, S.aureus and C.albicans. Optimal pH and salt concentration resemble the conditions in sweat.
Serotransferrin	Transferrins are iron binding transport proteins which can bind two Fe^{3+} ions in association with the binding of an anion, usually bicarbonate. It is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization. Serum transferrin may also have a further role in stimulating cell proliferation.
Membrane primary amine oxidase	Cell adhesion protein that participates in lymphocyte recirculation by mediating the binding of lymphocytes to peripheral lymph node vascular endothelial cells in an L-selectin-independent fashion. Has a monoamine oxidase activity. May play a role in adipogenesis.
Beta-actin-like protein 2	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
Fibrillin-1	Fibrillins are structural components of 10-12 nm extracellular calcium-binding microfibrils, which occur either in association with elastin or in elastin-free bundles. Fibrillin-1-containing microfibrils provide long-term force bearing structural support.
HLA class II histocompatibility antigen, DRB1	Binds peptides derived from antigens that access the endocytic route of antigen presenting cells (APC) and presents them on the cell surface for recognition by the CD4+ T-cells.
Neutrophil gelatinase-associated lipocalin	Iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development.
Alpha-actinin-4	F-actin cross-linking protein which is thought to anchor actin to a variety of intracellular structures.
Catalase	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide.
Vimentin	Vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells.
Basement membrane-specific heparan sulfate proteoglycan core protein	Integral component of basement membranes.
Fibronectin	Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin.
Histone H2B	Core component of nucleosome.
Dipeptidyl peptidase 4	Cell surface glycoprotein receptor involved in the costimulatory signal essential for T-cell receptor (TCR)-mediated T-cell activation.
Neurofilament heavy polypeptide	Neurofilaments usually contain three intermediate filament proteins: L, M, and H which are involved in the maintenance of neuronal caliber.
Neutrophil elastase	Modifies the functions of natural killer cells, monocytes and granulocytes.
Peroxiredoxin-1	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system
Ig lambda chain C regions	Complement activiation and innate immunity.
Ig kappa chain C regions	Complement activiation and innate immunity.

Table 4.2 continued

Protein	Biological function
Thioredoxin-dependent peroxide reductase, mitochondrial OS	Involved in redox regulation of the cell. Protects radical-sensitive enzymes from oxidative damage by a radical-generating system.
Complement C3	Plays a central role in the activation of the complement system.
Peroxiredoxin-2	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system
Annexin A2	Calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids.
Cathepsin G	Serine protease with trypsin- and chymotrypsin-like specificity. Cleaves complement C3. Has antibacterial activity against the Gram-negative bacterium *P. aeruginosa.*
Glial fibrillary acidic protein	Cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other cells.
Myeloblastin	Polymorphonuclear leukocyte serine protease that degrades elastin, fibronectin, laminin, vitronectin, and collagen types I, III, and IV (in vitro) and causes emphysema when administered by tracheal insufflation to hamsters.
Sulfide:quinone oxidoreductase	Catalyses the oxidation of hydrogen sulfide, with the help of a quinone.
Thioredoxin	Participates in various redox reactions through the reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions.
Voltage-dependent anion-selective channel protein 1	Forms a channel through the mitochondrial outer membrane and also the plasma membrane.
Transforming growth factor-beta	TGF-beta 2 has suppressive effects on interleukin-2 dependent T-cell growth.
78 kDa glucose-regulated protein	Probably plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
Proteasome subunit alpha type-4	Host virus interaction. The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH.
Alpha-amylase	Carbohydrate metabolism.
Nucleophosmin	Host virus interaction. involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors p53/TP53 and ARF.
Peroxiredoxin-4	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system
30S ribosomal protein S2	Spans the head-body hinge region of the 30S subunit and is loosely associated with the 30S subunit.
Annexin A6	Calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids.
Annexin A5	Calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids.
Cathelicidin antimicrobial peptide	Binds to bacterial lipopolysaccharides (LPS), has antibacterial activity.
Complement C5	Plays a central role in the activation of the complement system.
Cytochrome C	This is the heme-containing component of the cytochrome b-c1 complex, which accepts electrons from Rieske protein and transfers electrons to cytochrome c in the mitochondrial respiratory chain.

Table 4.2 continued

Protein	Biological function
Desmin	Desmin are class-III intermediate filaments found in muscle cells. In adult striated muscle they form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z-line structures.
Desmoplakin	Major high molecular weight protein of desmosomes. Involved in the organization of the desmosomal cadherin-plakoglobin complexes into discrete plasma membrane domains and in the anchoring of intermediate filaments to the desmosomes.
Elongation factor Tu	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
EMILIN-1	May be responsible for anchoring smooth muscle cells to elastic fibers, and may be involved not only in the formation of the elastic fiber, but also in the processes that regulate vessel assembly. Has cell adhesive capacity.
Lamin-A/C	Structural molecule activity.
Glutathione reductase	Maintains high levels of reduced glutathione in the cytosol.
Transaldolase	Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway.
Non-secretory ribonuclease	This is a non-secretory ribonuclease. It is a pyrimidine specific nuclease with a slight preference for U. Cytotoxin and helminthotoxin.
Erythrocyte band 7 integral membrane	Thought to regulate cation conductance. May regulate ACCN1 and ACCN3 gating.
Peptidyl-prolyl cis-trans isomerase B	PPIases accelerate the folding of proteins. It catalyses the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.
Proteoglycan-3	Possesses similar cytotoxic and cytostimulatory activities to PRG2/MBP. In vitro, stimulates neutrophil superoxide production and IL8 release, and histamine and leukotriene C4 release from basophils.
Tenascin	Extracellular matrix protein implicated in guidance of migrating neurons as well as axons during development
Nicastrin	Essential subunit of the gamma-secretase complex, an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (beta-amyloid precursor protein).
Integrin alpha-M	Integrin alpha-M/beta-2 is implicated in various adhesive interactions of monocytes, macrophages and granulocytes as well as in mediating the uptake of complement-coated particles.
Acid ceramidase	Hydrolyses the sphingolipid ceramide into sphingosine and free fatty acid.
Endoplasmin	Molecular chaperone that functions in the processing and transport of secreted proteins. Functions in endoplasmic reticulum associated degradation.
Scavenger receptor cysteine-rich type 1 protein M130	Molecular chaperone that functions in the processing and transport of secreted proteins.
Ceruloplasmin	Ceruloplasmin is a blue, copper-binding (6-7 atoms per molecule) glycoprotein.
Glyceraldehyde-3-phosphate dehydrogenase	Has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities, thereby playing a role in glycolysis and nuclear functions, respectively.
Beta-glucuronidase	Plays an important role in the degradation of dermatan and keratan sulfates.
Cystatin-S	This protein strongly inhibits papain and ficin, partially inhibits stem bromelain and bovine cathepsin C, but does not inhibit porcine cathepsin B.

The main functions of the 63 proteins found specific to Kveim reagent can also be classified as shown in figure 4.7 which shows the results as classified through GO and Kyoto Encyclopaedia of Genes and Genomes (KEGG) (version May 16, 2011) database searches. The largest group are proteins involved in immunity which can be subdivided into adaptive immunity (e.g. Dipeptidyl peptidase 4 which is a glycoprotein receptor involved in T-cell activiation) and innate immunity (e.g. Complement C5 which plays a major role in the activation of the complement system).

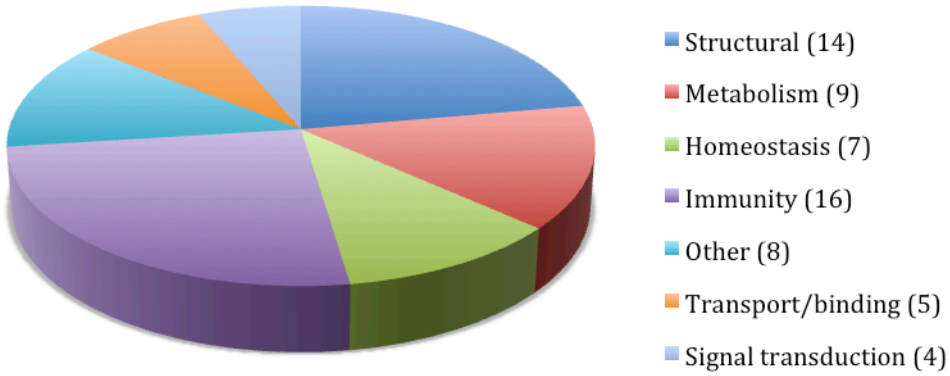


Figure 4.7 Functional classification (according to Gene Ontology and KEGG database searches) of proteins specific to Kveim reagent.

4.5 PATHWAY ANALYSIS AND PROTEIN-PROTEIN INTERACTIONS OF IDENTIFIED PROTEINS

A functional analysis of the identified proteins found through pathway analysis using the KEGG database reveals a number of pathways associated with proteins found specifically in Kveim reagent (table 4.3). As an example, the similarities between the proteins found specific to Kveim reagent and those known to be associated with SLE pathways is shown in figure 4.8. The proteins found specific to Kveim reagent can also be investigated for known and predicted protein-protein interactions using the STRING database (version 8.3) as shown in figure 4.9.

Table 4.3 KEGG database analysis of protein-protein interaction pathways associated with proteins found only in Kveim reagent

Pathway	n proteins involved	Protein(s) involved
Metabolic pathways	7	glyceraldehyde-3-phosphate dehydrogenase , cytochrome c , catalase , transaldolase , alpha-amylase, beta-glucuronidase , acid ceramidase
Systemic lupus erythematosus	4	cathepsin G , complement component 3, complement component 5, histone H2B
Lysosome	3	beta-glucuronidase , cathepsin G, acid ceramidase
Microbial metabolism in diverse environments	3	glyceraldehyde-3-phosphate dehydrogenase , catalase , transaldolase
Tuberculosis	2	complement component 5, cathelicidin antimicrobial peptide
Starch and sucrose metabolism	2	alpha-amylase , beta-glucuronidase
Complement cascades	2	complement component 3 complement component 5
Focal adhesion	2	fibronectin tenascin
ECM-receptor interaction	2	fibronectin tenascin
Oxidative phosphorylation	1	cytochrome c
Regulation of actin cytoskeleton	1	fibronectin 1
Tryptophan metabolism	1	catalase
Pentose and glucuronate interconversions	1	beta-glucuronidase
Pathways in cancer	1	Fibronectin
Pentose phosphate pathway	1	transaldolase
Bacterial invasion of epithelial cells	1	fibronectin
Drug metabolism - other enzymes	1	beta-glucuronidase

Table 4.4 continued

Pathway	n proteins involved	Protein(s) involved
Biosynthesis of secondary metabolites	1	transaldolase
Notch signaling pathway	1	NCSTN; nicastrin
Glutathione metabolism	1	glutathione reductase
Glycolysis / Gluconeogenesis	1	glyceraldehyde-3-phosphate dehydrogenase
Sphingolipid metabolism	1	acid ceramidase
Renin-angiotensin system	1	cathepsin G
Phagosome	1	complement component 3
Carbohydrate digestion and absorption	1	alpha-amylase
Methane metabolism	1	catalase
Proteasome	1	proteasome subunit alpha
Glycosaminoglycan degradation	1	beta-glucuronidase
Neuroactive ligand-receptor interaction	1	cathepsin G

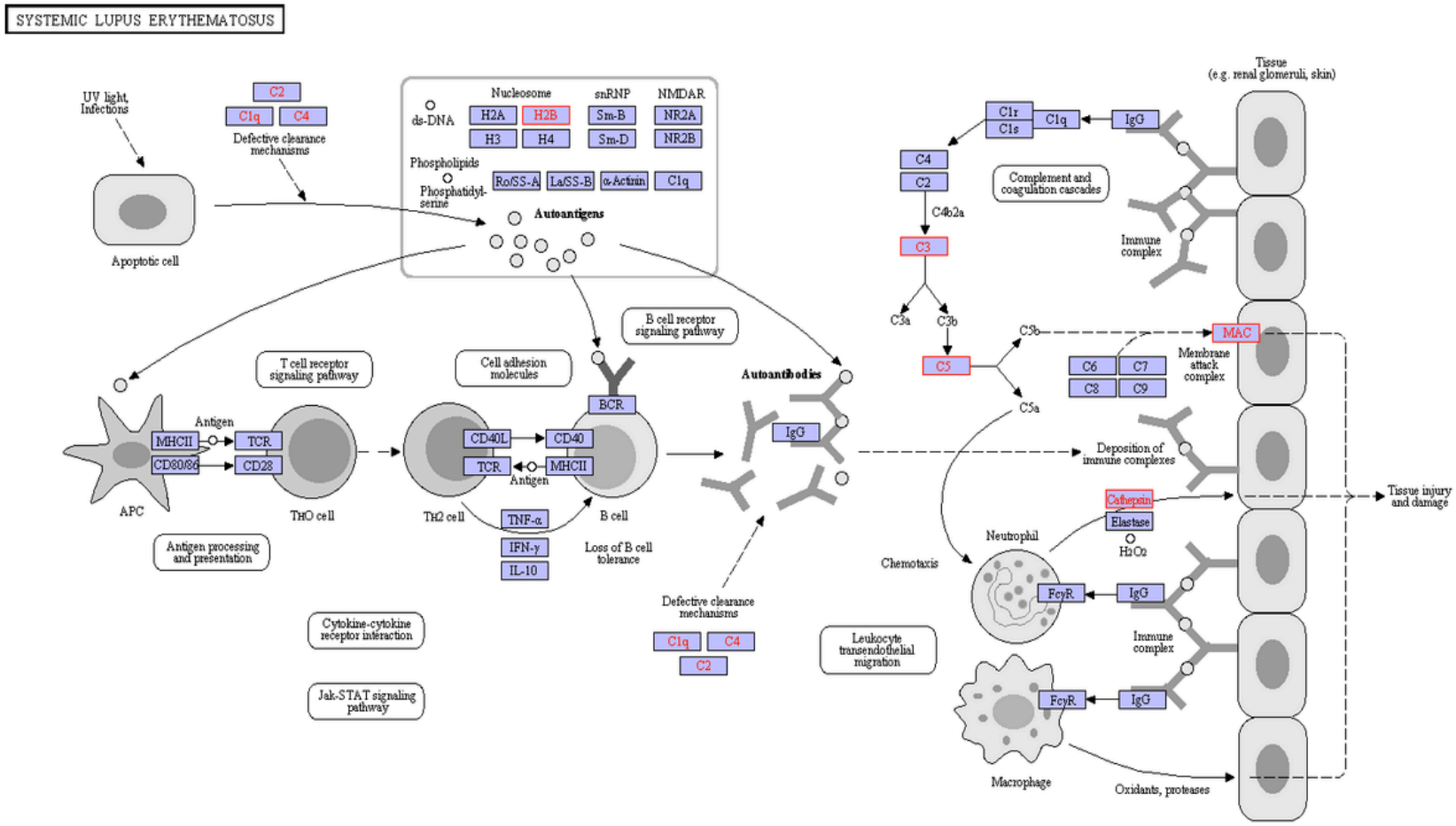


Figure 4.8 KEGG pathway of proteins known associated with Systemic Lupus Erythematosus. *The 4 Kveim reagent specific proteins found in this pathway (and detailed in table 4.3) are shown highlighted in red* (cathepsin G, complement component 3, complement component 5 and histone H2B). MAC= membrane attack complex (formed by Complement C5).

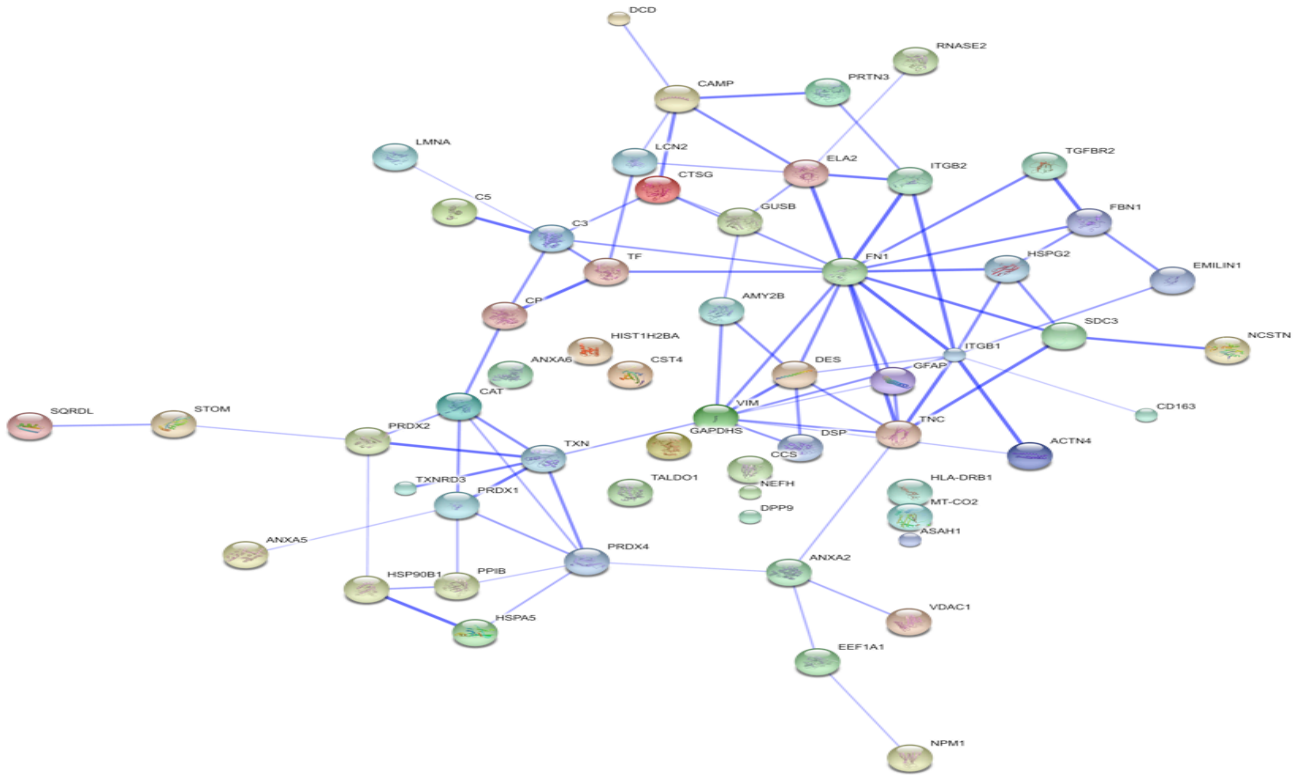


Figure 4.9 Known and predicted protein-protein interactions of all proteins found specific to Kveim reagent as investigated with STRING database (version 8.3, search carried out on 18/05/2011). Interactions are shown in the evidence view mode where thicker lines represent a greater level of evidence for a particular interaction (e.g. biochemical data, co-expression or concurrence amongst genomes). Proteins are named by the recommended term from the Uniprot database (version 2011_05)

CONCLUSIONS

This data represents the first comprehensive analysis of Kveim reagent by any proteomic method. 1D-SDS-PAGE has been used to identify proteins from 8 *in vivo* validated Kveim reagents and 6 *de novo* Kveim reagents prepared from fresh sarcoidosis spleens. A total of 63 proteins specific to these reagents have been identified i.e. not detected in any of 7 control spleens prepared to the exact specifications as Kveim reagent (Chase, 1961). A number of these 63 proteins are found in both old and new reagents e.g. superoxide dismutase was identified in 6 out of 8 *in vivo* validated and 3 out of 6 *de novo* Kveim reagents. The fact that many of the proteins were identified in the *in vivo* validated Kveim reagent suggests that the proteins described here are relatively stable during their long term storage at $4^{O}C$ and are relatively resistant to any residual protease activity (see section 7.2 for full discussion and implications of this finding).

A number of *in silico* classification methods have been adopted to investigate protein/pathway associations and protein-protein interactions specific to Kveim reagent proteins. It is perhaps not surprising that the top associated pathway (involving 7 of the identified proteins) by KEGG database search is that of 'metabolic pathways' as it assumed that general metabolism is increased in the diseased sarcoidosis state compared to the controls. A number of other associated pathways warrant further investigation e.g. proteins associated with Systemic Lupus Erythematosus (involving 4 proteins), microbial metabolism in diverse environments (3 proteins) and tuberculosis (2 proteins). The pathways and associations found here through KEGG database searches and STRING pathway analysis do not necessarily represent those simply found in the diseased state when compared to healthy tissue. This is as the physiochemical stresses involved with conversion of sarcoidosis tissue into Kveim reagent will have removed a number of viable identifiable proteins that were present before the conversion. However, several of these pathways are similar to those found

through the quantitative proteomics approach of unprocessed sarcoidosis spleen tissue when compared to controls (as detailed in chapter 5). For this reason, they are detailed here as being of valid interest. Further analysis of these pathways may provide avenues of research into sarcoidosis aetiology.

It is interesting that despite the use of human and non-human database searches, only 2 proteins were identified as being of microbial origin and both were found in the same Kveim reagent, K54. There is little clinical information as to the exact source of K54 but there is nothing to suggest that it is different in any particular way to the other Kveim reagents investigated. It is difficult to conclude as to whether these 2 proteins (both possibly from a *Pseudomonas* species) are involved in sarcoidosis pathogenesis. However, no evidence was found to indicate a mycobacterial involvement as has been previously hypothesised by other groups with mycobacterial proteins such as mKatG. If one or more proteins thus far identified are responsible for the Kveim reaction, it is possible that the reaction is due to the altered immune response towards a host protein rather than to a pathogen. If this is the case, it is important that sarcoidosis spleen tissue be additionally investigated for proteins that are found to show increased abundance in disease, rather than simply identifying proteins which are absent in controls. This is made possible through the approach of quantitative comparative proteomic analysis as detailed in the next chapter.

Chapter 5

Identification of proteins of differential abundance between sarcoidosis and control spleens

INTRODUCTION

Proteins identified from sarcoidosis spleen which may represent potential T-cell antigenic targets are likely to come from one of two categories. The first category contains those which are related to a potential pathogen(s) which may cause sarcoidosis. These proteins will be found only in sarcoidosis spleens and 1D-SDS-PAGE identifications of proteins in sarcoidosis tissue, as in chapter 4, will identify these proteins. Such non-host derived proteins are likely to be relatively straightforward to identify allowing simple proteomic techniques to be employed to differentiate diseased spleen from disease free spleen. The second category represents host proteins which may cause an altered and abnormal persistent T-cell response in patients with sarcoidosis that is not seen in those without the disease (Iannuzzi et al., 2007). Such proteins will be found in all spleens but may be of higher abundance in sarcoidosis. To identify proteins from this second group, a proteomics method is needed to directly quantify differences in the same proteins extracted from both sarcoidosis and healthy control spleens.

Gel based proteomics (two dimensional polyacrylamide gel electrophoresis, 2D-PAGE) has become a popular method of choice for comparative proteomics since first described more than 30 years ago (O'Farrell, 1975). This powerful analytical methodology involves the use of polyacrylamide gels to resolve proteins as spots which are initially separated by charge (as determined by position at which each protein resides in a neutral state i.e. the isoelectric point or pI) in the first dimension and by mass in the second dimension. The technique is well established and advances in immobilised pI strip technology allow for accurate running

conditions (Gorg et al., 1988). However, a direct comparison between two or more samples involves technical challenges and inaccuracies when using separate gels. Despite using identical voltage, current and time settings, protein extracts run on different gels will experience subtle differences in running conditions and hence proteins identical to two samples may not be resolved as spots in the same place on two different gels. In addition, reliable quantification of differences in protein expression is difficult when using SDS-PAGE only.

The development of two-dimensional difference in gel electrophoresis (2D-DIGE) using positively charged, amine reactive and molecular weight matched fluorescent cyanine dyes (Cy2, Cy3 and Cy5 which attach to 1-2% of lysine residues via amide linkages) has significantly improved accuracy and led to a more precise quantitation over a wider dynamic range. It allows for accurate quantification of proteins and can therefore demonstrate changes in protein abundance (Unlu et al., 1997). These dyes are also are sensitive than colloidal Coomassie or silver staining with detection limits of 2-10ng over 10-500ng dynamic range (Schrattenholz and Groebe, 2007). This increased dynamic detection range has increased the sensitivity of the technique with the added advantages of reduced inter-gel variability, reduction in the number of gels required, accurate spot matching and compatibility with the identification of protein spots using mass spectrometry. (Gygi et al., 1999, Unlu et al., 1997, Marouga et al., 2005b, Tonge et al., 2001).

2D-DIGE experiments require careful and robust reproducible design to ensure that statistically meaningful data can be obtained. The use of an internal standard permits high quality analysis and more precise quantitation. Labelling a pooled internal standard (a pool composed of equal aliquots of each sample used in the experiment) with Cy2 dye, while Cy3 and Cy5 are used to label disease and control samples, is the most popular experimental design in current use. Statistical analysis can be further improved by increasing the number of gels per experiment and performing biological replicates (with a minimum needed of three

per sample group). Differential expression is then calculated as the average fold-change (the average spot intensity ratio between differentially labelled spots matching across all gels) with statistical confidence provided by anova testing.

To date only one report on the use of 2D-DIGE in sarcoidosis research has been published. The technique was used to compare BAL fluid from patients with sarcoidosis (n=5) and chronic beryllium disease (n=5) to healthy controls (n=4). From a total 37 statistically valid differentially expressed spots 14 proteins were identified, of which peroxiredoxin 5, heat shock protein 70, complement C3, annexin A2 and transthyretin were measured in the sarcoidosis vs. controls groups (Silva et al., 2007). While these differentially expressed proteins may represent interesting biomarkers in the lungs, they do not advance the search for the candidate antigen(s) within sarcoidosis spleen tissue. In fact, there are no previously published data investigating any sarcoidosis tissue using any comparative proteomics method.

RESULTS

5.1 OPTIMISATION OF METHODOLOGY

A number of different aspects methodology needed extensive optimisation prior to producing a final set of clear high quality preparative 2D-SDS-PAGE gels for spot picking and subsequent protein identification. This optimisation included determining the most suitable method for protein extraction from spleen tissues into DIGE lysis buffer (use of a pre-frozen hand mortar and pestle), decision not to use additional destreak reagent (GE Healthcare) as this did not improve spot quality, decision to use cup-loading rather than in strip rehydration of proteins with immobiline strips (cup-loading produced clearer spot patterns) and the optimisation of focusing times for the first dimension of the gel electrophoresis. The final

protocol is detailed in materials and methods sections 2.207 & 2.208 but some of these steps are detailed in table 5.1 with the corresponding gel images shown in Appendix 8.

Table 5.1 Summary of some sample steps for optimisation of 2D-DIGE (gels shown in appendix 10)

Gel	Sample preparation	Comment	Number of detectable spots
8.1	Sample grinding and protein extraction only (coomassie stain)	some smearing	<100 spots
8.2	Detergent R3 (Biorad), sample grinding and protein extraction (coomassie stain)	some smearing	<200 spots
8.3	Protein extraction + 2d clean up kit (coomassie stain)	some smearing	>300 spots
8.4	Protein extraction + 2d clean up kit + destreak reagent into DIGE lysis buffer (typhoon laser image)	clearly defined	>600 spots
8.5	Protein extraction into DIGE lysis buffer (typhoon laser image)	clearly defined	>600 spots

5.2 DIGE WORK FLOW

Proteins extracted from 6 sarcoidosis spleens were directly compared with those from 6 histologically normal control spleens. The demographic and clinical characteristics of all spleens used are the same as detailed in table 3.3. In order to achieve the best resolution for subsequent quantitative analysis, methods were optimised as previously detailed and 6 DIGE gels were run with 24 cm immobiline strips at pI range of 3-7 and 6 gels run at a range of 7-11. For each run of 6 gels, the sarcoidosis and control samples were split with 3 from each group labelled with Cy3 dye and the other 3 from each group with Cy5 to exclude the bias towards a particular fluorescent marker. The 2D-DIGE experimental work flow is detailed in figure 5.1 Samples were assigned to gels as detailed in table 5.2.

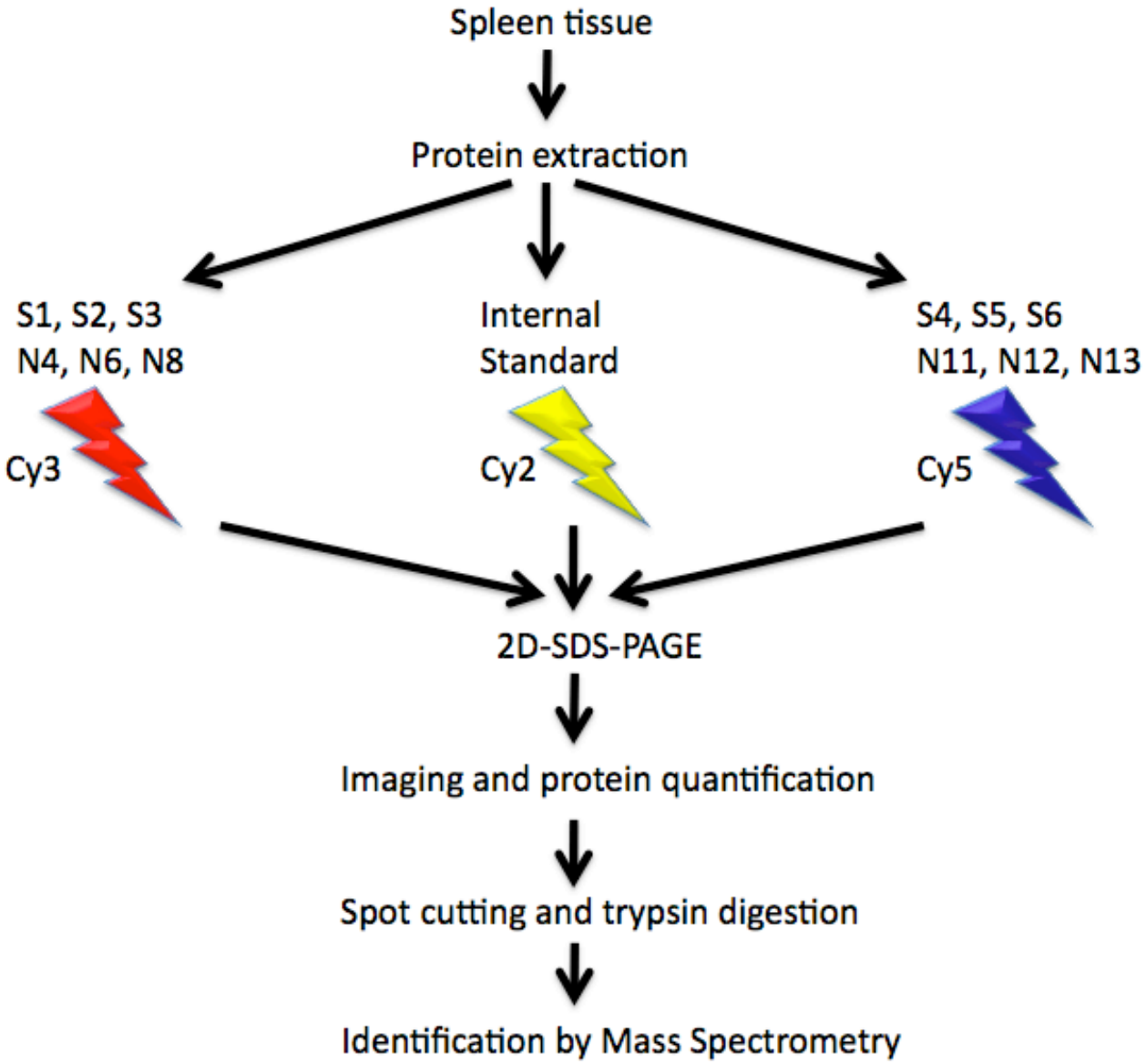


Figure 5.1 Representation of work flow for 2D-DIGE. The final experiment was repeated at two pI ranges (3-7 and 7-11). Sarcoidosis and control spleen protein extracts were labelled with Cy3 or Cy5 dye. Cy2 labelling was reserved for the internal standard. Samples were combined for the 2D-SDS-PAGE step and each gel contained an internal standard, sarcoidosis spleen protein extract and control spleen extract.

Table 5.2 Layout of 12 gels used in the final 2D-DIGE experiment. Each gel contained 50 µg of sarcoidosis spleen (S) protein extract and 50 µg of control spleen (N) protein extract. Each 50 µg protein was labelled with 400 pM of Cy dye. The dyes were switched between samples as shown in the table to avoid bias towards one type of fluorescence. Each gel contained an additional equivalent amount of internal standard (a combination of all 12 spleen protein extracts) labelled with Cy2 dye.

Gel	pI	Cy3 labelled sample	Cy5 labelled sample	Cy2 labelled sample
1	3-7	S1	N4	Pooled internal standard
2	3-7	S2	N6	Pooled internal standard
3	3-7	S3	N8	Pooled internal standard
4	3-7	N11	S4	Pooled internal standard
5	3-7	N12	S5	Pooled internal standard
6	3-7	N13	S6	Pooled internal standard
7	7-11	S1	N4	Pooled internal standard
8	7-11	S2	N6	Pooled internal standard
9	7-11	S3	N8	Pooled internal standard
10	7-11	N11	S4	Pooled internal standard
11	7-11	N12	S5	Pooled internal standard
12	7-11	N13	S6	Pooled internal standard

5.3 2-D GEL SEPARATION OF PROTEINS

The images produced by laser scanning each contained 900-1200 protein spots per acidic (pI 3-7) and 250-400 protein spots per alkaline (pI 7-11) gel. Figure 5.2 a and b show the Cy2 labelled reference gels used at the 2 different pI gradients. Figure 5.3 shows an example of (a) Cy3 labelled gel containing sarcoidosis spleen proteins, (b) Cy5 labelled gel containing control spleen proteins and (c) the overlay image of both gels plus the Cy2 labelled internal standard all in the pI range 3-7. Differences in spot volume representing potential differentially expressed proteins are shown in 5.3 d. Figure 5.4 shows the same results as figure 5.3 but run on a pI range 7-11.

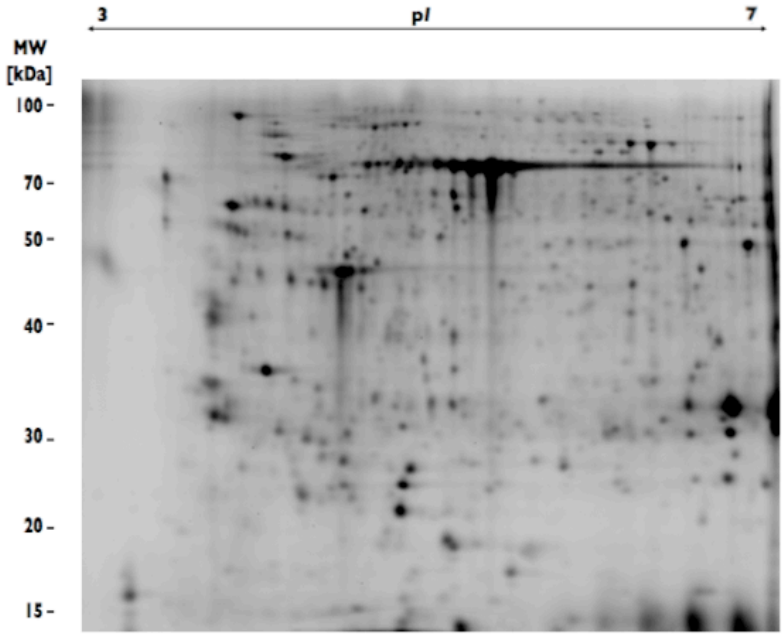


Figure 5.2a Cy2 Reference gel at pI 3-7

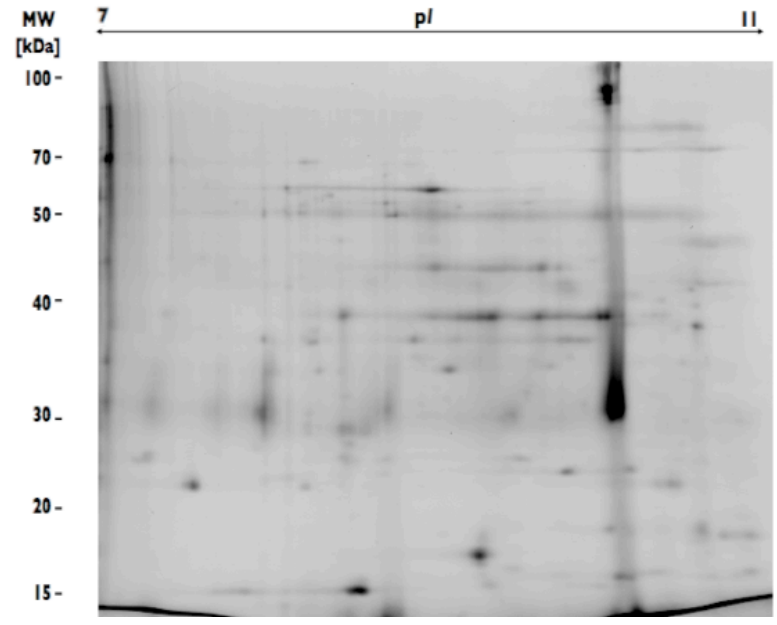


Figure 5.2b Cy2 Reference gel at pI 7-11

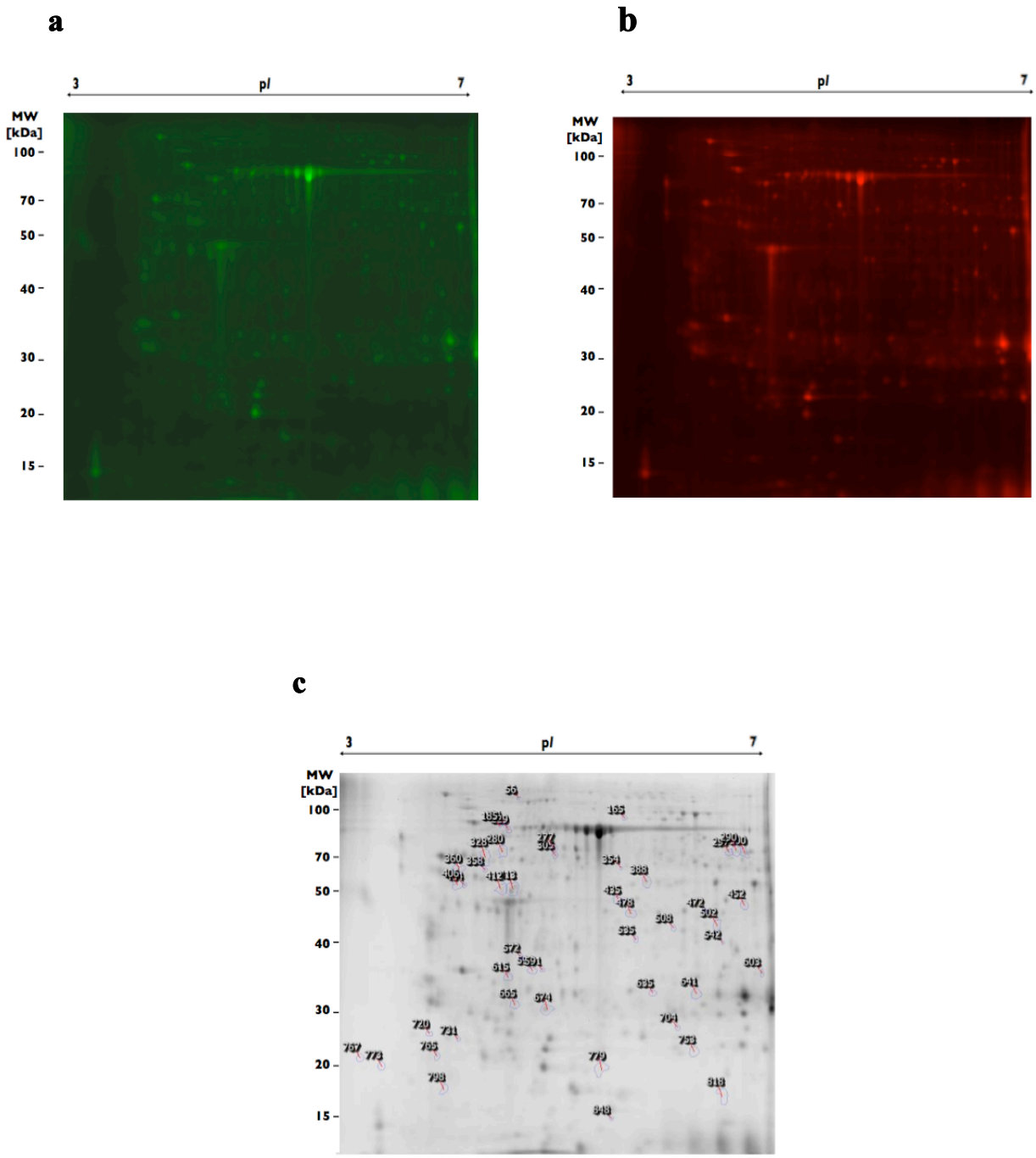


Figure 5.3 An example of Cy3 labelled sarcoidosis proteins are shown in figure (a) with Cy5 labelled control proteins in figure (b). All gels were run at a pI of 3-7 on 12.5% SDS-PAGE gels. Figure (c) represents 48 proteins spots of interest which were subsequently excised from the preparative gel (with 450 µg protein loaded in total), trypsin digested and analysed by Mass Spectrometry.

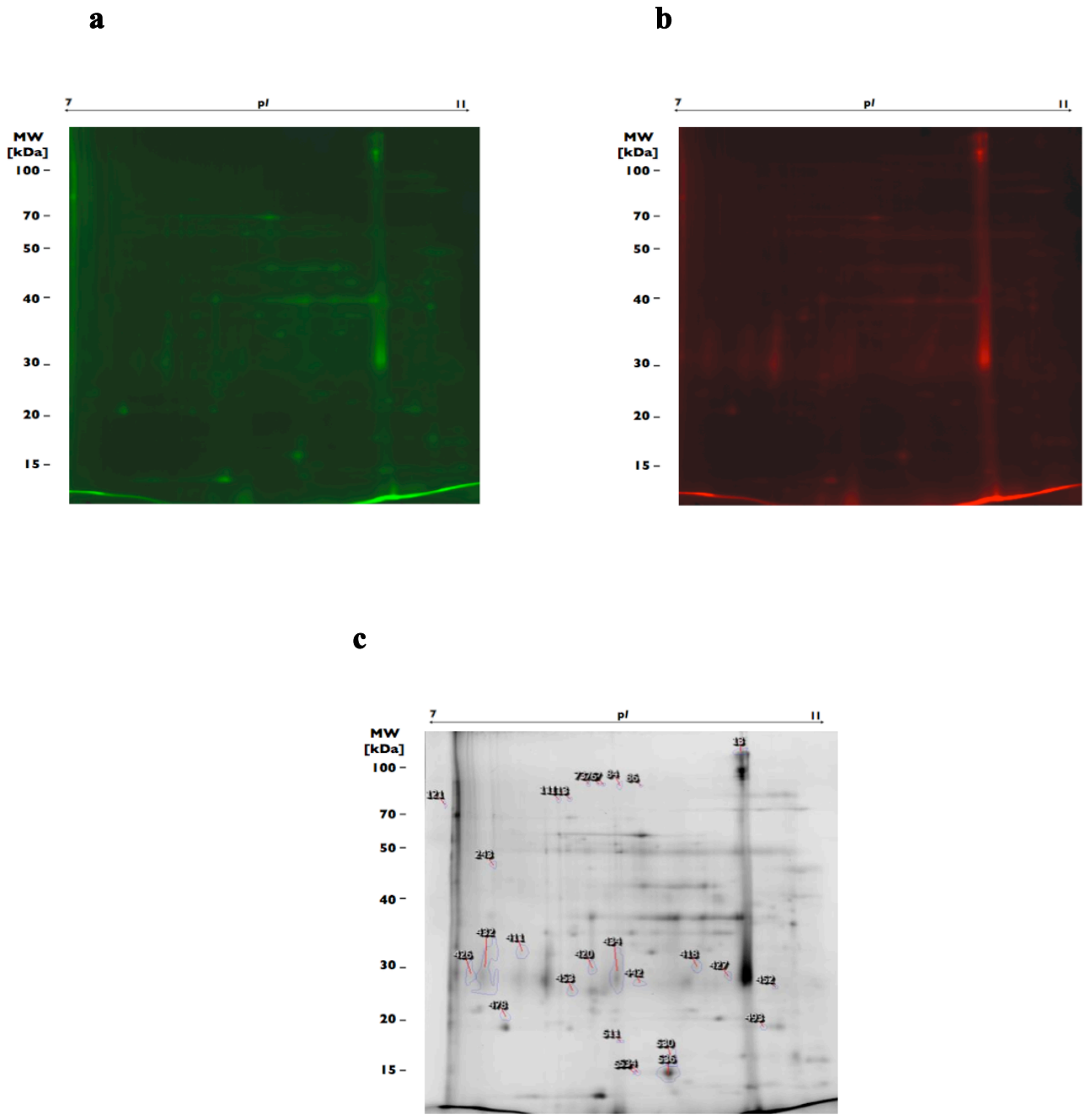


Figure 5.4 An example of Cy3 labelled sarcoidosis proteins are shown in figure (a) with Cy5 labelled control proteins in figure (b). All gels were run at a pI of 7-11 on 12.5% SDS-PAGE gels. Figure (c) represents 27 proteins spots of interest which were subsequently excised from the preparative gel (with 450 µg protein loaded in total), trypsin digested and analysed by Mass Spectrometry.

5.4 QUANTITATION AND IDENTIFICATION OF DIFFERENTIAL PROTEIN EXPRESSION

SameSpots software (Progenesis Ltd, UK) was used to analyse all gel images and identify statistically valid differences in spot volume between the sample groups at pI 3-7 and pI 7-11 when compared to the internal standard (table 5.3). In total, 31 spots with a statistically significant ($p < 0.05$ measured by ANOVA) increase/decrease in abundance (≥1.5 fold change) in the sarcoidosis group compared to controls were identified (18 at pI 3-7 and 13 at pI 7-11). Subsequent MS analysis of these spots identified 7 unique proteins with increased abundance and 7 unique proteins of decreased abundance as seen in tables 5.4 & 5.5 One example of this is the protein Catalase which is detailed in figure 5.5 and was identified as having a 1.7 fold upregulated expression ($p=0.008$) in the sarcoidosis group compared to controls.

In addition to the statistically valid proteins identified, a significant number of spots were found strongly associated with 1 or more sarcoidosis spleens with little or no presence (as determined by spot volume) in the control group. Although the fold increase in the sarcoidosis group did not reach significance, it is known candidate proteins of interest may not be found in all of the sarcoidosis spleens and this limitation may mean that fold increases as determined by SameSpots did not reach statistical significance. The SameSpots software (and the main alternative software by GE Healthcare called 'Decyder') compares spots across gels using ANOVA testing but the software does not have the ability to compare spot abundance between single gels. This is a major limitation of 2D-DIGE analysis software for the purposes of this project. One example of such a protein (which did not reach statistical significance) is vimentin which is found strongly expressed in 2 sarcoidosis spleens and no control spleens as shown in figure 5.6 and was identified as having a 2.2 fold upregulated expression ($p=0.365$) in the sarcoidosis group compared to controls. The 43 spots in this category yielded 16 unique protein identifications detailed in table 5.6. All proteins were searched with Spectrum Mill (Agilent Technologies) and confirmed using parameters detailed

in materials and methods section 2.215. The identities were confirmed with MASCOT (Matrix Sciences) (data not shown). Full Spectrum Mill data is shown in appendix 11.

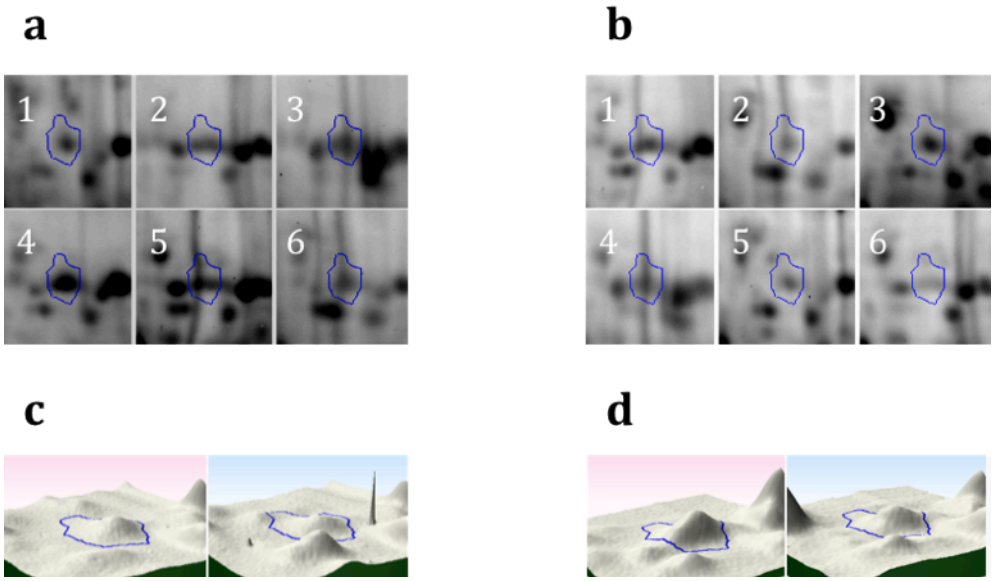


Fig 5.5 Catalase is found in both (a) sarcoidosis and (b) control spleens but shows increased spot volume in the sarcoidosis group. SameSpots software was used to create 3D images with the peak intensity representing the spot volume relative to the internal standard. Figure (c) shows the relative protein spot volumes for gel 4 and figure (d) represents gel 5. The left hand side peaks are from the sarcoidosis group with a larger volume in both gels 4 and 5 compared to the control peaks shown on the right hand side.

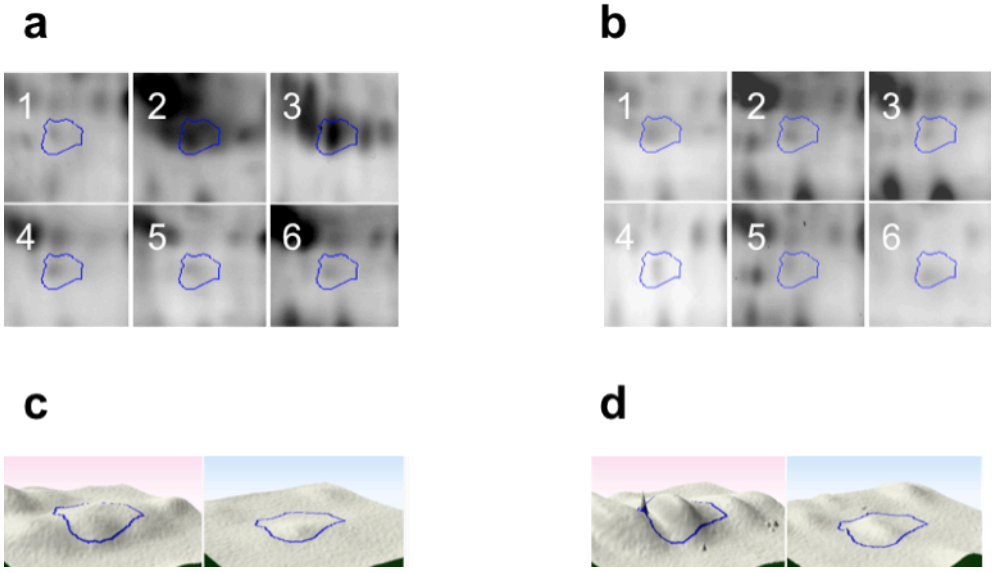


Fig 5.6 Vimentin is found strongly expressed in gels 2 and 3 in the sarcoidosis spleens (a) but shows little or no expression in the control spleens (b). SameSpots software was used to create 3D images with the peak intensity representing the spot volume relative to the internal standard. Figure (c) shows the relative protein spot volumes for gel 2 and figure (d) represents gel 3. The left hand side peaks are from the sarcoidosis group with a larger volume in both gels 2 and 3 compared to the control peaks shown on the right hand side.

Table 5.3 Protein spots excised out of a preparative gel are categorised as those which potentially showed significant over or under expression in sarcoidosis spleens compared to controls (based on changes in spot volume) or those which were found strongly expressed in at least one sarcoidosis spleen with minimal or no expression in control spleens (again based on spot volumes).

Category for further investigation	Number of spots cut out pI 3-7)	Number of spots cut out (pI 7-11)	Number of unique proteins identified
>1.5x fold over expression in sarcoidosis	11	9	7
>1.5x fold under expression in sarcoidosis	8	4	7
Found in 1 sarcoidosis spleen but in no controls	23	11	13
Found in 2 sarcoidosis spleen but in no controls	6	3	3

Table 5.4 Proteins identified from spots with significantly increased expression (based on spot volume) in the sarcoidosis group compared to controls.

Spot no	Protein	Fold increase	p value	MS/MS score	Peptide matches	% Amino acid coverage
A665	Actin type 2 cytoplasmic	2.3	0.05	106.9	8	18
A665	Beta-actin-like protein 2	2.3	0.05	51.89	8	1
B432	Haemaglobin subunit beta	1.9	0.05	50.2	3	23
A300	Catalase	1.7	0.01	117.51	8	16
A354	Selenium binding protein 1	1.8	0.02	21.82	2	3
A452	Medium-chain specific acyl-CoA dehydrogenase	1.8	0.04	62.92	6	15
A452	Carbonic anhydrase 1	1.8	0.04	35.49	3	10

Table 5.5 Proteins identified from spots with significantly decreased expression (based on spot volume) in sarcoidosis group compared to controls.

Spot no	Protein	Fold decrease	p value	MS/MS score	Peptide matches	% Amino acid coverage
A674	Serum amyloid P-component	2.2	0.03	148.1	9	31
B530	Cofilin-1	2.1	0.03	23.01	2	15
A280	Plastin-2	1.7	0.03	76.39	7	10
A280	Plastin-3	1.7	0.03	39.92	3	4
A280	Plastin-1	1.7	0.03	25.35	2	3
A280	60 kDa heat shock protein, mitochondrial	1.7	0.03	77.52	6	9
A305	Dihydropyrimidinase-related protein 2	1.5	0.02	69.94	5	10

Table 5.6 Proteins identified from spots showing a potentially stronger expression (based on spot volume) in at least one sarcoidosis spleen but with minimal or no expression in all control spleens.

Spot no	Protein	Found strongly in (n) sarcoid spleens	p value	Fold increase	MS/MS score	Peptide matches	% Amino acid coverage
A406	Vimentin	2	0.37	2.7	214.26	14	26
A406	Desmin	2	0.37	2.7	61.73	4	6
A406	Peripherin-2	2	0.37	2.7	31.77	2	3
A413	Eukaryotic initiation factor 4A-II	1	0.13	4.9	38.88	4	9
A541	Annexin A5	1	0.09	2	24.68	2	5
A753	Peroxiredoxin-2	1	0.11	1.9	35.52	4	18
A535	Transaldolase	1	0.08	1.8	61.31	5	13
A056	Alpha-actinin-4	1	0.10	1.7	51.47	4	5
A360	Tubulin beta	1	0.44	1.6	60.62	4	9
A508	Aldose 1-epimerase	1	0.51	1.6	87.82	7	8
A502	Fructose-1,6-bisphosphatase 1	1	0.92	1.6	174.38	12	40
A472	Mannose-1-phosphate guanyltransferase beta	1	0.14	1.5	41.64	4	9
A765	Proteasome subunit beta	1	0.34	1.4	35.24	3	14
B493	Neutrophil gelatinase-associated lipocalin	1	0.68	1.4	53.19	4	17
A593	Glyoxalase domain-containing protein 4	1	0.28	1.1	57.14	4	12
A478	Phosphatidylethanolamine-binding protein 1	1	0.93	1.1	35.16	3	13

In total, 78 proteins of interest have been identified through 1D-SDS-PAGE (chapter 4) and 2D-DIGE (this chapter) analysis as being specific to or showing increased expression in sarcoidosis spleen and Kveim reagent compared to controls. Of these 78 proteins, 8 are common to both analytical methods and are shown in figure 5.7.

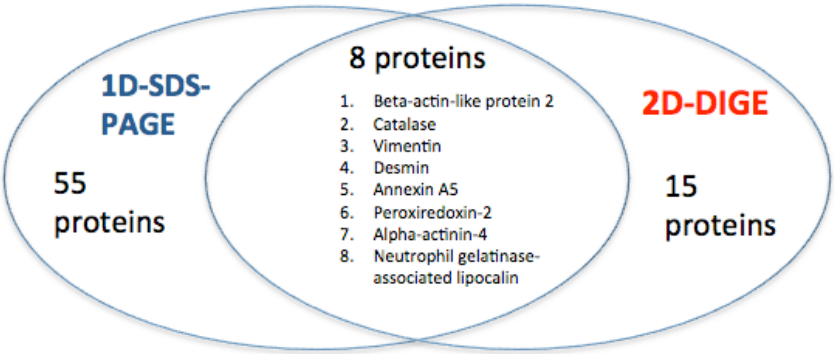


Figure 5.7 Proteins of interest found through 1D-SDS-PAGE and 2D-DIGE showing 8 proteins found through both methodologies

5.5 SUBCELLULAR CHARACTERISATION OF IDENTIFIED PROTEINS

A Uniprot (Universal Protein Resource version 2011_05) database search showed that the majority of the proteins identified as showing significantly increased expression in the sarcoidosis group (or that are found only associated with this group) are primarily located in the cell cytoplasm (transaldolase and proteasome subunit beta are additionally found in the cell nucleus) as shown in figure 5.8. Two proteins are located in the mitochondrion (Medium-chain specific acyl-CoA dehydrogenase and Glyoxalase domain-containing protein 4) two in the membrane (Peripherin-2, Selenium binding protein 1), one in the peroxisome (catalase) and one protein is secreted from the cell (Neutrophil gelatinase-associated lipocalin).

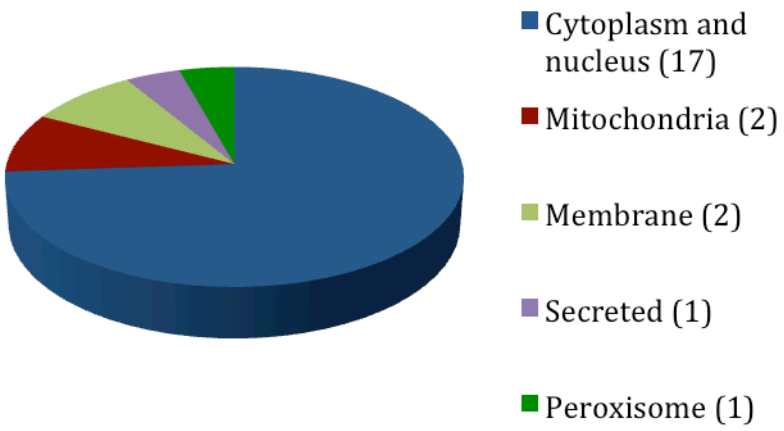


Figure 5.8 Subcellular location of proteins identified as specific to or showing increased expression in sarcoidosis tissue compared to controls as identified through 2D-DIGE.

5.6 FUNCTIONAL CLASSIFICATION OF IDENTIFIED PROTEINS

Each protein found has a number of functions as detailed by the Gene Ontology or GO (AMIGO version 1.8) database but the main biological function as determined by Uniprot database search (version 2011_05) is given in table 5.7.

Table 5.7 Main biological function of proteins showing increased or specific expression in sarcoidosis as determined by Uniprot database searches

Protein	Biological Function
Actin type 2 cytoplasmic	Highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
Beta-actin-like protein 2	Highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
Heamaglobin subunit beta	Involved in oxygen transport from the lung to the various peripheral tissues.
Catalase	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide.
Selenium binding protein 1	Selenium-binding protein which may be involved in the sensing of reactive xenobiotics in the cytoplasm. May be involved in intra-Golgi protein transport.
Medium-chain specific acyl-CoA dehydrogenase	Fatty acid and lipid metabolism.
Carbonic anhydrase 1	Reversible hydration of carbon dioxide. Can hydrate cyanamide to urea.
Vimentin	Class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells.
Desmin	Class-III intermediate filaments found in muscle cells.
Peripherin-2	May function as an adhesion molecule involved in stabilization and compaction of outer segment disks or in the maintenance of the curvature of the rim.
Eukaryotic initiation factor 4A-II	ATP-dependent RNA helicase which is a subunit of the eIF4F complex involved in cap recognition and is required for mRNA binding to ribosome.
Annexin A5	Anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade.
Peroxiredoxin-2	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system.

Table 5.7 continued

Protein	Biological Function
Transaldolase	Important for the balance of metabolites in the pentose-phosphate pathway.
Alpha-actinin-4	F-actin cross-linking protein which is thought to anchor actin to a variety of intracellular structures.
Tubulin beta	The major constituent of microtubules.
Aldose 1-epimerase	Converts alpha-aldose to the beta-anomer.
Fructose-1,6-bisphosphatase 1	Gluconeogenesis and carbohydrate metabolism.
Mannose-1-phosphate guanyltransferase beta	GDP-mannose biosynthesis.
Proteasome subunit beta	The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH.
Neutrophil gelatinase-associated lipocalin	Trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development.
Glyoxalase domain-containing protein 4	Mitochondrial processes.
Phosphatidylethanolamine-binding protein 1	Binds ATP, opioids and phosphatidylethanolamine.

The main functions of the 23 proteins found specific to or of increased expression in sarcoidosis spleen can also be classified as shown in figure 5.9 which shows the results as classified through GO (AMIGO version 1.8) and Kyoto Encyclopaedia of Genes and Genomes or KEGG (version May 16, 2011) searches. The largest group are proteins involved in metabolism and structural proteins.

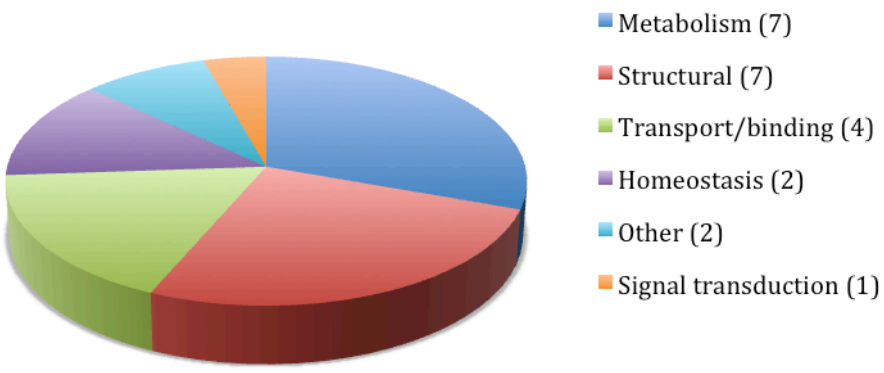


Figure 5.9 Functional classification (based on GO and KEGG database searches) of proteins found specific to or with increased expression in sarcoidosis spleen

5.7 PATHWAY ANALYSIS AND PROTEIN-PROTEIN INTERACTIONS OF IDENTIFIED PROTEINS

An analysis of identified proteins through pathway analysis using the KEGG database revealed a number of pathways associated with proteins found significantly increased or specifically expressed in the sarcoidosis group. These are detailed in table 5.8. The dominant pathways found (each associated with two proteins) are biosynthesis of secondary metabolites, microbial metabolism in diverse environments and metabolic pathways. In addition, the only protein found significantly downregulated in sarcoidosis which has KEGG pathway associations is Cofilin-1 which is found in Fc gamma R-mediated phagocytosis, Axon guidance and regulation of actin cytoskeleton. The proteins found specific to sarcoidosis tissue can also be investigated for known and predicted protein-protein interactions using the STRING database (version 8.3) as shown in figure 5.10.

Table 5.8 KEGG database analysis of protein-protein interaction pathways associated with proteins found significantly increased or specifically expressed in the sarcoidosis group.

Pathway	n proteins involved	Protein(s) involved
Biosynthesis of secondary metabolites	2	Mannose-1-phosphate guanyltransferase beta Aldose 1-epimerase
Microbial metabolism in diverse environments	2	Catalase Aldose 1-epimerase
Metabolic pathways	2	Catalase Mannose-1-phosphate guanyltransferase beta
Proteosome	1	Proteasome subunit beta
Gap junction	1	Tubulin beta
Phagosome	1	Tubulin beta
Pathogenic Escherichia coli infection	1	Tubulin beta
Fructose and mannose metabolism	1	Mannose-1-phosphate guanyltransferase beta
Amino sugar and nucleotide sugar metabolism	1	Mannose-1-phosphate guanyltransferase beta
Tryptophan metabolism	1	Catalase
Methane metabolism	1	Catalase
Nitrogen metabolism	1	Carbonic anhydrase

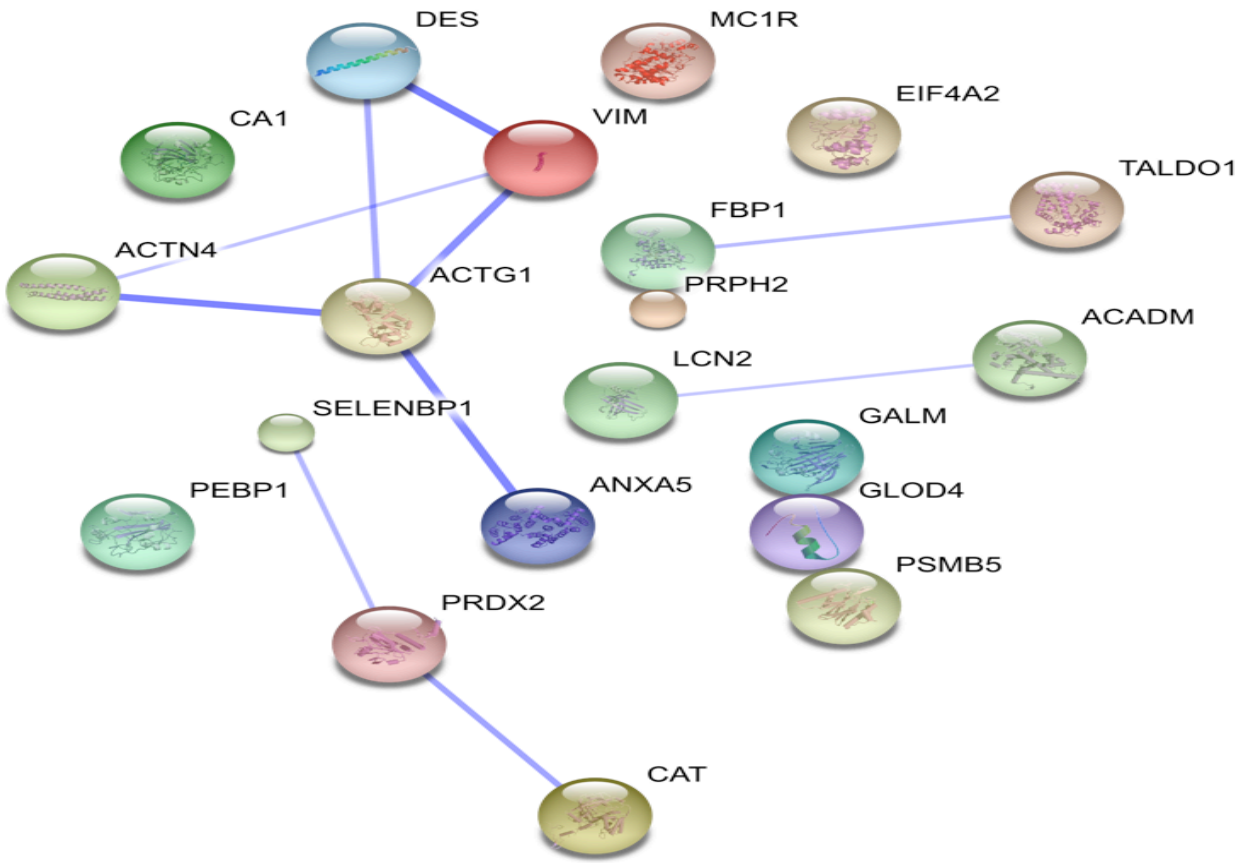


Figure 5.10 Known and predicted protein-protein interactions of all proteins found specific to or showing increase expression in sarcoidosis spleen as investigated with STRING database (version 8.3, search carried out on 22/05/2011). Interactions are shown in the evidence view mode where thicker lines represent a greater level of evidence for a particular interaction (e.g. biochemical data, co-expression or concurrence amongst genomes). Proteins are named by the recommended name from the Uniprot database (version 2011_05).

CONCLUSIONS

The data presented represent the first known comparative proteomic investigation of sarcoidosis tissue and controls. The use of 6 samples within each of the two groups represents an adequate sample size to find statistically significant differences in protein abundance (see section 7.2 for full discussion). The analysis of independently fluorescent labelled protein samples from both the sarcoidosis and control groups eliminates the need for technical replicates and is one of the advantages of using DIGE methodology (Unlu et al., 1997). The use of 2D-DIGE at two pI gradients (3-7 and 7-11) provides a more comprehensive protein separation than if gels of single pI at wider gradients (e.g. at 3-10) had been used. The investigation of whole spleen-extracted protein allows for as comprehensive a proteomic analysis as possible and avoids the problems associated with potential protein degradation after long term storage if only *in vivo* validated Kveim reagent had been used. The differential level of protein abundance has been accurately quantified and this data details for the first time identified proteins of differential abundance in a sarcoidosis spleen group when compared to controls. In addition, a number of proteins have been identified as being found with increased abundance in 1 or more sarcoidosis spleens with minimal or no presence in controls.

A number of these identified proteins have also been found specific to Kveim reagent using 1D-SDS-PAGE (as detailed in chapter 4) including the structural proteins vimentin (found specific to both Kveim reagent and to sarcoidosis spleen whole protein extract) and the enzyme catalase which was identified specific to Kveim reagent through 1D-SDS-PAGE and found upregulated 1.7 fold through 2D-DIGE in the sarcoidosis spleens compared to controls. In addition, a number of proteins found to be upregulated in or specific to sarcoidosis spleen through 2D-DIGE share similar biological functions with those found specific to Kveim through 1D-SDS-PAGE e.g. proteins involved in host-pathogen interaction as part of the

innate immune response (Cathelicidin antimicrobial peptide found through 1D-SDS-PAGE and Eukaryotic initiation factor 4A-II through 2D-DIGE). On analysis of protein-protein interactions it was again found that similar networks are found upregulated in or specific to sarcoidosis spleen through 2D-DIGE analysis to those found through 1D-SDS-PAGE of Kveim reagent including metabolic pathways and tryptophan metabolism.

The use of 2D-DIGE in this context does have limitations. The technique represents one single method for quantification of differential abundance and the proteins of difference found may need to be confirmed through alternate methods of proteomic comparative analysis e.g. isobaric tag labelling with subsequent shotgun mass spectrometry (Treumann and Thiede, 2010) or Westernblot confirmation. The use of a second technique would not simply be to confirm the protein identities found here. Rather, isobaric tags have two main advantages over DIGE. Firstly, there is global labelling (rather than the minimal lysine labelling used here) which may results in more proteins being identified. Secondly, isobaric labelling avoids the gel step of 2D-DIGE therefore avoiding any potential co-migration of more than one protein in the same gel position. For these reasons, further work using another quantitative approach may prove useful in the search for candidate antigens.

The sarcoidosis spleen samples used were not able to be tested *in vivo* for their granuloma causing capacity, so unlike the 1D-SDS-PAGE analysis of validated Kveim as shown in chapter 4, it is unknown as to how many of the new spleens contain the candidate antigen(s). However, the data from chapter 3 show that some of these spleens do have an ability to specifically induce a Th1 response from sarcoidosis PBMCs.

Another limitation is that the control spleens used are from patients with no diagnosed splenic disease. Although diseased patient tissue is difficult to acquire (e.g. spleens with other infiltrative diseases or those with splenic cancer) a comparison with non-sarcoidosis diseased

tissue would have been important to draw the most accurate possible conclusions for proteins that display differential abundance in sarcoidosis.

However, Aim 1 of this project is primarily to investigate sarcoidosis tissue and Kveim reagent for protein candidates which may be able to elicit an antigen specific T-cell response in patients with diagnosed sarcoidosis. Kveim reagent has been historically validated *in vivo* using normal healthy spleen tissues as controls. This historical comparison is exactly the same as used here to compare protein candidates with 2D-DIGE. The identification of proteins of interest through 2D-DIGE, some of which are also found specific to Kveim reagent through 1D-SDS-PAGE is paramount to Aim 1 of this project. Future work can now focus on investigating individual proteins for their potential to cause antigen-specific T-cell responses in sarcoidosis.

Chapter 6

Investigation of BAL and serum cytokine profiles in sarcoidosis and tuberculosis

INTRODUCTION

The previous three results chapters have shown work towards Aim 1 of this project. Aim 2 involves the identification of specific cytokine profiles in sarcoidosis that can help distinguish it from diseases which are similar in terms of clinical presentation. There are numerous anecdotal case reports of patients with a shared respiratory exposure who subsequently develop different diseases. One such intriguing report involves monozygotic twins. One twin presented with dyspnoea, a pleural effusion, strongly positive Mantoux and negative Kveim test, and subsequently responded appropriately to anti-tuberculous therapy. The other twin denied respiratory symptoms but a chest radiograph revealed bilateral hilar lymphadenopathy, a Kveim test was positive and the clinical course was in keeping with sarcoidosis (Mikhail et al., 1970). Mycobacterial involvement in sarcoidosis pathogenesis remains a contentious issue. Supporting evidence includes similarities in clinical and radiographic picture (Iannuzzi et al., 2007), identification of mycobacterial DNA in sarcoid granulomas which had otherwise stained negative for acid fast bacilli (Popper et al., 1997) and the finding in some sarcoidosis cohorts of peripheral blood and BAL INFγ-mediated T-cell responses towards the mycobacterial proteins Antigen 85A (Hajizadeh et al., 2007) and mKatG (Chen et al., 2008). Conversely, evidence against a relationship includes the marked differences in extra-pulmonary disease sites and treatment strategy (Iannuzzi et al., 2007), and the absence of INFγ-mediated T-cell responses towards the immunodominant mycobacterial proteins ESAT-6 and CFP-10 in large worldwide sarcoidosis studies (Inui et al., 2008, Horster et al., 2009).

The hypothesis of a shared aetiological trigger but separate disease outcomes can be probed by examining immune responses in demographically matched patients. The T-cell profile in

sarcoidosis BAL cells is biased towards Th1 cytokines (Moller, 1999) with similar findings in tuberculosis (Taha et al., 1997). This picture is distinct from that seen in atopic asthma which exhibits a Th2 bias (Robinson et al., 1992) or hypersensitivity pneumonitis which displays a strong Th17 response (Scott et al., 1989). There is recent evidence that Th17 cytokines may also play a role in both sarcoidosis (Facco et al., 2011) and tuberculosis (Torrado and Cooper, 2010). In contrast to BAL, there are few studies of serum cytokine profile in either disease.

For this work, pulmonary tuberculosis samples were taken from culture confirmed or highly probable cases (Dosanjh et al., 2008). Pulmonary sarcoidosis samples were taken from patients with a strong clinical diagnosis, supportive histology where available and a subsequent clinical course in keeping with the original diagnosis. Tuberculosis and sarcoidosis samples were taken from Imperial College Healthcare Trust and matched to the extent possible for age, sex and ethnicity. Healthy volunteer samples were taken from two unrelated studies within the Centre for Respiratory Infection (Acknowledgements to Dr. Saranya Sridhar and Dr. Jon Macintrye for this provision). All diseased BAL samples were taken prior to or within two weeks of starting treatment. Sarcoidosis serum samples were taken from patients on minimal steroid therapy (≤10mg prednisolone daily) and on no other immunosuppressants. Demographic data for all patients is displayed in table 6.1. Examples of the standardised (and ethically approved clinical information sheets, consent forms and data collections sheets) are given in appendix 7. Full clinical record forms for a large selection of sarcoidosis and tuberculosis patients (5 acute cases per group) are given in appendix 7. Information for healthy volunteers (other than basic demographic details such as age, sex and ethnicity as shown in table 6.1) was not available to this project as samples were donated from two unrelated studies as detailed above and no further information was available.

BAL and serum samples in pulmonary sarcoidosis, pulmonary tuberculosis and healthy controls were investigating by measuring a cytokine panel which best represented the spectrum of immune process involved in both diseases including the Th1 (INFγ, TNα) vs.

Th2 (IL-4, IL-5, IL-13) balance, T-cell stimulation (IL-2, IL-12), macrophage activation (Il-1b), granuloma formation (IL-8), and limitation of inflammation (IL-10). Multiplexed protein analysis is increasingly used for immune profiling and biomarker identification where accurate rapid diagnosis is paramount (Balboni et al., 2006) with promising data in a number of diseases including Systemic Lupus Erythematosus (Carlsson et al., 2011) and prostate cancer (Christensen et al., 2009). For each comparison (sarcoidosis vs. tuberculosis , sarcoidosis vs. healthy and tuberculosis vs. healthy) a p-value was calculated using a Mann-Whitney test for each cytokine. Within each of the comparisons, p-values were adjusted for multiple comparisons using a Bonferroni correction (multiplying each p-value by 10). Data analysis was performed using GraphPad Prism version 5.04 for Macintosh (La Jolla, California, USA). To create a classification rule to distinguish between diseases, partial least squares (PLS) logistic regression was carried out using the gpls package in R version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria). The within sample mis-classification rate was calculated for the logistic regression model found by PLS. Leave-one-out cross-validation was used to obtain an estimate of prediction error of the PLS logistic regression model.

Table 6.1 Demographic and clinical characteristics of patients with sarcoidosis, tuberculosis, and healthy volunteers . Examples of clinical record forms are given in appendix 7.

	Pulmonary sarcoidosis	**Pulmonary tuberculosis**	**Healthy volunteers**
BAL STUDY POPULATION			
Number	18	12	16
Median age (range)	47.5 (27-72)	39 (24-77)	27 (18-42)
Female gender (%)	8 (44)	6 (50)	10 (63)
Ethnic origin: Caucasian/Indian subcontinent/Black	9/2/7	6/3/3	15/0/1
Time since diagnosis	0-2 weeks	0-2 weeks	N/A
SERUM STUDY POPULATION			
Number	40	15	40
Median age (range)	48 (27-73)	33 (25-71)	31 (20-60)
Female gender (%)	21 (53)	5 (33)	26 (65)
Ethnic origin: Caucasian/Indian subcontinent/Black	19/8/13	4/10/1	33/4/3
Time since diagnosis	0-5 years	0-6 months	N/A

RESULTS

6.1 THE BAL CYTOKINE PROFILE IN ACUTE PULMONARY SARCOIDOSIS IS INDISTINGUISHABLE FROM ACTIVE PULMONARY TUBERCULOSIS

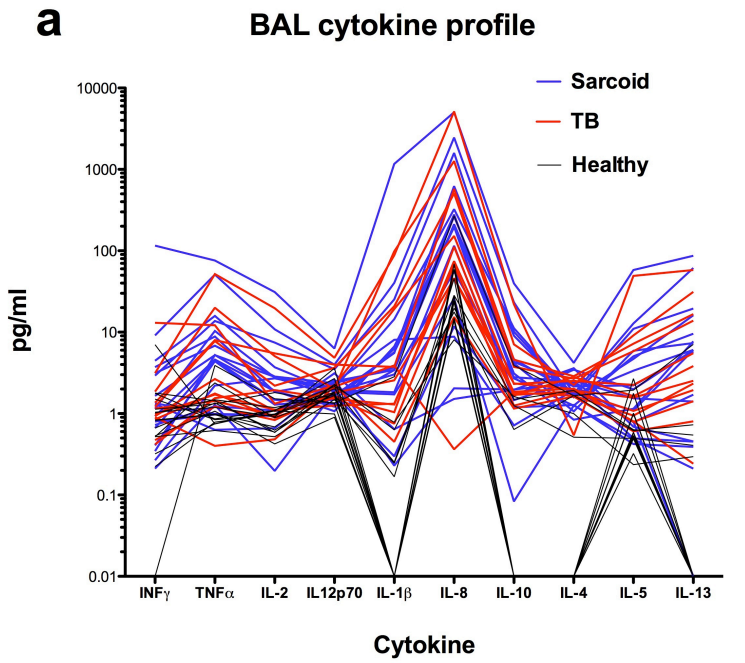
The cytokine profile in acute pulmonary sarcoidosis and active pulmonary tuberculosis BAL are indistinguishable from each other (table 6.2 and figure 6.1)**.** The individuals within these groups all have evidence of acute or active lung involvement and BAL fluid was taken for diagnostic purposes prior to or at the same time as starting presumptive therapy. The median levels of the Th1 cytokines INFγ and TNFα were higher in both diseases compared to healthy BALs and this elevation was significant (adjusted p-value < 0.05) with TNFα (figure 6.2a).

In addition to TNFα, the level of the pro-inflammatory cytokine IL-1β was significantly elevated in both sarcoidosis and tuberculosis groups over healthy individuals (aP < 0.001 in both cases). Similar levels of IL-1β were found in the sarcoidosis group (median of 4.919, IQR = 1.801 – 9.659) compared to patients with tuberculosis (median of 3.152, IQR = 1.101 – 20.42).

Median BAL levels of the Th2 cytokines IL-4 and IL-13 (figure 6.2b) were found significantly higher in sarcoidosis and tuberculosis compared to healthy BALs (adjusted p-value < 0.001 in all cases). IL-5 levels were found higher in the diseased states but this increase reached adjusted statistical significance in tuberculosis only. Levels of IL-10 were found significantly elevated in both diseases compared to healthy BALs whereas levels of IL-2 were significantly elevated in sarcoidosis only. Levels of IL-12p70 and IL-8 were not significantly different between any group.

Table 6.2 BAL and serum cytokine levels in pulmonary sarcoidosis, pulmonary tuberculosis and healthy volunteers with Bonferroni-adjusted p-values for each of the possible comparisons.

Cytokine	Sarcoidosis BAL n=18 median (range) pg/ml	Sarcoidosis Serum n=40 median (range) pg/ml	Tuberculosis BAL n=12 median (range) pg/ml	Tuberculosis Serum n=15 median (range) pg/ml	Healthy BAL n=16 median (range) pg/ml	Healthy Serum n=40 median (range) pg/ml	Sarcoidosis vs. tuberculosis BAL P value	Sarcoidosis vs. tuberculosis Serum P value	Sarcoidosis vs. Healthy BAL P value	Sarcoidosis vs. healthy Serum P value	Tuberculosis vs. Healthy BAL P value	Tuberculosis vs. healthy Serum P value
INFγ	1.23 (0.21-115.5)	3.26 (0.22-42.4)	1.07 (0.42-13.03)	5.20 (0.80-74.56)	0.83 (0.01-7.01)	1.94 (0.91-37.06)	1	1	1	0.001	1	0.045
TNFα	4.92 (0.63-75.93)	8.73 (2.47-76.92)	2.21 (0.40-51.74)	8.92 (4.55-19.96)	1.03 (0.62-3.90)	5.31 (2.41-17.43)	1	1	0.008	<0.001	0.037	<0.001
IL-2	1.89 (0.20-31.12)	1.01 (0.31-3.08)	1.22 (0.48-19.67)	0.84 (0.25-1.61)	0.94 (0.42-1.81)	0.46 (0.01-10.03)	1	0.385	0.016	<0.001	0.485	0.063
IL-12 p70	2.01 (1.07-6.32)	3.19 (2.01-12.32)	2.08 (1.25-4.86)	2.43 (0.83-194.4)	1.81 (0.90-3.62)	2.73 (0.72-776.5)	1	1	1	1	1	1
IL-1β	4.62 (0.23-1167)	0.21 (0.01-11.59)	3.15 (0.45-98.48)	0.11 (0.01-1.45)	0.01 (0.01-2.84)	0.48 (0.09-180.8)	1	1	<0.001	<0.001	<0.001	0.059
IL-8	202.9 (1.51-5036)	10.10 (0.99-50.96)	93.20 (0.36-5155)	11.02 (3.63-22.3)	24.19 (8.07-267.8)	6.11 (1.18-2606)	1	1	0.218	<0.001	0.087	0.575
IL-10	6.90 (0.08-39.7)	3.45 (1.25-18.76)	4.29 (1.15-21.95)	2.33 (0.50-51.47)	0.56 (0.01-3.91)	2.86 (1.45-54.41)	1	0.838	<0.001	1	0.001	1
IL-4	1.77 (0.84-4.20)	0.61 (0.01-21.02)	2.01 (0.54-2.88)	0.01 (0.01-0.82)	0.01 (0.01-1.93)	1.93 (0.43-13.76)	1	0.004	<0.001	<0.001	<0.001	<0.001
IL-5	1.767 (0.42-58.13)	1.20 (0.58-7.92)	1.60 (0.60-49.27)	0.91 (0.01-57.91)	0.59 (0.24-2.69)	1.32 (0.39-41.81)	1	1	0.083	1	0.032	1
IL-13	6.03 (0.01-86.85)	0.8 (0.01-6.2)	3.17 (0.24-57.97)	0.01 (0.01-34.37)	0.01 (0.01-3.90)	1.49 (0.25-26.24)	1	0.157	<0.001	0.293	<0.001	0.014



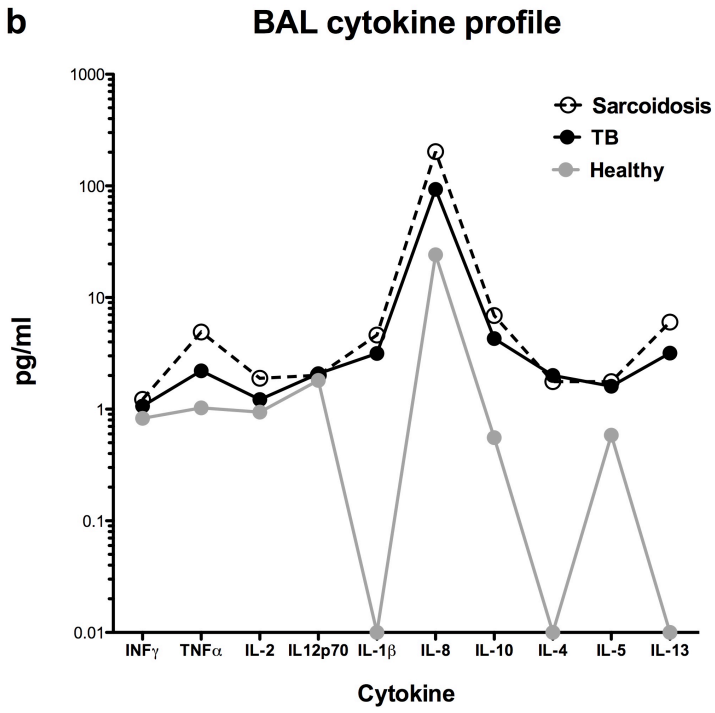


Figure 6.1 BAL cytokine profile observed in the different groups studied. Each line represents one patient with sarcoidosis (blue), tuberculosis (red) or healthy volunteers (black) as shown in 6.1a. Figure 6.1b displays the same data showing median cytokine concentrations only for each investigated group.

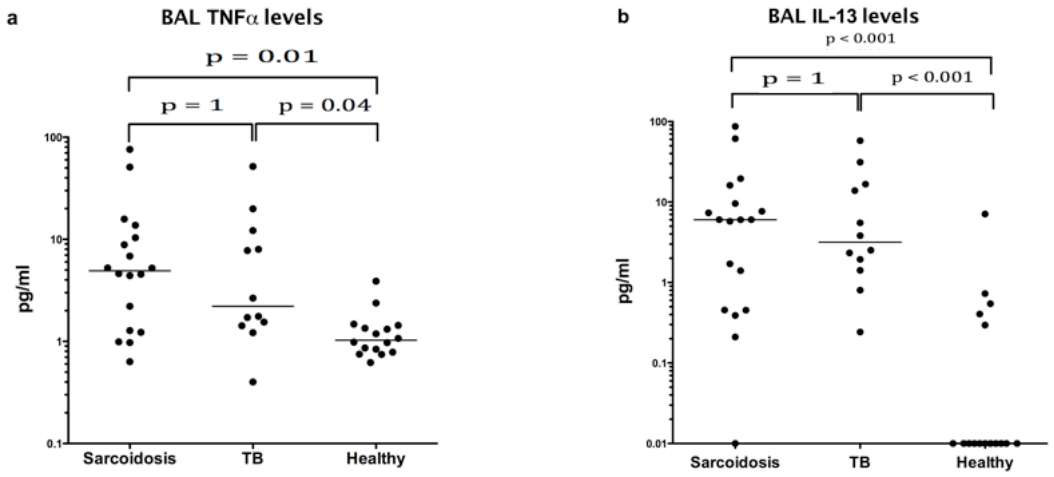


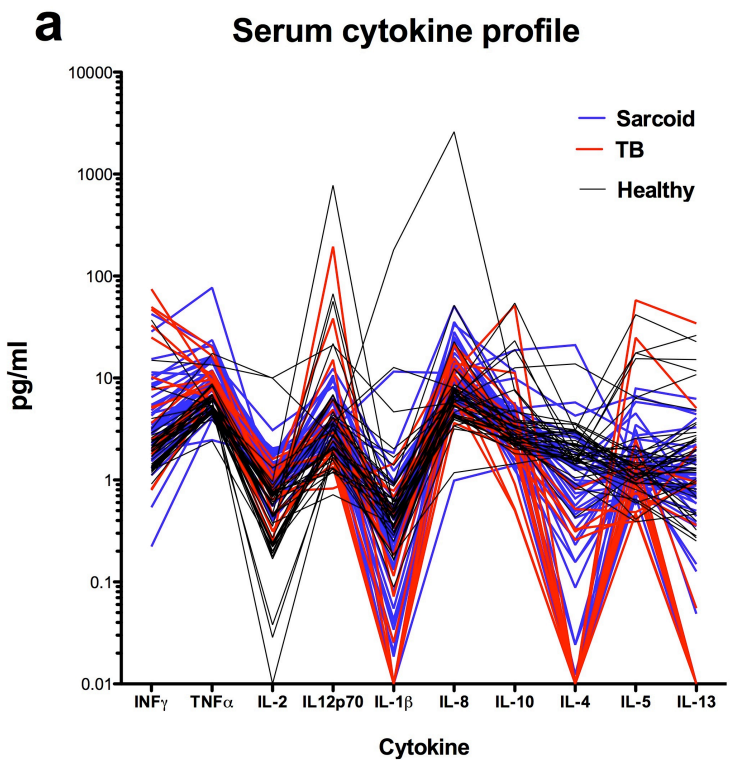
Figure 6.2 BAL levels of (a) TNFα and (b) IL-13 in the different patient groups (sarcoidosis, tuberculosis and healthy volunteers). Each data point represents the measurement of one patient sample.

6.2 Serum IL-4 levels are significantly higher in pulmonary sarcoidosis than in pulmonary tuberculosis

Serum levels of the Th1 cytokines INFγ and TNFα were found significantly elevated in both pulmonary sarcoidosis and pulmonary tuberculosis compared to healthy volunteers (table 6.2 and figure 6.3). Serum levels of IL-2 and IL-8 were also elevated in both diseases compared to healthy volunteers but these increases only reached adjusted statistical significance in sarcoidosis. Levels of IL-1β were found lower in both diseases compared to healthy serum with adjusted significance in sarcoidosis and just below adjusted significance in tuberculosis (adjusted p-value = 0.059). Serum levels of IL12p70 and IL-10 were not significantly different between any group.

In contrast, serum Th2 cytokines IL-4, IL-5 and IL-13 were found reduced in both diseases compared to the healthy volunteers. Within both diseases, serum levels of Il-4 were significantly higher (adjusted p-value = 0.004) in sarcoidosis compared to tuberculosis (figure 6.4a). Levels of IL-5 and IL-13 (figure 6.4b) were also higher in sarcoidosis although these differences did not reach statistical significance when adjusting for multiple comparisons. In fact, only five patients with pulmonary tuberculosis had measurable serum IL-4 levels. Two of these five had evidence of extra pulmonary (abdominal) disease but there was nothing else clinically specific about them compared to the majority with undetectable levels.

In order to ensure that the differences seen in serum IL-4 existed in the acute stage as well as in all cases of pulmonary sarcoidosis, patients with acute pulmonary sarcoidosis only (known duration of disease < 2 weeks) were re-analysed against all cases of active pulmonary tuberculosis (also of known duration of disease < 2 weeks). Upon this further analysis (figure 6.5) when performing a single statistical analysis of IL-4 in patients with acute pulmonary sarcoidosis only (n=16) compared with active pulmonary tuberculosis (n=15), the significant differences remained ($p = 0.0418$). When performing an analysis of IL-13 levels between these two groups the picture is similar with lower levels in tuberculosis but the difference is just above the significance level ($p = 0.0817$).



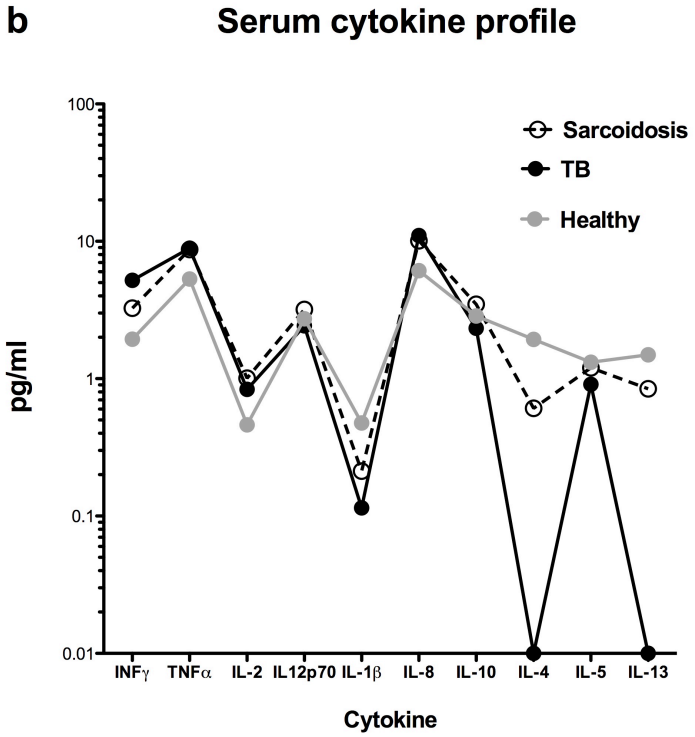


Figure 6.3 Serum cytokine profile observed in the different groups. Each line represents one patient with sarcoidosis (blue) tuberculosis (red) or healthy volunteers (black) as shown in (a). Figure (b) displays the same data showing median cytokine concentrations only for each group.

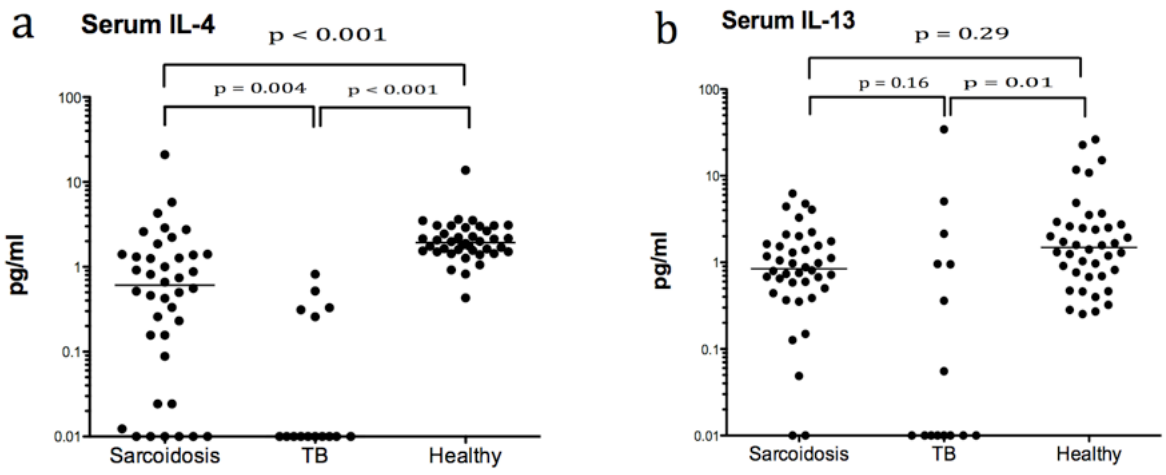


Figure 6.4 Serum levels of (a) IL-4 and (b) IL-13 in the different patient groups (sarcoidosis, tuberculosis and healthy volunteers). Each data point represents one patient sample.

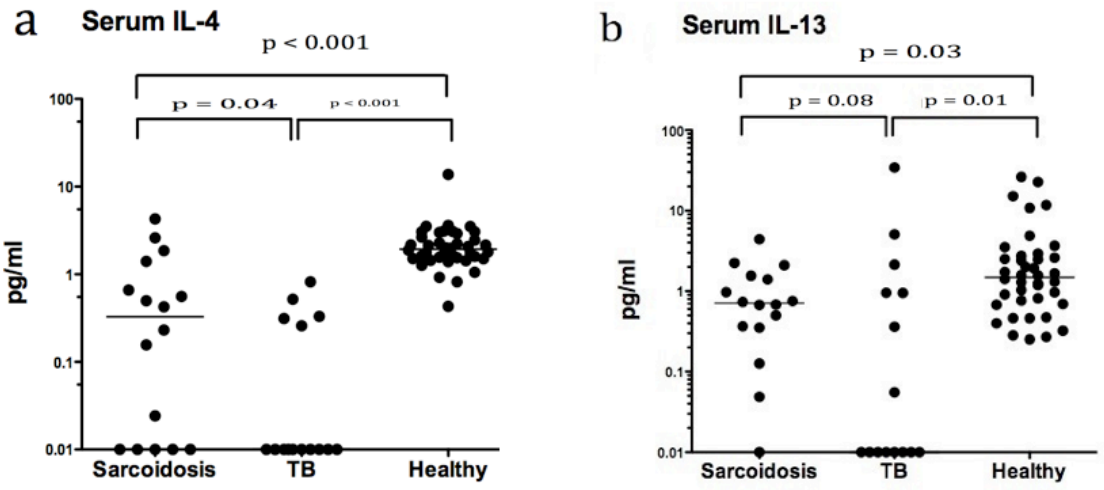


Figure 6.5 Serum levels of (a) IL-4 and (b) IL-13. The healthy volunteer and tuberculosis data is the same as that shown in figure 6.4 but only cases of acute untreated pulmonary sarcoidosis is displayed alongside (rather than all cases of pulmonary sarcoidosis as in figure 6.4). This allows a direct comparison to be made between acute untreated cases of both sarcoidosis and tuberculosis. Each data point represents one patient sample.

6.3 DIFFERENCES IN SERUM CYTOKINE PROFILE ALLOW FOR ACCURATE DIFFERENTIATION BETWEEN PULMONARY SARCOIDOSIS AND PULMONARY TUBERCULOSIS

A targeted bioinformatic approach to exploit these serum cytokine differences was employed to differentiate between the two diseases. Partial least squares was used to create a logistic regression model comparing the values of all serum cytokines. The best fit model found was log(P(Tuberculosis)/P(sarcoidosis)) = 1.214 + 8.884 ∗ IL1 + 0.075 ∗ IFN + 0.32 ∗ IL12p70 + 0.039 ∗ TNF - 0.097 ∗ IL5 − 0.015 ∗ IL8 - 0.73 ∗ IL13 − 0.606 ∗ IL10 - 2.404 ∗ IL2 − 4.231 ∗ IL4. The classification rule was to classify a sample as tuberculosis if log(P(tuberculosis)/P(sarcoidosis)) was greater than zero, or as sarcoidosis otherwise.

By applying this model to the data, the observed and predicted disease groups for the sera were recorded (figure 6.6). The created model had a within-sample mis-classification rate of 9% (5/55 sera) i.e. the model could predict whether a serum sample was sarcoidosis or tuberculosis with 91% accuracy. Of the five patients misclassified by the model (two with sarcoidosis and three with tuberculosis), there were no clinical indications found that would separate them from the correctly classified patients. The within-sample error rate is probably an under-estimate of the true prediction error of this model, as the data was used to fit the model and assess its accuracy. Leave-one-out cross validation was used to obtain a better estimate of prediction error; this was found to be 27% (15/55).

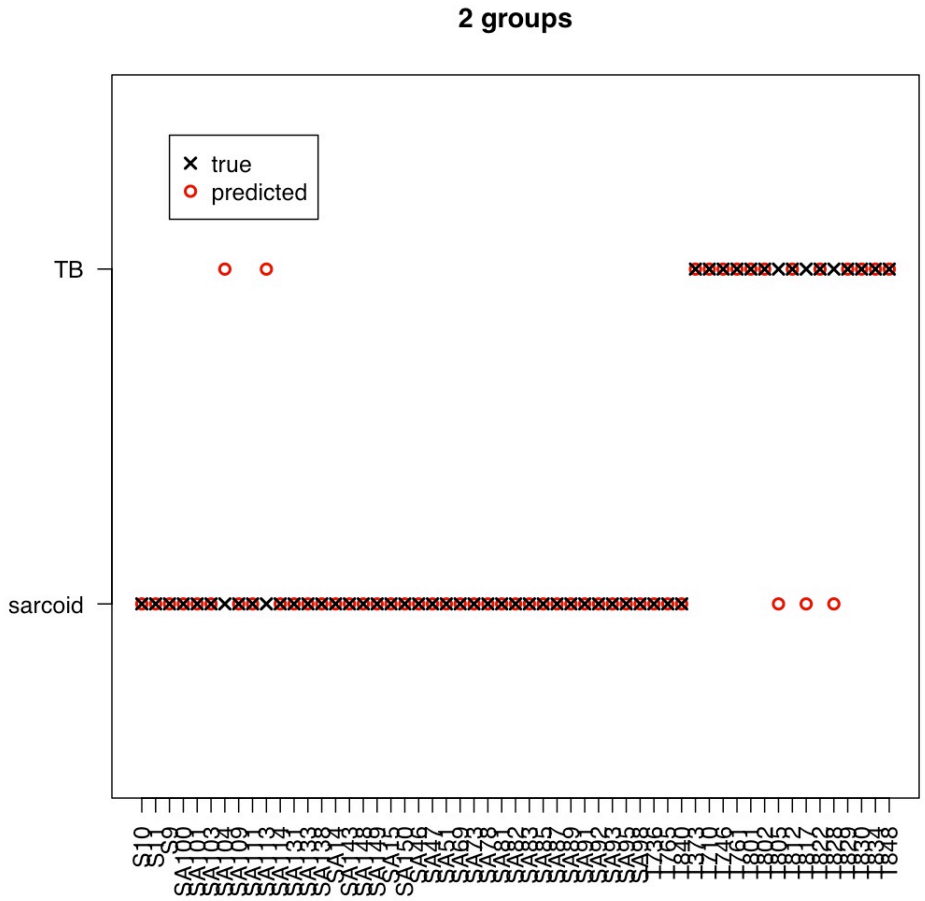


Figure 6.6 Partial Least Squares was used to create a linear regression model which takes into account differences in serum cytokine levels between pulmonary sarcoidosis and pulmonary tuberculosis and is able to accurately classify investigated serum samples with over 90% accuracy. PLS model, cross-validation and leave one out validation was created by Dr. Alex Lewin, Department of Biostatistics at Imperial College London.

6.4 Compartmentalisation of cytokines between BAL fluid and serum in pulmonary sarcoidosis is similar to pulmonary tuberculosis

Figure 6.7 and table 6.3 show that the pattern of compartmentalisation of cytokines between the BAL and serum is similar in the sarcoidosis and tuberculosis groups and differences are apparent when compared to the healthy group. For example, as shown in figure 6.8a-c, the median levels of the Th2 cytokine IL-4 are higher in the BAL than in the serum in both sarcoidosis (ratio of BAL : serum is 5.39) and tuberculosis (ratio of 2.01). This pattern is

reversed in the healthy volunteers group where almost no IL-4 is seen in the BAL (median 0.01, IQR 0.01 – 0.8772) and therefore ratio of BAL: serum IL-4 in this group is recorded as 0.01.

Th2 cytokines are preferentially found in the BAL compared to the serum in both sarcoidosis and tuberculosis. Healthy volunteers demonstrate the opposite pattern with higher Th2 cytokines found in the serum. This is evident with IL-4 as previously discussed, IL-5 (sarcoidosis = 1.44 ; tuberculosis = 1.67 ; healthy = 0.44) and IL-13 (sarcoidosis = 8.51 ; tuberculosis = 3.17 ; healthy = 0.01). This pattern of raised Th2 cytokines in the BAL compared to the serum of patients with both sarcoidosis and tuberculosis is not seen with INFα, TNFα or IL-2.

The pattern seen in BAL and serum cytokine compartmentalisation is also observed in individual matched patient samples. A number of matched serum and BAL samples were taken at the same time point from patients with acute pulmonary sarcoidosis and as seen in figure 6.8d, the general trend for higher IL-4 levels in the BAL compared to the serum is continued when examining cytokine levels within individual patients.

Table 6.3 Ratio of median BAL : median serum cytokine concentrations for each group (sarcoidosis, tuberculosis and healthy volunteers). A ratio of 0.01 indicates that there was no measurable cytokine in the BAL.

Cytokine	Sarcoidosis BAL (n= 18): serum (n= 40) ratio	TB BAL (n= 12): serum (n= 15) ratio	Healthy BAL (n= 16): serum (n= 40) ratio
INFγ	0.13	0.21	0.43
IL-2	1.75	1.46	2.04
IL-12 p70	0.56	0.86	0.67
IL-1b	19.00	27.48	0.01
IL-8	23.44	8.46	3.96
TNFα	0.46	0.25	0.19
IL-10	0.82	0.89	0.01
IL-4	5.39	2.01	0.01
IL-5	1.44	1.76	0.44
IL-13	8.51	3.17	0.01

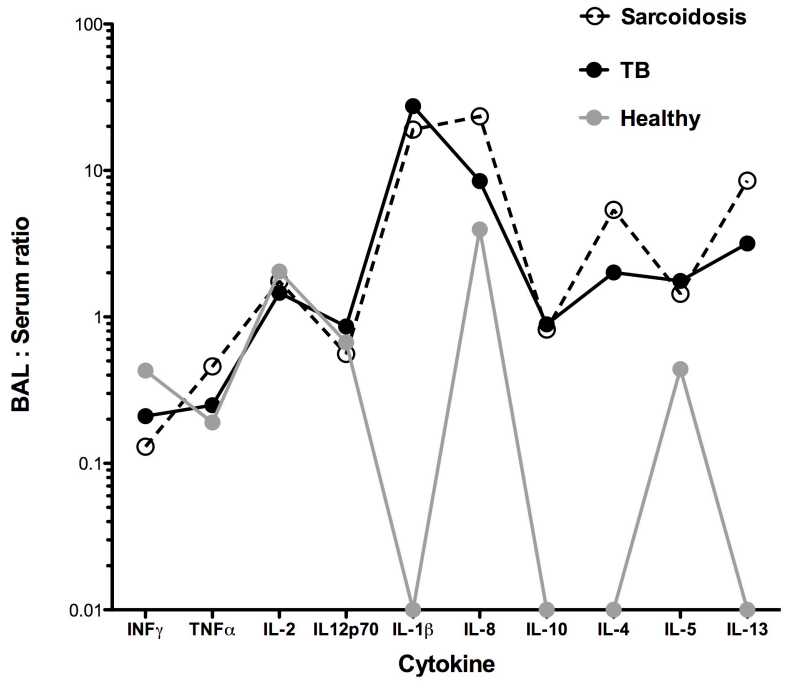


Figure 6.7 Profile of median BAL : median serum cytokine ratios for each of the three groups: sarcoidosis, tuberculosis and healthy volunteers.

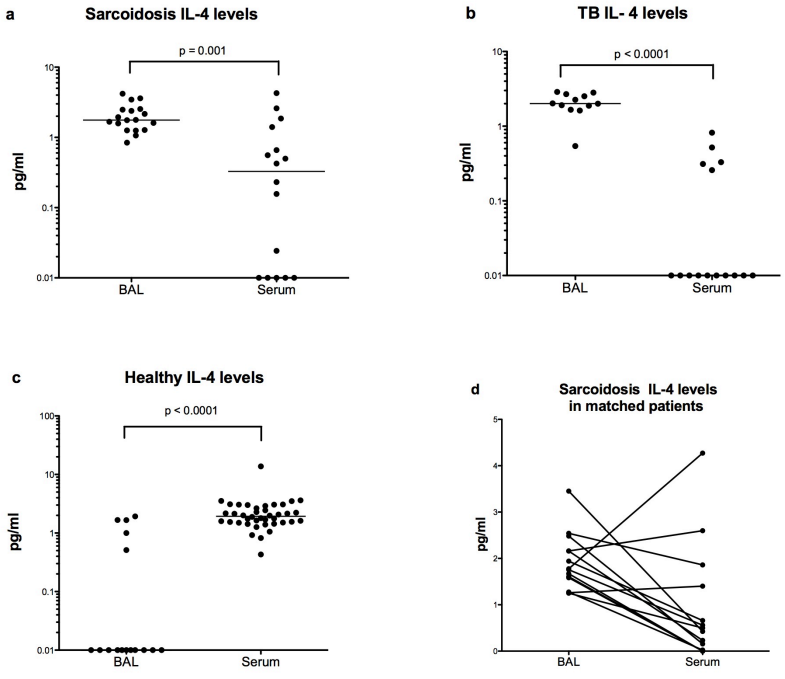


Figure 6.8 BAL and serum levels of IL-4 in each of the groups studied: (a) sarcoidosis, (b) tuberculosis and (c) healthy volunteers. Each data point represents one patient sample. Figure (d) details the BAL and serum cytokine levels of IL-4 in sarcoidosis patients where matched BAL and serum samples were available. Lines are used to indicate the BAL and serum relationships between individual patients.

6.5 CYTOKINE PROFILES IN SARCOIDOSIS: SUB-GROUP ANALYSIS

No significant differences were found between the acute and chronic pulmonary sarcoidosis groups e.g. levels of the Th1 cytokines INFγ (acute median 4.55, IQR: 3.07-8.61 ; chronic median 3.18, IQR: 1.64-4.52) and TNFα (acute median 10.81, IQR: 8.64-14.86 ; chronic median 7.18, IQR: 6.25-11.35). In addition, levels of Th2 cytokines are are also found similar i.e. IL-4 (acute median 0.33, IQR: 0.01-1.22 ; chronic median 0.90, IQR: 0.28-1.4) and IL-13 (acute median 0.708, IQR: 0.36-1.52 ; chronic median 1.02, IQR: 0.61-1.72). However, the numbers in this sub-group analysis (table 6.3) are perhaps too small and a larger study (with appropriate power) will be needed to determine if there are any differences.

A second analysis examined those patients with radiological evidence of fibrosis compared to those who did not (table 6.4). The numbers with proven fibrotic sarcoidosis in this study are probably too small (n=3) to draw accurate comparisons with the remaining patients (n=37). Additionally, no significant differences were found in any cytokine between stage of disease as classified by chest radiograph or presence of extra-pulmonary disease (data not shown).

Table 6.4 Serum cytokine levels in sarcoidosis with Bonferroni-adjusted p-values for each of the possible comparisons.

Cytokine	Acute n=16 median (range) pg/ml	Chronic n=24 median (range) pg/ml	Fibrosis n=3 pg/ml	No fibrosis n=37 pg/ml	Acute vs. chronic aP value	Fibrosis vs. no fibrosis aP value
INFγ	4.55 (1.60-11.38)	3.18 (0.22-42.4)	3.66 (2.80-28.53)	3.26 (0.22-42.4)	1	1
TNFα	10.81 (2.47-23.63)	7.18 (3.94-76.92)	8.97 (8.45-76.93)	8.45 (2.47-23.63)	0.62	1
IL-2	1.08 (0.78-1.82)	1.01 (0.40-3.08)	0.88 (0.40-1.94)	1.03 (0.41-3.08)	1	1
IL-12 p70	3.6 (2.01-12.32)	3.13 (2.16-10.61)	2.86 (2.26-9.48)	3.21 (2.01-12.32)	1	1
IL-1b	0.24 (0.01-1.23)	0.209 (0.01-11.59)	0.46 (0.140-1.84)	0.212 (0.01-11.59)	1	1
IL-8	8.67 (0.99-35.55)	11.01 (4.57-50.96)	33.34 (14.96-50.96)	9.79 (0.99-35.55)	1	0.18
IL-10	3.60 (1.40-9.98)	3.48 (1.25-18.76)	5.0 (1.44-11.55)	3.45 (1.25-18.76)	1	1
IL-4	0.33 (0.01-4.27)	0.90 (0.01-21.02)	0.256 (0.01-5.77)	0.67 (0.01-21.02)	0.72	1
IL-5	1.22 (0.67-6.67)	1.12 (0.58-7.92)	2.73 (2.20-7.20)	1.18 (0.58-6.67)	1	0.27
IL-13	0.71 (0.01-4.42)	1.02 (0.01-6.25)	3.29 (0.44-6.25)	0.81 (0.01-1.77)	0.81	1

CONCLUSIONS

The data shows for the first time through direct comparisons that the cytokine profile in the BAL of patients with acute pulmonary sarcoidosis (as well as the compartmentalisation of cytokines between BAL and serum) is identical to that in active pulmonary tuberculosis when patients are matched as best as possible for age, sex and ethnicity (see section 7.3 for full discussion). The similarities seen show marked differences compared to that seen in healthy volunteers. The patterns seen in sarcoidosis BAL are as previously described with elevation of Th1 cytokines compared to healthy volunteers. The levels of Th2 cytokines are shown also elevated compared to normal lungs. This contrasts with some sarcoidosis studies suggesting that Th2 producing T-cells are not found at elevated levels in the lungs (Hoshino et al., 1995). However, other studies show that Th2 cytokines such as IL-13 may be found at higher levels in the lungs (Hauber et al., 2003) and so the findings here warrant further investigation.

The analysis of serum reveals that there is a significant increase in Th1 (IFNγ and TNFα) and decrease in Th2 cytokine levels (IL-4) in both sarcoidosis and tuberculosis compared to the healthy volunteers. In addition, there are significant differences with respect to serum IL-4 levels between patients with pulmonary sarcoidosis and pulmonary tuberculosis and these differences remain when analysing a subset of patients with acute pulmonary sarcoidosis and comparing these to active pulmonary tuberculosis. The differences in serum IL-4 (as well as possibly with IL-5 and IL-13) in the sarcoidosis group compared to the tuberculosis group contribute to the construction of a linear regression model which exploits these differences to differentiate between the groups with over 90% accuracy after within group cross-validation, a number that drops to 73% after leave one out validation. Detailed analysis of these results may provide further insights into the relationship between sarcoidosis and tuberculosis and allow identification of unique cytokine biomarker signatures centred around serum Th2 cytokines for differentiation between both diseases.

Chapter 7

Discussion

7.1 KVEIM REAGENT-SPECIFIC T-CELL RESPONSES IN SARCOIDOSIS

The data from chapter 3 show that a precise clinical phenotyping of sarcoidosis patients without tuberculosis infection is possible through clinical risk analysis and interferon-gamma release assay (IGRA) testing (section 7.11). However the mycobacterial antigen PPD (and perhaps ESAT-6) may warrant further investigation in patients with sarcoidosis (7.12). Investigation of historic *in vivo* validated Kveim reagent reveals specific interferon gamma secretion from sarcoidosis PBMCs (7.13) and further experimentation with fractions of *de novo* Kveim reagent reveal a specific Th1 response with significant secretion of INFγ and TNFα from sarcoidosis PBMCs when compared to controls (7.14). The results from the protein fractionation process may also give clues as to the identities of the candidate antigens within Kveim reagent.

7.11 PRECISE CLINICAL PHENOTYPING OF SARCOIDOSIS PATIENTS WITHOUT TUBERCULOSIS INFECTION

The aetiology of sarcoidosis is yet to be fully explained but there is some evidence for the association of mycobacteria with the disease, perhaps as an inciting factor in some cases (section 1.22). However, this potential causation is made confusing by the fact that some patients with sarcoidosis may have a latent mycobacterial infection unlreated to their

sarcoidosis, a critical confounder which has not been excluded in previously published studies (section 1.65). In order to avoid any confusion over co-infection, a phenotypically 'clean' population of sarcoidosis patients who were specifically at low clinical risk of tuberculosis infection were chosen. Of the 14 patients who fitted this category, 11 had negative INFγ ELISpot responses to both of the mycobacterial immunodominant proteins ESAT-6 and CFP-10 (figure 3.1). These 11 patients were then selected for further investigation with Kveim reagent.

The data show that sarcoidosis patients at low clinical risk of tuberculosis infection do not respond to ESAT-6 and CFP-10 to the same levels as seen in patients with diagnosed tuberculosis infection (figure 3.1). This in keeping with the only two studies published to date which investigated sarcoidosis patients using commercially available IGRAs used for routine diagnosis of tuberculosis. In Japan, a commercially available ELISA kit (QuantiFERON, Cellestis) was used to test for the same 2 proteins (ESAT-6 and CFP-10) in 90 patients with sarcoidosis and found that only 3 patients were positive to the assay, a percentage comparable with that of the healthy Japanese controls (Inui et al., 2008). A second study from Germany used the commercially available ELISpot (T-Spot.*TB*, Oxford Immunotec) in a smaller number of patients and again found that T-cell responses from both PBMCs and BAL mononuclear cells were significantly lower than that seen in patients with diagnosed tuberculosis infection and similar to rates seen in healthy volunteers (Horster et al., 2009). Neither study risk stratified sarcoidosis patients nor did they pre-select those at low clinical risk of tuberculosis infection as in this work. The majority of other studies which have identified T-cell responses towards mycobacterial proteins have used patients recruited from areas of higher endemic latent tuberculosis infection and no prior attempt has been made to rule out a co-existing infection e.g. the Drake group's work all involve patients recruited from Texas, USA (Drake and Newman, 2006) which currently has the second highest incidence of tuberculosis infection in all 50 states (Gerberding et al., 2008).

However, it is important to note that mycobacterial proteins such as the previously described mKatG (section 1.64) may play a role in some cases of sarcoidosis and there are three hypotheses which may explain the findings from previous studies in context with the present work. Firstly, if sarcoidosis has a multifactorial aetiology, the individuals pre-selected in this data set simply represent a sub-group of disease with an aetiology that is not mycobacterial in origin. Such patients may simply have been under represented in previous studies from areas of the world where the majority of sarcoidosis is mycobacterial in origin. A second explanation is that mKatG may be specifically involved in the pathogenesis of disease as a mycobacterial protein which is unrelated to the other immunodominant proteins such as ESAT-6 and CFP-10. This may explain why mKatG-specific T-cell responses previously studied have not corresponded with ESAT-6-specific T-cell responses in the same patients (Oswald-Richter et al., 2010). A third explanation is that mKatG and other mycobacterial proteins are not involved in sarcoidosis pathogenesis to the extent previously postulated and that some of the patients previously studied (particularly those from regions of high TB endemicity) have simply had mycobacterial co-infection unrelated to their sarcoidosis. This last point is, however, difficult to reconcile in a study with some sarcoidosis patients recruited from Sweden, where TB incidence is lower than North Amerca (Chen et al., 2008).

7.12 ESAT-6 AND PPD RESPONSES IN PATIENTS WITH SARCOIDOSIS MAY WARRANT FURTHER INVESTIGATION

It is interesting to note that whilst none of 14 patients with sarcoidosis (who were at low clinical risk of tuberculosis infection) had positive responses towards CFP-10 (figure 3.1c), three patients exhibited a response to ESAT-6 and only one of these three displayed a positive response to PPD (figure 3.1b). Peripheral blood T-cell responses towards ESAT-6 have been previously investigated in sarcoidosis with one study showing that 8 of 26 patients exhibited a

strongly positive response (Drake et al., 2007). The same group in a later study showed that responses to ESAT-6 were from CD4+ T-cells and that the mechanism of action involved antigen-presenting cells and expression of the sarcoidosis susceptibility allele DRB1*1101 (Oswald-Richter et al., 2010). An earlier study by the same group found ESAT-6 specific T-cell responses from the BAL in 32 of 44 patients with pulmonary sarcoidosis compared with just 1 of 27 controls and that blocking of Toll-like receptor 2 reduced the strength of the observed immune response, suggesting that the response found was due to the induction of innate immunity by a causal mycobacteria (Oswald-Richter et al., 2009). The BCG vaccination and PPD status (measured by tuberculin skin test or by ELISpot response to PPD) of the patients in these previous studies is unknown and therefore the ESAT-6 responders may simply represent individuals co-infected with latent tuberculosis infection. However, these studies, along with the data shown in figure 3.1b indicate that if mycobacteria are involved in some cases of sarcoidosis, T-cell responses to ESAT-6, like mKatG, may warrant further study.

It is also of interest that despite at least 10 of 14 sarcoidosis patients having definite evidence of prior BCG vaccination, only 5 of these same patients displayed a PPD-specific T-cell response (which has a significant antigenic cross reactivity with proteins found in BCG) as seen in figure 3.1a. On further investigation, it was found that the level of responses were significantly lower compared to that found in BCG vaccinated healthy controls (figure 3.2). It is well recognised that patients with sarcoidosis who are BCG vaccinated show a decreased response to the tuberculin skin test (which uses PPD injected intracutaneously to produce a skin response) used in diagnosis of tuberculosis infection (Hartston, 1964). A number of studies have also shown that patients with sarcoidosis display a reduced skin recall response to other protein antigens including a reduced sensitivity to candida and mumps skin test antigens (Friou, 1952).

PPD-specific T-cell responses in patients with sarcoidosis have not previously been specifically investigated in sarcoidosis. The data shown here suggest that the reduction in PPD-specific peripheral blood T-cells (when compared to BCG vaccinated healthy controls) may be responsible for the phenomenon of depressed TST sensitivity seen in sarcoidosis. It would be interesting to investigate other cytokines (in addition INFγ) to see if there is a different PPD-specific cytokine secretion signature in sarcoidosis and also to investigate the BAL PPD-specific T-cell responses in sarcoidosis patients, which may provide interesting information about the compartmentalisation of T-cell responses in the disease.

7.13 SARCOIDOSIS PATIENTS EXHIBIT INFγ-SECRETING T-CELL RESPONSES IN THE PERIPHERAL BLOOD IN RESPONSE TO KVEIM REAGENT

The present data show for the first time that proteins or peptides can be precipitated from *in vivo* validated Kveim reagents which are more than 10 years old, and specifically elicit a cytokine secretion from cells in peripheral blood of patients with sarcoidosis (figure 3.3). This data shows that 9 of 11 sarcoidosis patients displayed an INFγ response as measured by ELISpot to at least one *in vivo* validated Kveim reagent, compared to just 1 of 9 tuberculosis patients. There has been no previously published work investigating Kveim-specific cytokine responses in patients with sarcoidosis but a few studies have investigated other immune aspects of Kveim reagent. One study found a reduction in proliferation (measured by [3H]-thymidine incorporation) at some concentrations of Kveim reagent and IL-2 compared to control PBMCs incubated with IL-2 only (Lyons et al., 1991). The same group had earlier found an inhibition of leucocyte migration in the presence of Kveim reagent when investigating cells from both sarcoidosis patients and those with Crohn's disease, suggesting a similar immune response in both diseases (Willoughby and Mitchell, 1971). These studies together suggest that Kveim reagent may have an inhibitory effect on cell proliferation but

they do not impact or give particular insight into the data shown in figure 3.3. Aside from these two studies, little else is known about *ex vivo* cellular responses towards Kveim reagent.

In the 6 patients with sarcoidosis tested against all 4 validated Kveim protein extracts, all displayed an INFγ response as measured by ELISpot to at least one Kveim reagent (figure 3.4). It is interesting to note that individuals had different levels of response to different Kveim reagents with patient S46 eliciting a response to 3 of 4 Kveim reagents. Different Kveim reagents also varied in their ability to produce a response with both K12 and K51 producing a response in 4 out 6 patients as seen (figure 3.5). These findings are consistent with numerous *in vivo* studies showing that the level of skin response depends on a number of factors including time since presentation and extent of disease (Mikhail and Mitchell, 1970) and that not all sarcoidosis spleens have the ability to form a successful Kveim reaction (Mitchell et al., 1976). Kveim reagents may have similar high specificity but large variations in diagnostic sensitivity (Israel et al., 1958, Danbolt, 1951a). It is also possible that the long term storage conditions may have resulted in some Kveim reagents tested retaining a greater amount of the candidate antigen(s) than others as although they have been shown to be generally resistant to long term storage at 4^0C, some Kveim reagents can lose their *in vivo* diagnostic capabilities after a short period of time (Nelson and Schwimmer, 1957).

There was nothing clinically distinct about patient S46 who produced an INFγ response to 3 of 4 Kveim reagents tested. However, S15 (the patient who produced the highest response far to an individual Kveim reagent at more than double the next highest response as shown in figure 3.5) was a patient with diagnosed sarcoidosis who was 3 months pregnant at the time of sample donation. Sarcoidosis in pregnancy is a well recognised phenomenon (Chapelon Abric et al., 1998) but the symptoms can be dampened, perhaps due to the elevated levels of circulating steroids, hence the need for careful post-partum surveillance for resurgence of

disease. Another mechanism for the reduction in disease symptoms may be due to the altered regulatory T-cell profile in pregnancy which is known to contribute to tolerance to the foetus (Kahn and Baltimore, 2010). This may result in a reduced effector T-cell function i.e. tolerance of the foetus may be a similar mechanism to tolerance to the antigen(s) which cause disease in sarcoidosis. If this hypothesis is correct, a reduced ex-vivo T-cell response towards Kveim reagents would be expected, not the increased response seen in figure 3.5. In contrast, other case series have found that pregnancy in existing sarcoidosis has exacerbated the disease (Agha et al., 1982). As there is no published literature on *in vivo* Kveim responses in pregnancy, interpretation of this particularly high response is difficult.

In addition to INFγ, a small but detectable IL-2 response was seen in response to 3 of the 4 Kveim reagents in 1 of 4 patients (figure 3.6). All 4 patients had pulmonary sarcoidosis of longer than 1 month duration and all 4 had previously demonstrated INFγ responses to at least 1 Kveim reagent. The patient who responded (S36) had the longest duration of disease of all 4 patients, with a diagnosis more than 5 years previously. There is evidence from infective diseases such as such as CMV and HIV that an acute stage with high disease burden is associated with a dominance of CD4+ T-cells secreting INFγ (from effector T-cells) with a pattern which switches to dual secretion of INFγ and IL-2 or IL-2 only (from central memory T-cells) in chronic disease with a constant but low antigen burden (Harari et al., 2005, Emu et al., 2005). A similar phenomenon has more recently been discovered at work in tuberculosis (Millington et al., 2007a). If this same model is applied to sarcoidosis it may explain why some (albeit low levels) of IL-2 response are found in this data set, and particularly in a patient with a long duration of disease. It would be interesting to further investigate IL-2 Kveim-specific secretion in patients with established or resolved sarcoidosis to look for potential responses. This phenomenon of switching of T-cell profile within the context of

effector and memory T-cell populations in acute and chronic sarcoidosis does not appear to have been previously discussed in the literature.

Fresh sarcoidosis spleen can be used to create *de novo* Kveim reagent which is able to elicit an INFγ T-cell response in patients with sarcoidosis at significantly higher levels than in controls (figure 3.7). Historically, only 1 in 5 sarcoidosis spleens yielded enough high quality reagent to cause a strong *in vivo* granulomatous response and there was no relationship seen between presence of spleen granulomas and ability to cause Kveim reactions (Mitchell et al., 1976). It is interesting that the ex-vivo INFγ responses for *de novo* Kveim reagent are substantially higher than that seen when using validated *in vivo* Kveim reagent. The long term storage conditions may have adversely affected the *in vivo* validated reagent and thus reduced its ability to cause an antigen-specific response. An alternative explanation for the higher INFγ responses seen with *de novo* Kveim reagent is that the creation of material from fresh sarcoidosis spleen has resulted in the retaining of a number of impurities which non-specifically cause a cytokine response from peripheral blood T-cells incubated with the Kveim reagent. This may explain why large responses (compared to the data shown with validated Kveim reagent in figure 3.3) are still seen in the control patients with tuberculosis. An important limitation of this experiment is that healthy patients were not investiagated. They would have been an important control to see whether they too mounted a T-cell responses, further strengthening the argument for a contaminant casuing a non-specific cytokine secretion.

7.14 SARCOIDOSIS PATIENTS EXHIBIT POLARISED Th1-RESPONSES TOWARDS KVEIM REAGENT

Proteins pooled from fractionation of *de novo* Kveim material appear to retain the candidate antigen(s) which are able to elicit a specific Th1 secretion from PBMCs after overnight incubation (figure 3.8). Whilst the concentrations of cytokine as measured by MSD are not directly comparable with SFCs seen in an ELISpot, it is interesting to note that none of the three control PBMC samples (2 apparently healthy volunteers and 1 patient with hypersensitivity pneumonitis) appeared to exhibit INFγ responses towards fractionated *de novo* Kveim reagent (figure 3.8a). This is in contrast to all 6 patients with tuberculosis who displayed a response in the INFγ ELISpot, albeit a response that was significantly lower than seen in sarcoidosis patients as seen (figure 3.7). One explanation is that the fractionation process may selectively retain the antigen(s) of interest whilst removing any of the impurities from the protein pool that may have caused a non-specific activation of T-cells. The fact that protein fractions 1 and 24 (which contain most of the non-protein impurities becoming apparent during fractionation) had been removed prior to use supports this theory.

Although the fractionation process produces protein pools which are capable of eliciting a cytokine response from sarcoidosis T-cells, it was unable to identify a particular isoelectric fraction of interest which contained a greater proportion of the candidate antigen(s) (figures 3.10 a & b). Four possibilities exist for this finding. Firstly, there may be multiple protein candidates with different pIs which therefore exist in all fractions. However, this is unlikely as previous work showing oligoclonal T-cell infiltration indicates only a limited number of T-cell candidate antigens (Klein et al., 1995). Secondly, the fractionation process may not have been completely successful as proteins may have leaked out during the process into all pools. However, the data generated from the experiment shows a holding step of 100V occurring between 24 and 100 hours which is set to maintain all proteins in their fractionated pools which argues against this possibility (appendix 4).

Thirdly, they may be proteins which exist in numerous isoforms which can therefore be found in multiple pools through the fractionation process as has been previously found by another group using a similar method (Michel et al., 2003). Finally, it is possible that the candidate antigens cannot be isolated into one single fraction due to their size e.g. large structural proteins which may exist as complexed conglomerates (Alberts, 1998) and which cannot therefore be separated into pools (due to the size of the membrane pores acting as a barrier to fractionation). This final hypothesis is intriguing as large structural host proteins serve as autoantigens in a number of other diseases e.g. vimentin-specific T-cells (Feitsma et al., 2010a) and antibodies (Wagner et al., 2009) have been identified in Rheumatoid Arthritis and fibronectin antibodies have been found specific to Systemic Lupus Erythematosus (Atta et al., 1995). Vimentin itself has been identified as a possible auto-antigen in sarcoidosis (see section 7.21).

The multiplex cytokine data also show that cytokines other than INFγ may be of importance in antigen specific recognition when incubated directly with Kveim reagent extracted proteins. It is well recognised that there is a strong Th1 response seen with an accumulation of T-cells secreting Th1 cytokines at the site of disease but all previous work involving ELISpot and flow cytometry analysis of PBMCs and T-cells incubated with potential antigenic targets in sarcoidosis has investigated T-cell secretion of INFγ only (Drake and Newman, 2006), with one just study one study demonstrating IL-2 specific T-cell responses towards mKatG (Oswald-Richter et al., 2010).

The data presented here shows that as well as INFγ, TNFα may play a role in antigen recognition in disease with significantly higher levels seen when sarcoidosis PBMCs are incubated with fractionated Kveim protein extracts than in the two control experiments (figure 3.8j). TNFα has been studied before with reports of spontaneous production from

sarcoidosis BAL cells (Bachwich et al., 1986). TNFα secretion from T-cells has been investigated in related diseases, most notably in tuberculosis where TNFα secreting CD4+ T-cells towards mycobacterial proteins enable distinction between active and latent tuberculosis (Harari et al., 2011). Each of the 3 patients investigated in this experiment had acute pulmonary sarcoidosis but it would be interesting to see whether TNFα secretion from PBMCs of patients with chronic sarcoidosis is lower than that found here.

The levels of production of the cytokine IL-2 in the SS condition are relatively comparable to that seen in the control groups (figure 3.8b). As already discussed, one explanation for the low levels of IL-2 (despite high levels of INFγ and TNFα secretion) is that effector rather than memory T-cells may be responsible for antigen recognition in acute sarcoidosis. There is no previous published work investigating this phenomenon in sarcoidosis and, as with TNFα measurements, it would be interesting to repeat the multiplex cytokine analysis of responses towards fractionated *de novo* Kveim reagent using patients with chronic long standing pulmonary sarcoidosis to determine whether IL-2 responses in these patients are higher (due to memory T-cell antigen recognition) than those seen in the data presented here from patients with acute pulmonary disease.

It is interesting to see that there is also significantly more secretion of IL-1b and IL-10 in the SS group than in the controls (figures 3.8b & g). Interleukin 1 is a pro-inflammatory cytokine and there is evidence that both IL1α and β are expressed in bronchoalveolar lavage fluid and from within sarcoidosis granulomas (Yamaguchi et al., 1988). IL-10 is a key cytokine involved in autoimmunity and T-cell regulation but there is no conclusive evidence to date that it is produced at different levels in sarcoidosis to that seen in healthy lungs (Zissel et al., 1996, Hoshino et al., 1995). Interestingly, the presence of IL-10 has been shown in a number of studies to induce antigen-specific T-cell anergy during antigen priming, with one study

showing that the IL-10-induced anergic state was irreversible as T-cell anergy could not be reversed after stimulation of the cells with anti-CD3 and anti-CD28 monoclonal antibodies (Groux et al., 1996). The high levels of IL-10 seen secreted by sarcoidosis PBMCs in specific response to Kveim reagent extracted proteins may therefore be related to maintaining the anergic state seen in sarcoidosis.

The increased INFγ and TNFα secretion from sarcoidosis PBMCs incubated with sarcoidosis spleen fractions support the finding that sarcoidosis is generally a disease of heightened Th1 expression (Moller, 1999). However, the unexpected finding that IL-13 is significantly and specifically elevated in the control experiment of sarcoidosis PBMCs incubated with control spleen protein fractions is difficult to interpret (figure 3.8j). One study has shown increased IL-13 mRNA expression from unstimulated PBMCs in sarcoidosis (Hauber et al., 2003) but another study did not find this (Prasse et al., 2000). IL-13 is an important Th2 cytokine involved with regulating inflammatory and immune responses (Minty et al., 1993) and is known to be a critical cytokine in the initial granulomatous response to schistosomal infections such as *S. mansoni* (Chiaramonte et al., 1999). The high levels of IL-13 found here may similarly be related to an initial immune response in sarcoidosis (all 3 patients investigated had acute pulmonary disease within 2 weeks of onset of symptoms). There is also evidence that IL-13 inhibits TNFα secretion in the blood (Cosentino et al., 1995) and that this is also true specifically in sarcoidosis (Hauber et al., 2003). Indeed, the high levels of IL-13 correspond with reduced levels of TNFα in the control CS experiment (figure 3.8i). However, the presence of a 'sarcoid antigen' (SS experiment) appears to switch cytokine production away from the anti-inflammatory effects of high IL-13 levels towards Th1 cytokine production (figure 3.8j). The reason for this remains unclear. Despite the small number of patients investigated here, the possible differences in IL-13 between CS and SS experiments is very interesting and merits further investigation given the conflicting data published to date.

In addition, when the data for individual patients is displayed separately, it is seen that the sarcoidosis patients respond differently with differing levels of cytokine response, despite al 3 having biopsy proven newly diagnosed pulmonary sarcoidosis e.g. SA69 produces a higher INFγ response to all protein fractions but a lower TNFα response than SA92 (figures 3.9a & b). This data is consistent with the idea that different patients can evoke different levels of cytokine to the same Kveim reagents and is consistent with *in vivo* work showing that patients with pulmonary sarcoidosis who are clinically very similar will produce very different levels of skin test reaction to Kveim reagent (Mikhail and Mitchell, 1970).

7.2 PROTEOMIC ANALYSIS OF KVEIM REAGENT AND SARCOIDOSIS SPLEEN TISSUE

The data from chapters 4 & 5 suggest that the candidate antigens within Kveim reagent may be human host proteins (section 7.21) and can be classed as structural (7.22) or non-structural (7.23). Additionally, a number of proteins (which are less likely to represent candidate antigens) have been identified through these methods as being part of the immune response to foreign agents (7.24). Proteins involved in the co-stimulation during the T-cell response towards antigens have been identified with decreased expression in sarcoidosis (7.25). Finally, the proteins identified here show associations with other diseases and provide avenues for further work (7.26).

7.21 IDENTIFICATION OF HOST PROTEINS AS CANDIDATE ANTIGENS

The data generated from 1D-SDS-PAGE and ESI-TOF-MS (chapter 4) represent the first comprehensive analysis of Kveim reagent by any proteomic approach. It also represents the first identification of proteins isolated from validated *in vivo* and freshly prepared *de novo* Kveim reagents which are not found in control spleens from healthy individuals prepared in the same manner as clinically used Kveim reagent. This comparative analysis was taken further by the use of 2D-DIGE and ESI-TOF-MS (chapter 5) to identify for the first time proteins which appear both with increased and decreased abundance in sarcoiodosis tissue compared to healthy controls. It is interesting that a number of proteins are identified from multiple Kveim reagents (table 4.1) and that some of these same proteins are also found over-expressed or specific to sarcoidosis spleen through 2D-DIGE (figure 5.7). This use of two different proteomic methodologies has enabled a more comprehensive analysis of sarcoidosis tissue which has resulted in the identification of more than 80 proteins for further study. 1D-SDS-PAGE analysis of validated *in vivo* Kveim reagent revealed a number of protein identities despite the long term storage of this material at $+4^{O}C$ which would have usually

resulted in the degradation of whole proteins into peptides rendering them difficult to analyse by gel-based proteomic means.

The ESI-TOF-generated mass spectral data was evaluated with two complementary proteomic search engines and searched against both human and non-human databases e.g. micro-organism and viral databases (materials and methods section 2.215). The vast majority of the identifications achieved were retrieved from the human database which suggests that if the candidate T-cell protein antigens have been identified within this data set, they are human host not pathogen-derived proteins. The two complete non-human proteins found (Elongation factor tu and 30S ribosomal protein S2) were both from a single validated Kveim reagent and were shown as possibly arising from a *Pseudomonas* species. In each case, both search engines returned the same identifications , each with high significance scores (table 4.1).

There is one case report in the literature of a patient with known airway colonization from *Pseudomonas aeruginosa* in the setting of bronchiolitis who then developed sarcoidosis (Trisolini et al., 2008). Additionally, there is one report of *P. aeruginosa* causing pulmonary granulomas in an experimental rabbit model of bronchiolitis (Takahashi et al., 2001) but this needs to be considered in the context of the large number of different organisms known to cause pulmonary granulomas in animal models (Swaisgood et al., 2011, Ichiyasu et al., 1999, Rand et al., 2003). It is not inconceivable that proteins from a *Pseudomonal* species indicate the presence of this organism in this particular Kveim reagent and that sarcoidosis may have developed in that patient due to its presence. However given the age of the reagent and the storage conditions, the result could also be considered spurious due to microbial contamination after harvesting.

Much research in sarcoidosis has focused on looking for pathogens within diseased samples with contrasting findings from different parts of the world e.g. the finding of mycobacterial DNA from patients in Europe (Popper et al., 1997), propionibacterial DNA within sarcoid granulomas from Japanese patients (Ishige et al., 1999), Chlamydophila-specific antibodies in sarcoidosis serum and BAL fluid from patients in Germany (Gaede et al., 2002), or the identification of borrelia like organisms in the skin biopsies of patients from Western Australia with sarcoidosis (Derler et al., 2009). Additionally, there is suggestive evidence that some cases of sarcoidosis are initiated by dust or pollen particles (Barnard et al., 2005). Indeed, the different organic and non-organic components identified from patients from around the world make for a confusing picture in understanding the pathogenesis of sarcoidosis. These differing findings, and the fact that despite much investigation no single cause of sarcoidosis worldwide has been found to date, suggest that multi-factoral aetiologies exist with a dependence on geographical location.

However, if sarcoidosis does in fact have multiple initiating factors leading to the same pathology, this would be difficult to reconcile with proteins of a pathogen-derived origin found in Kveim reagent. It has been shown that well-validated Kveim reagents have a high specificity which is constant in different settings across the world. If this historical information is correct and Kveim reagent does contain proteins from pathogens causing disease, it would have to contain proteins from multiple pathogens in order to be effective worldwide. This is not possible. In addition, it seems unlikely that a mycobacterial protein such as mKatG isolated from sarcoidosis lymph node tissue in a patient from North America (Song et al., 2005) in whom the disease is caused by mycobacteria, would elicit an antigen-specific T-cell response in patients from Japan with sarcoidosis caused by propionibacteria.

It can only be possible for validated Kveim reagent from one patient with sarcoidosis to elicit a skin reaction in multiple patients worldwide if they all share a T-cell response to the same protein antigen. If we discard the hypothesis of a unifying (but as yet unfound) single pathogenic cause of sarcoidosis, the candidate proteins within Kveim reagent must be human host proteins to which there is a dysregulated and persistent autoimmune T-cell response which is not observed in patients without sarcoidosis. Evidence to support this hypothesis include the fact that there is a persistent T-cell activity in sarcoidosis which may be due to a loss of immunoregulation due to CD1d-restricted NKT cells (Ho et al., 2005).

This persistent cellular activity might be explained if the target of such activity was a host protein(s) present throughout the human body. This host protein would not be cleared by the immune system in the manner seen with pathogen-related antigens and hence result in the chronic and abnormal immune response in some cases of sarcoidosis. Further support for a host protein as the causative agent within Kveim reagent comes from a recent study investigating potential autoimmune responses in sarcoidosis. A total of 78 unique amino acid sequences (from 44 different proteins) were identified by mass spectrometry after affinity purifying the HLA-DR molecules of lung cells from patients with sarcoidosis and eluting the bound peptides (Wahlstrom et al., 2007). A number of these were identified as host proteins which may have played a role in sarcoidosis.

It is interesting that of the 78 proteins identified through chapters 4 & 5 as being specific to (or of increased abundance in) Kveim reagent and sarcoidosis spleen tissues compared to controls, 8 identicial (or near identical e.g. in the case of β-actin and β-actin like protein) proteins were similarly identified by Wahlstrom et al in 2007 as shown in figure 7.1. It is diffuclt to calculated the probability of these 8 proteins being similar to both this work and that by Wahlstrom et al. However, given the 20,500 protein encoding genes throught to exist

in the human proteome (2010), the calculation can be made using a combinatorics approach (calculation by Dr. Manish Pareek, School of Public Health, Imperial College London) as follows:

Probability = (78 choose 8)*(20422 choose 36)/(20500 choose 44) = 4.755182e-12

This somewhat arbitary calculation makes a number of assumptions (e.g. does not take into account abundance of protein in different body compartments). However, it is exteremely interesting that the proteomics approach taken here to investigate processed sarcoidosis spleen has found 8 proteins of commonality with a method used to isolate T-cell antigens in sarcoidosis lungs. The similarities between these 2 differing approaches in 2 different body compartments lends weight to the argument that a number of the proteins identified in chapters 4 & 5 represent a true proteomic 'signal' in sarcoidosis. It is of additional interest that 3 of these 8 are large structural proteins which are hypothesised as being the reason for the inability to fractionate the antigens by pI (discussed in detail in section 7.14).

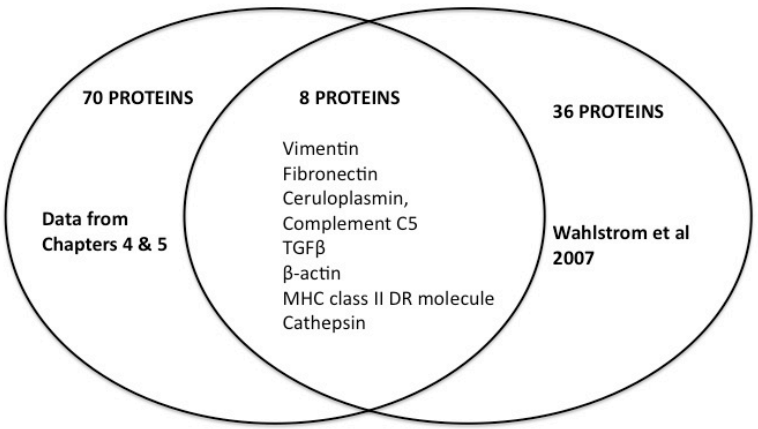


Figure 7.1 Cross over of proteins between data presented here and Wahlstron et al 2007

7.22 CANDIDATE ANTIGENS – STRUCTURAL PROTEINS

Structural proteins comprise the largest group of proteins in the human body and a number of structural proteins including collagen (types 1-3) and keratin were found in all Kveim reagents and control spleens. However, a number of structural proteins were identified as being either specific to Kveim reagent through means of 1D-SDS-PAGE (table 4.1) or present in one or more sarcoidosis spleens at higher abundance than that seen in control spleen through analysis by 2D-DIGE (table 5.6). Many structural proteins are relatively resistant to degradation, structural proteins are generally of high molecular weight and their primary function is to provide a cell or organelle with a scaffolding that helps to maintain the shape. The very nature of this function means that they are inherently stable. Collagen for example (found in all spleens and not sarcoidosis-specific) is the most abundant protein in the human body and has an extensive hydrogen-bonded water network which makes it highly resistant to degradation and changes in temperature. This molecular network is a major contributor to the stabilisation of collagen's triple helices which are subsequently arranged into fibrils (Brodsky and Shah, 1995).

If structural protein(s) are responsible for the Kveim reaction, it is consistant with the fact that some Kveim reagents can remain stable after several years of storage at 4^0C (Siltzbach and Ehrlich, 1954) and indeed why the proteins have survived the actual process of making Kveim reagent itself which included centrifuging, irradiating, phenolisation and heating the material to 56^0C for 2 hours on 2 successive days (Chase, 1961). The active components within Kveim reagent were also found to be relatively protease resistant (Chase, 1961) and whilst this may reflect proteins being cleaved into peptide epitopes which remain antigenic, it may also be due to the proteins themselves being structurally stable. Protease resistance is the nature of a number of the structural proteins identified here e.g. Lamin A/C (Traub et al., 1988) which was found through 1D-SDS-PAGE.

The structural proteins specific to Kveim reagent (through 1D-SDS-PAGE) or found with increased abundance in sarcoidosis spleen (through 2D-DIGE) of interest are listed in table 7.1. A key is given to denote the presence in the literature of associations with sarcoidosis or immune responses towards that protein. Each protein which has been identified in this work has been assigned a level of priority reflecting the summed evidence herein and in the literature suggesting a higher level of interest as a potential protein antigen in sarcoidosis.

Table 7.1 Host structural proteins found specific to Kveim reagent or increased abundance in sarcoidosis spleen when compared to healthy controls. * = published evidence for involvement in sarcoidosis (** indicates published evidence for involvement in sarcoidosis immune response), ^ = published evidence for auto-antibody or autoimmune T-cell responses in other diseases.

Priority	Protein	Method of identification
1	Vimentin ** ^	1D-SDS-PAGE + 2D-DIGE
2	Fibronectin ** ^	1D-SDS-PAGE
3	Tenascin ** ^	1D-SDS-PAGE
4	Beta-actin-like protein 2 ** ^	1D-SDS-PAGE + 2D-DIGE
5	Tubulin beta ** ^	2D-DIGE
6	Desmin * ^	1D-SDS-PAGE + 2D-DIGE
7	Histone H2B *	1D-SDS-PAGE
8	Peripherin-2 *	2D-DIGE
9	Fibrillin-1 *	1D-SDS-PAGE
10	Lamin-A/C *	1D-SDS-PAGE
11	Desmoplakin *	1D-SDS-PAGE
12	Actin type 2 cytoplasmic ^	2D-DIGE
13	Alpha-actinin-4 ^	1D-SDS-PAGE + 2D-DIGE

Table 7.1 does not represent all structural proteins found specific to Kveim reagent (1D-SDS-PAGE) or of increased abundance in sarcoidosis spleen (2D-DIGE) however it does represent potential candidate proteins involved in sarcoidosis or autoimmune responses in other diseases based on published literature. Vimentin, a cytoskeletal component which is a member of the intermediate filament set of proteins, has been found to be both specific to Kveim reagent (1D-SDS-PAGE) and with increased abundance in sarcoidosis spleen (2D-DIGE). Immunofluorescence has previously shown that Vimentin filaments are the major constituents of asteroid bodies (Cain and Kraus, 1983) and Schaumann bodies (Ang and Moscovic, 1996) which are both found within multinucleate giant cells in sarcoid granulomas.

Moreover, antibodies to vimentin have also been used to identify the protein within myofibroblast-type cells in sarcoidosis granulomas (Kaarteenaho-Wiik et al., 2007).

The protein has also been identified by mass spectrometry after affinity-purifying the HLA-DR molecules of lung cells from patients with sarcoidosis and eluting the bound peptides (Wahlstrom et al., 2007). Vimentin was further investigated by the same group who used ELISpot assays to detect INFγ T-cell responses to vimentin in 55% patients with sarcoidosis compared to 13% of healthy controls (Wahlstrom et al., 2009). However, just the one peptide which they had discovered in 2007 (comprising 15 amino acids in length) was used in this investigation, whereas human vimentin has a total length of 466 Amino Acids. It may be that further work to investigate T-cell responses towards peptides compromising the entire structure of vimentin detects a higher percentage of responses. T-cell responses towards a form of structurally altered vimentin have also been demonstrated in one other disease where epitope mapping was used to identify citrullinated vimentin peptides that were presented by HLA-DRB1*0401 molecules to T-cells in patients with rheumatoid arthritis (RA) (Feitsma et al., 2010a).

Vimentin has been found by immunohistochemical staining methods within granulomas of other diseases including synovial metaplasia (Gonzalez et al., 1987), granuloma annulare (Mullans and Helm, 1994), synovial-like hyperplasia around mammary implants (Hameed et al., 1995), angiosarcoma (Sorrentino et al., 2003), epithelioid sarcomas (Kato et al., 2004) and capilliary haemangiomas (Teankum et al., 2008). Vimentin has also been investigated in tuberculosis infection with one study finding significantly greater expression on the surface of infected monocytes, compared with uninfected cells. Mass Spectrometry was subsequently used to show that the protein was involved in binding to the NKp46 receptor which is involved in the lysis of *Mycobacterium tuberculosis* H37Ra-infected monocytes (Garg et al., 2006).

In addition to T-cells, B-cell responses towards vimentin have been demonstrated in a number of diseases, the most prominent of which is RA. Serum antibodies towards citrullinated vimentin have been demonstrated in RA in a number of studies e.g. in one study 193 patients were investigated and the antibody was found to diagnose disease with over 70% sensitivity and 80% specificity and antibody levels were shown related to the level of disease intensity (Wagner et al., 2009). A separate study showed that measurement of serum vimentin antibodies may be more useful in diagnosis of RA than the more widely used anti-cyclic citrullinated peptide antibodies (Wagner et al., 2009). Serum antibodies towards vimentin have also been demonstrated within Reed-Sternberg cells in patients with Hodgkin's disease (Carbone et al., 1990) and within the aqueous humour in patients with glaucoma (Joachim et al., 2007). Vimentin antibodies have also been discussed as a marker of renal transplantation rejection as it is found in endothelial cells and is exposed to the immune system following surgery or by chronic allograft rejection due to endothelial cell breakdown (Carter et al., 2005) (Mi et al., 2005). Antibodies to vimentin have also been found in the spleen of a patient with autoimmune thrombocytic purpura (Varon et al., 1990).

It is interesting to note that studies involving Kveim reagent have shown that a number of patients with rheumatoid arthritis and Hodgkin's lymphoma (but no clinical evidence of sarcoidosis) have tested falsely positive with Kveim reagent (Hurley et al., 1975) (Szpilman and Pazdur, 1974). As anti-vimentin antibodies and T-cells have been described in both these diseases, it is tempting to suggest that vimentin is a cross-reactive protein causing an autoimmune response in a number of diseases, sarcoidosis being one of them. Future studies could examine sarcoidosis serum, spleen and lymph node tissue specifically for anti-vimentin antibodies. Kveim reagent has been investigated for antibodies in the past and a small number of potential anti-Kveim antibodies were found through 1D-SDS-PAGE and subsequent immunoblotting but none have been identified (Stahl and Veien, 1981).

In addition to investigations for antibodies and T-cell responses towards vimentin, it would be interesting to look for T-cell responses towards modified vimentin e.g. proteins which have undergone citrullination (post-translational modification of arginine into citrulline) as seen in RA. Such modifications need more sophisticated interpretation than available by the type of Mass Spectrometry analysis performed here. Citrullination was not specifically investigated in previous work identifying vimentin as a potential target of the adaptive immune response in sarcoidosis (Wahlstrom et al., 2007). Autoimmune T-cell responses towards modified vimentin which has undergone post-tranlastional modifications, as well as to peptides from the entire protein itself, may warrant further investigation.

In addition to vimentin, a number of other structural proteins (table 7.1) may have relevance in sarcoidosis disease pathogenesis. One of particular interest fibronectin is a large (400 KDa in size) glycoprotein that resides in the cell's extracellular matrix. It has been identified specifically from BAL of patients with sarcoidosis (O'Connor et al., 1988) and it has also been postulated that BAL levels of the glycoprotein may be used as a surrogate marker of disease activity as detected levels were higher in sarcoidosis patients than in matched patients with tuberculosis (Albera et al., 1991). Alveolar macrophages from patients with sarcoidosis have also been shown to have increased levels of fibronectin (Spiteri et al., 1992) and the glycoprotein has also been found consistently at the periphery of sarcoidosis granulomas in a concentric pattern (Shigehara et al., 1998). One peptide from fibronectin was also identified through mass spectrometry after affinity purifying the HLA-DR molecules of lung cells from patients with sarcoidosis and eluting the bound peptides (Wahlstrom et al., 2007).

The presence of serum antibodies towards fibronectin have been investigated in several diseases including scleroderma (Taova and Kurdanov, 1996) and SLE where levels were found to be higher in disease than controls and related to disease activity (Atta et al., 1995). If fibronectin is a target of the adaptive immune system in sarcoidosis it may explain some of the clinical similarities seen between the disease and SLE including the multisystem nature

and unknown aetiology of disease. Mutated fibronectin has been demonstrated as a CD4+ T-cell autoantigen in tumour metastasis (Wang et al., 2002) and in RA, synovial T-cells have been found which selectively bind to fibronectin when compared to T-cells from the peripheral blood (Rodriguez et al., 1992). This binding suggests a mechanism for the ongoing chronic joint inflammation seen in RA and if fibronectin is involved in sarcoidosis, the mechanism of antigen-induced chronic inflammation may be similar in both diseases. If it is a candidate antigen, the very large size of fibronectin (like vimentin) may also explain why fractionation by pI using the offgel fractionator was unable to selectively fractionate the antigen (figure 3.9).

Tenascin, like fibronectin is an extracellular matrix glycoprotein. Through immunohistochemical staining methods it has also been found in lung tissue of patients with sarcoidosis and fibrosing alevolitis (Wallace et al., 1995). Tenascin has also been found specifically elevated in the pleural fluid of patients with sarcoidosis compared to the serum, suggesting active synthesis of tenascin in the lower respiratory tract in the disease (Kaarteenaho-Wiik et al., 1998). Importantly, antibodies directed towards tenascin have been demonstrated through western blotting of lung biopsies in sarcoidosis and interstitial lung disease where the glycoprotein has been shown associated with collagen fibers and within type 2 pneumocytes. The authors of this study also suggest tenascin as a prognostic marker as it appeared related to the levels of fibrosis in lung disease (Kaarteenaho-Wiik et al., 1996).

Aside from findings in sarcoidosis, the isoform of tenascin-C is found in the tumor extracellular matrix of a number of solid cancers and tenascin antibodies have been demonstrated in lung cancers (Silacci et al., 2006). Conversely, tenascin has been shown to inhibit (rather than activate) proliferation of T-cells (Hemesath et al., 1994) as well as suppress CD3-mediated T-cell activation (Hibino et al., 1998) and inhibit the polarisation and transmigration of T-cells (Huang et al., 2010). One could speculate that the potential role of tenascin in sarcoidosis (in addition to being a candidate antigen) may be to suppress systemic

T-cell activation and hence be responsible for the systemic immunosuppression seen in patients where there is a decrease in delayed type hypersensitivity reactions (Mathew et al., 2008). This inhibition of transmigration and proliferation of T-cells concurs with previous work showing a reduction of IL-2 mediated proliferation when PBMCs are incubated with Kveim reagent (Lyons et al., 1991) as well as the inhibition of leucocyte migration in the presence of Kveim reagent (Willoughby and Mitchell, 1971).

Desmin, like vimentin, is a member of the intermediate filament set of proteins and has been demonstrated within multinucleated giant cells which constitute the granulomatous response seen in sarcoidosis (Drut and Drut, 1986) as well as nonspecific cytoplasmic bodies in sarcoidosis (Caron et al., 1999). Desmin is associated with other granulomatous diseases such as granulomatous myopathy where the protein has been shown expressed by atrophic muscle fibres (Tews and Pongratz, 1995). Serum antibodies to desmin have been demonstrated in a number of diseases including coeliac disease (Teesalu et al., 2001), pulmonary lymphangioleiomyomatosis (Matthews et al., 1993), and rhabdomyosarcomas (Osborn et al., 1986). Kveim reactions have been found falsely positive in patients with coeliac disease and this could potentially be explained by autoimmune responses to desmin in both diseases (Karlish et al., 1972).

Beta-actin has previously been postulated as an autoantigen in sarcoidosis as 4 different peptides from the protein were independently identified as potential T-cell antigenic targets through Mass Spectrometry after affinity purifying the HLA-DR molecules of lung cells from patients with sarcoidosis and eluting the bound peptides (Wahlstrom et al., 2007). Numerous studies have demonstrated antibodies to actin at high levels in the serum of patients with autoimmune hepatitis (Liaskos et al., 2007) and coeliac disease where levels have shown correlation with the extent of intestinal mucosal damage (Carroccio et al., 2005). As previously mentioned, there is evidence of Kveim false-positive reactions in patients with

coeliac disease and no clinical evidence of sarcoidosis possibly attributable to a link between the two diseases through the cross reactivity of proteins, one of which may be beta-actin.

Tubulin has been found in one study at high concentrations within Schaumann bodies in sarcoidosis granulomas (Ang and Moscovic, 1996). More importantly, it has been postulated as an auto-antigen in a number of diseases including sarcoidosis where antibodies to the protein were detected using western blotting in patients with sarcoidosis related uveitis (Okunuki et al., 2008). Antibodies against tubulin have been identified in a number of other diseases including elevated levels in the cerebrospinal fluid of patients with multiple sclerosis (Svarcova et al., 2008) and SLE (Cullum et al., 1991). More recently, CD4+ T cells specific for β-tubulin have been hypothesised as being responsible for autoimmune disorders of the inner ear as seen in murine models (Zhou et al., 2011).

A number of the remaining structural proteins identified in table 7.1 show evidence for autoimmune responses in other diseases, despite there being no published literature of an association with sarcoidosis. For example, antibodies to desmoplakin have been found in erythema multiforme (Cozzani et al., 2011) and pemphigus (Jiao and Bystryn, 1998). Antibodies towards histone H2B have been identified in procainamide-induced lupus (Rubin et al., 1985), scleroderma (Wallace et al., 1994) and in HIV where levels have been found related to disease activity (Williams et al., 1996). Antibodies to fibrillin 1 have been found at higher levels in the serum of women with recurrent pregnancy loss than in controls (Atanasova et al., 2011), antibodies to lamin have been found elevated in the serum of patients with SLE (Senecal et al., 1999). Murine models of autoimmune diabetes show associations with T-cells (Boitard et al., 1992) and antibodies (Strom et al., 2010) directed towards peripherin. All the structural proteins identified in table 7.1 have the ability to cause autoimmune antibody or T-cell responses in a number of diseases. Many have been implicated at some level in sarcoidosis and some such as vimentin, fibronectin and tubulin

have themselves been identified as potential self antigens in sarcoidosis. All warrant further investigation in the disease.

7.23 CANDIDATE ANTIGENS – NON-STRUCTURAL PROTEINS

In addition to structural proteins, a number of other proteins found specific to Kveim reagent (1D-SDS-PAGE) or with increased abundance in sarcoidosis spleen (2D-DIGE) have potential as candidate antigens in sarcoidosis. These proteins are listed in table 7.2. A key is given to denote the presence in the literature of associations with sarcoidosis or immune responses towards that protein. Each protein which has been identified in this work has been assigned a level of priority reflecting the summed evidence herein and in the literature suggesting a higher level of interest as a potential protein antigen in sarcoidosis.

Table 7.2 Host non-structural proteins found specific to Kveim reagent or increased abundance in sarcoidosis spleen when compared to healthy controls. * = published evidence for involvement in sarcoidosis (** indicates published evidence for involvement in sarcoidosis immune response), ^ = published evidence for auto-antibody or autoimmune T-cell responses in other diseases.

Priority	Protein	Method of identification
1	Superoxide dismutase ** ^	1D-SDS-PAGE
2	Ceruloplasmin ** ^	1D-SDS-PAGE
3	Cathepsin G ** ^	1D-SDS-PAGE
4	Complement C3 / C5 ** ^	1D-SDS-PAGE
5	Transforming growth factor-beta ** ^	1D-SDS-PAGE
6	Thioredoxin *^	1D-SDS-PAGE
7	Serotransferrin ** ^	1D-SDS-PAGE
8	Annexin A2/A5/A6 *^	1D-SDS-PAGE
9	Peroxiredeoxin 2/4 *^	1D-SDS-PAGE
10	Carbonic anyhydrase ^	2D-DIGE
11	Nucleophosmin ^	1D-SDS-PAGE

A number of enzymes have been discovered specific to Kveim reagent by means of 1D-SDS-PAGE. One of these is superoxide disumatase (Sod) which is primarily responsible for the conversion of oxygen radicals into hydrogen peroxide. The enzyme was specifically found in

9 out of 14 Kveim reagents and in no controls (table 4.2), making it the top protein in terms of the number of Kveim reagents within which it was found. It is an extremely common protein found in almost all living cells but interestingly, a mycobacterial form of the enzyme (SodA) has been investigated in sarodosis before. Experiments on SodA are based on the knowledge that it elicits strong immune responses in tuberculosis infected mice (Andersen, 1994). With a background of this finding, researchers identified SodA from sarcoidosis tissue using Polymerase Chain Reaction (Drake et al., 2002). They later detected SodA amplicons in 12 of 17 (71%) sarcoidosis specimens, compared to 2 of 16 (13%) controls ($p = 0.001$). Peripheral blood IFNγ ELISpot analysis found that 6 of 12 (50%) sarcoidosis subjects recognised sodA peptides, compared to 1 of 26 (4%) PPD negative controls ($p = 0.002$) and six out of 11 (55%) of PPD positive subjects (Allen et al., 2008).

A follow up to this work has been the development of a mouse model of pulmonary sarcoidosis (see section 1.63) where SodA was injected to cause mouse pulmonary granulomas and subsequent analysis demonstrated CD4+ T-cell responses against SodA in the mice BAL (Swaisgood et al., 2010). One explanation for the data found here is that a human form of Sod, rather than a mycobacterial form, may serve as an autoantigen in sarcoidosis.

There is little other work on assessing T-cells towards Sod but managanese superoxide dismutase has been found at increased levels in sarcoidosis granulomas compared to controls (Lakari et al., 2000). Antibodies towards superoxide disumatase have been postulated as potential biomarkers in a number of unrelated diseases including autoimmine hepatitis in humans (Miyata et al., 1995) and brucella in cattle (Tabatabai and Hennager, 1994).

Another enzyme of interest identified here is the copper carrying enzyme ceruloplasmin. Ceruloplasmin was previously identified after eluting the bound peptides from HLA-DR molecules of lung cells from patients with sarcoidosis (Wahlstrom et al., 2007). Elevated

levels of the enzyme have also been found in the serum of patients with sarcoidosis (Koskelo et al., 1966) but there are few disease associations published elsewhere. Carbonic anhydrase, another identified enzyme, is expressed on alveolar epithelium and is involved in carbon dioxide metabolism. It was found here significantly elevated (1.8 fold, $p = 0.043$) in sarcoidosis spleen compared to controls (table 5.5). There appear to be no other reported associations of carbonic anhydrase in sarcoidosis but serum antibodies towards the enzyme have been reported with lung involvement in systemic sclerosis (Alessandri et al., 2003). Serum antibodies have also been described in a number of other non-respiratory diseases including autoimmune pancreatitis (Aparisi et al., 2005) and recurrent pregnancy loss (Karahan et al., 2009).

Cathepsins are proteases that have a vital role in mammalian cellular turnover and are distinguished by their substrate specificities. There are at least twelve members in this enzyme class, which are distinguished by their structure, catalytic mechanism, and which proteins they cleave. Cathepsin G, which was found specific to Kveim reagent (1D-SDS-PAGE) is a serine protease. Serum antibodies towards the protein have been found significantly elevated in inflammatory bowel disease and the level of anti-cathepsin G has been shown to be related to severity of disease in ulcerative colitis (Kuwana et al., 2000). Inflammatory bowel disease (IBD) has a number of described clinical similarities with sarcoidosis including the hallmark granulomas and the unknown aetiology of disease. In addition, there are reports of false positive Kveim reactions in patients with Crohn's disease who have no evidence of sarcoidosis (Karlish et al., 1972) and cathepsin could theoretically serve as a self antigen in both diseases.

In humans, peroxiredoxins represent a class of 6 enzymes which are involved in redox regulation of the cell through hydrogen peroxide metabolism. Peroxiredoxins 1, 2 and 4 have been shown specific to Kveim reagent (1D-SDS-PAGE). Peroxiredoxins 1 and 3 have shown prominent and cell specific expression in sarcoidosis granulomas and alveolar macrophages

and are hypothesised as contributing towards lung damage (Kinnula et al., 2002). Serum antibodies towards peroxiredoxin-2 have been found significantly higher in systemic vasculitis than in controls with collagen disorders but no vasuclitis and the levels of antibody correlated strongly with other markers of disease such as serum d-dimers (Karasawa et al., 2010).

In addition, some of the proteins identified herein (table 7.2), are involved with both innate and adaptive immune processes. These proteins may simply represent the ongoing immune activation pathways seen in sarcoidosis (which is why they are found specific to Kveim reagent and not identified from controls) but interestingly, a number of them may be targets of the immune system itself in sarcoidosis. These include Complement C3 and C5, which are intricately involved in the complement pathway as the first line of defence against pathogens. A number of studies have unsurprisingly found high levels of Complement in sarcoidosis e.g. elevated levels in the BAL (Lambre et al., 1986b) and the pleural fluid (Lambre et al., 1986a). However, Complements have been hypothesised themselves as targets of the adaptive immune system in sarcoidosis. Multiple peptides from Complement proteins C2, C4, C5 and C6 were identified by Mass Spectrometry after affinity purifying the HLA-DR molecules of lung cells from patients with sarcoidosis and eluting the bound peptides (Wahlstrom et al., 2007). Serum antibodies towards Complement proteins have been identified as being specific to SLE when compared to controls (Daha, 2008). Another immune mediator found by Wahlstrom et al is Transforming Growth Factor Beta (TGFβ) which is responsible for differentiation and proliferation of a number of human cells. TGFβ was found specific to Kveim reagent (1D-SDS-PAGE) but aside from sarcoidosis, T-cell responses and antibodies directed towards TGFβ do not appear to have been described in any other disease.

The remaining proteins identified herein have a number of diverse functions. Nucleophosmin for example, a protein was found specific to Kveim reagent (1D-SDS-PAGE) has a number of functions including host-virus interactions, ribosome biogenesis and protein chaperoning. It

has not been previously associated with sarcoidosis but antibodies towards the protein have been demonstrated in a mouse model of SLE where the levels have been found correlated with the more traditionally used anti-cardiolipin antibodies (Lartigue et al., 2005). In addition to antibody responses, an increased expression of nucleophosmin has been found in some tumours and the protein has been recognised by a cytotoxic T-cell line derived from a rectal carcinoma patient (Swoboda et al., 2010).

Serotransferrin is an iron binding protein and levels have been found elevated in the BAL of patients with sarcoidosis compared to controls (Shigemura et al., 2010). Investigations of alveolar macrophages in sarcoidosis have revealed that serotransferrin receptor expression on these cells may be related to disease as higher expression appears related to lower lung function (Haslam et al., 1990).

Annexins are membrane binding proteins with multiple functions. Annexins 2,5 and 6 have all been identified as specific to Kveim reagent (1D-SDS-PAGE). There is one genetic study involving annexins and sarcoidosis which found a single nucleotide polymorphism (SNP) in annexin 11 as being associated with the disease (Fatimathas and Moss, 2009). Serum antibodies towards annexin have been described in a number of studies and purported as potential biomarkers in both systemic sclerosis (Habeeb et al., 2010) and SLE (Kretz et al., 2010). Another protein found specific to Kveim reagent, thioredoxin, acts primarily as an antioxidant and has been shown to play a role in sarcoidosis as antibody staining has been used to identify thioredoxin in sarcoidosis lung and lymph node granulomas but not in nodes of patients with other granulomatous diseases. The same study hypothesised that thioredoxin might act as a local inducing factor for IL-2 receptor expression on T-cells (Koura et al., 2000). In atopic dermatitis, T-cells specific for thioredoxin have been implicated as a cause of skin inflammation due to cross reactivity of these cells with fungal thioredoxin (Balaji et al., 2011). It is possible that one, or several, of the proteins described here may serve as the antigens at the heart of the Kveim reaction.

7.24 PROTEINS INVOLVED IN INNATE AND ADAPTIVE IMMUNE RESPONSES

Although Aim 1 of this work sought to identify candidate antigenic proteins from Kveim reagent, a number of other proteins of interest were found through 1D-SDS-PAGE and 2D-DIGE. Although these proteins are unlikely to represent candidate antigens (based on litrature searches of their function and previous involvement in other diseases), some are of potential interest in further understanding the immune processes involved and the aetiology of sarcoidosis. A number of proteins identified are associated with a general disease state e.g. enzymes such as catalase, metabolic proteins such as Fructose-1,6-bisphosphatase and Medium-chain specific acyl-CoA dehydrogenase. These identifications are of limited value as they will be associated with most diseased tissues. Of the remainder, a significant proportion of proteins of interest are those associated with the immune response and can be subdivided into three major groups as detailed in table 7.3.

Table 7.3 Proteins of immunological interest found specific to Kveim reagent or over-expressed in sarcoidosis spleen compared to healthy controls. All proteins identified through 1D-SDS-PAGE only aside from Neutrophil gelatinase-associated lipocalin which was additionally identified through 2D-DIGE. Number in brackets represents number (out of 14) of Kveim reagents in which the protein was found through 1D-SDS-PAGE.

Direct response to pathogens	Innate immune response	Adaptive immune response
Dermicidin (8)	Neutrophil gelatinase-associated lipocalin (5)	Membrane primary amine oxidase (6)
Proteasome subunit alpha type-4 (2)	Complement proteins C3 (1), C5 (1)	Dipeptidyl peptidase 4 (4)
Nucleophosmin (2)	Cathepsin G (2)	Neutrophil elastase (4)
Cathelicidin antimicrobial peptide (1)	Ig lambda (3) / kappa chain C regions (3)	TGF-β (3)
		HLA class II histocompatibility antigen, DRB1 (5)

Four of the proteins in table 7.3 are directly involved with pathogen interaction with the immune system but none of them have been previously described as being associated with sarcoidosis. Dermicidin appears to be highly specific to Kveim reagent having been identified

in 8 out of 14 Kveim reagents but in 0 out of 7 control spleens. The protein displays antimicrobial activity in the skin against potential pathogens in the first few hours after bacterial colonisation and is highly effective against a number of organisms including *E. coli, E. faecalis, S. aureus* and *C. albicans* (Uniprot database version 2011_05). One possible method of the protein's antimicrobial action has been identified through a mutant screen showing the interaction of dermicidin with bacterial membrane phospholipids (Li et al., 2009). Cathelicidin antimicrobial peptide is another protein with similar actions to dermicidin which binds directly to bacterial lipopolysaccharides (LPS). As with dermicidin, it has been shown to be effective against a number of pathogens, particularly those which colonise the skin (Lee et al., 2005) but additionally it has been shown that serum levels of the protein correlate with acid fast bacilli sputum smear positivity and weight loss in patients with active pulmonary tuberculosis (Yamshchikov et al., 2010). Both proteasome subunit alpha type-4 and nucleophosmin are proteins involved in host virus interaction e.g. Hepatitis B virus core has been shown to interact with nucleophosmin (Lee et al., 2009).

In addition to those proteins directly engaged with pathogenic infections, 6 proteins identified as being specific to Kveim reagent are primarily involved with the innate immune response. Neutrophil gelatinase-associated lipocalin (also called lipocalin-2) has no published association with sarcoidosis but it has been investigated in a number of other diseases. It's main mechanism of action is thought to be through the Toll-like receptors on immune cells which on encountering bacteria stimulate transcription, translation and secretion of the protein which then limits bacterial growth by sequestrating the iron-laden siderophore (Flo et al., 2004). Cathepsin G is a serine protease which has been investigated in sarcoidosis as previously discussed (7.23) and one of its many actions is the cleavage of complement 3 as part of the complement cascade in innate immunity (Matsuda et al., 1985). The Complement proteins themselves (C3, C5) have been identified as being unique to Kveim reagent (1D-SDS-PAGE) and are a series of proteins involved in multiple pathways of the innate immune system. Their relevance to sarcoidosis has been previously discussed (7.23). Immunoglobulin

light chains have also been found specific to Kveim reagent suggesting a heightened B-cell driven antibody response at the centre of Kveim reagent which is not found in control tissues.

In addition, 5 proteins associated with adaptive immunity were identified as being specific to Kveim reagent (1D-SDS-PAGE). Membrane primary amine was specifically identified in 6 out of 14 Kveim reagents but there is no previous published association of the protein with sarcoidosis. It functions as a cell adhesion protein that participates in lymphocyte recirculation by mediating the binding of lymphocytes to peripheral lymph node vascular endothelial cells (Smith et al., 1998). Dipeptidyl peptidase 4 is a cell surface glycoprotein receptor involved in activation of T-cells through the T-cell receptor (TCR) (Ikushima et al., 2000). Animal models of asthma have postulated a role for Dipeptidyl peptidase 4 in allergic responses in the lung through allergen-specific T-cell recruitment (Schade et al., 2008). This same method may be applicable to the T-cell recruitment identified in sarcoidosis (Iannuzzi et al., 2007). Neutrophil elastase is a protein known to modify the functions of natural killer cells, monocytes and granulocytes (Duan et al., 2004). It has been investigated in a number of diseases including sarcoidosis where BAL levels were found higher in patients than in controls and levels were related to extent of pulmonary disease (Peros-Golubicic et al., 2001). TGF-β is a signaling molecule with a large number of functions in the immune system as described in detail in section 7.23. Finally, an HLA-DR antigen was also specifically found within Kveim reagent suggesting antigen presentation to CD4+ T-cells. This is well recognised as being the immune hallmark of sarcoidosis.

These 15 proteins identified as being specific to Kveim reagent represent the first proteomic evidence in sarcoidosis to reveal proteins that are associated with multiple arms of the immune system. They support the widely held assumption of an abnormal immune response towards an as yet unknown pathogen(s) in sarcoidosis (Iannuzzi et al., 2007). Proteins such as cathelicidin antimicrobial peptide potentially suggest a response to bacterial products within

the spleen tissue at some point in the pathogenesis of sarcoidosis whilst the identification of neutrophil gelatinase-associated lipocalin, complement and dipeptidyl peptidase suggest a defence process that involves both the innate and adaptive arms of the immune system.

7.25 PROTEINS OF DECREASED ABUNDANCE IN SARCOIDOSIS ARE INVOLVED IN T-CELL CO-STIMULATION

2D-DIGE analysis identified a total of 7 of proteins that were downregulated in sarcoidosis spleen tissue compared to healthy controls (table 5.6). Of these, plastins 1,2 and 3 and cofilin 1 are involved in the effector T-cell response through the binding of actin. Plastins play a key role in adaptive immunity through actin binding and the activation of T-cells in response to co-stimulation through TCR/CD3 and CD2 or CD28 (Kindt et al., 2006). Actin rearrangement is critical for T-cell receptor engagement and a recent mouse model study using LPL (-/-) mice found that T-cells from the animals were markedly defective in TCR-mediated cytokine production and proliferation and the mice also showed a delayed rejection of skin allografts after release from immunosuppression (Wang et al., 2010). A related study using photon microscopy showed that LPL (-/-) mice had T-cells of reduced velocity and motility in lymph nodes and ascribed this to Plastin being critical for CCR7-mediated cell motility (Morley et al., 2010).

Cofilin-1 is also responsible for actin binding, cofilins have been identified as key proteins in co-stimulation-dependent reorganisation of the actin cytoskeleton which is responsible for T-cell activiation through proliferation and cytokine production (Eibert et al., 2004). There is no previously published data on the role of Plastin or Cofilin in sarcoidosis or other granulomatous diseases. Additional evidence for defects in T-cell co-stimulation come from genetic studies. A recent genome wide association study showed an association of the BTNL2

(butyrophylin-like 2) gene with sarcoidosis (Valentonyte et al., 2005). BTNL2 is a B7 family member which functions as a negative co-stimulatory molecule in antigen presentation to T-cells.

One possible explanation for the finding of decreased abundance of these proteins in sarcoidosis is that defects in Plastin and Cofilin associated genes may lead to a defect in co-stimulation and hence problems in the appropriate T-cell activation when antigens are presented as part of the adaptive immune response. An inadequate response to specific inciting antigens may be responsible for failure to clear a pathogen and the formation of granulomas at the site of pathogen entry and the subsequent development of sarcoidosis in susceptible individuals. Work to investigate this further could inlucde measurement of plastin and cofilin-1 mRNA levels in sarcoidosis PBMCs and avidity assays to measure the strength of T-cell receptor binding in sarcoidosis cells compared to controls.

7.26 PROTEOMIC EVIDENCE FOR SIMILARITIES OF SARCOIDOSIS WITH OTHER DISEASES

A number of proteins identified herein have other disease associations. One such disease is systemic lupus erythematosus (SLE) which is an autoimmune connective tissue disorder. SLE is a multi-system disorder of unknown aetiology but with a potential (unknown) environmental trigger in genetically susceptible individuals (Hess and Farhey, 1995). Antibodies such as serum anti-fibronectin and serum anti-nucleophosmin (the proteins themselves are identified here in Kveim reagent) play a key role as targets of the immune system in SLE. In addition, KEGG pathway analysis shows that 4 of the specific Kveim identified proteins are involved in a biological pathway of known association with SLE (figure 4.8). Unlike sarcoidosis, the molecular mechanisms of SLE have been intensively

investigated and there are a number of clinically useful biomarkers and quantitative antibody tests used for diagnosis and prognosis.

There is little information on Kveim skin test reactions in patients with SLE but there are numerous reported associations of both diseases in the same patient groups (Wesemann et al., 2009). It may be that both diseases share a similar pathogenic trigger(s) in some cases or that the immune responses, in terms of antibody or T-cell response, are similar in both diseases. There is little published work on diagnostic biomarkers of SLE in patients with sarcoidosis but one study found anti nuculear antibodies and anti-double-stranded DNA antibodies (both recognised disease markers in SLE) in a significant proportion of sarcoidosis patients with no clinical features of SLE (Weinberg et al., 2000). Given the potential degree of overlap it seems clear that new studies using a variety of recognised SLE biomarkers in sarcoidosis patients is needed.

Some Kveim reagent proteins have also been associated with disease processes in Rheumatoid Arthritis (RA) (see section 7.23) and Inflammatory Bowel Disease (IBD). RA is a multisystem inflammatory disorder which primarily affects the joints and like sarcoidosis there is much speculation about possible environmental triggers in the disease (Becker and Winthrop, 2010). In IBD and ulcerative colitis significantly elevated serum levels of anti-cathepsin G antibodies have been reported along with false positive Kveim reactions in patients with IBD but no clinical evidence of sarcoidosis. IBD, in particular Crohn's disease, shares a number of clinical traits with sarcoidosis including the granulomatous nature of the lesions in both diseases and the possible but as yet unidentified infectious agent trigger(s) which may be inhaled to cause pulmonary sarcoidosis or ingested to cause Crohn's disease.

The similarities between the proteins found in Kveim reagent and that seen in RA and IBD may point towards a shared aetiology in some cases or perhaps a similar immune response to different pathogens. SLE, RA, IBD and sarcoidosis are all examples of so called 'autoimmune' or 'immune-mediated inflammatory' disorders which are due, in theory, to a failure in the normal balance between immune activation and control (Kindt et al., 2006). This can happen for a number of reasons, one of which is the theory of molecular mimicry whereby the sequence similarities between pathogen-derived antigens and self antigens result in an ongoing immune activation long after an initial infection has been cleared, as has been hypothesised in SLE (Agmon-Levin et al., 2009)**.** This possibility would lead to the ongoing B- and T-cell activation seen in sarcoidosis, as well as in other diseases, to a self antigen.

The discovery of primairly human host proteins as being specific to Kveim reagent means that immune responses to self proteins may be involved in sarcoidosis. In particular, structural host proteins which persist at the site of inflammation may be the targets of the Kveim reaction itself. These proteins may be common across a number of diseases which would explain the false positive Kveim responses in patients with diseases such as RA and IBD who have no clinical manifestations of sarcoidosis (Karlish et al., 1972). Furthermore, there is the possibility of epitope spreading i.e. reactions against one self-antigen results in further antigens being released and B- and T-cells beginning to react to these new antigens. This phenomenon has been detailed in RA with the identification of serum antibodies to multiple citrullinated proteins before the onset of clinical disease (van der Woude et al., 2010) and may also explain why diseases such as sarcoidosis begin as acute but in some cases become chronic and self-perpetuating.

The only widespread biomarkers for sarcoidosis in general use are serum ACE levels which are unhelpful for diagnosing disease (due to false positives in a number of other diseases) but

may be useful as part of a range of investigations e.g. serial lung function and chest radiographs in assessing prognosis or response to treatment. T-cell responses and serum antibodies towards some of the proteins identified here as being specific to Kveim reagent need to be further investigated for their potential to diagnose and monitor disease.

7.3 CYTOKINE ANALYSIS OF SARCOIDOSIS BAL AND SERUM

The data from chapter 6 show that the profiles of investigated cytokines in the BAL are indistinguishable between acute pulmonary sarcoidosis and active pulmonary tuberculosis (section 7.31). Profiles in serum differ significantly with respect to IL-4 and allow for reliable differentiation of diseases (7.32).

7.31 BAL CYTOKINE PROFILES IN ACUTE PULMONARY SARCOIDOSIS AND ACTIVE PULMONARY TUBERCULOSIS ARE IDISTINGUISHABLE

Cytokine profiles in acute pulmonary sarcoidosis and active pulmonary tuberculosis BAL appear indistinguishable from each other (figure 6.1). Significantly elevated TNFα (compared to healthy BAL) was expected from previous studies (Baughman et al., 1990, Tsao et al., 2000) but is shown here for the first time to correlate closely between both diseases. This may represent an acute phase response not seen in healthy lungs but TNFα has a number of specific functions including activation of macrophages and granuloma formation (Chensue et al., 1994).

The Th2 cytokines IL-4 and IL-13 were significantly raised in both diseases compared to healthy BALs. To date, sarcoidosis BAL T-cell studies have been inconsistent with levels of

spontaneous IL-4 secretion found similar (Hoshino et al., 1995) but mRNA expression of IL-13 found significantly increased, compared to healthy controls (Hauber et al., 2003). There appears to be limited previous work investigating IL-13 BAL levels in tuberculosis but one study found increased IL-4 secretion from both CD4+ and CD8+ T-cells in active pulmonary tuberculosis (van Crevel et al., 2000).

The compartmentalisation of cytokines shows identical patterns between the sarcoidosis and tuberculosis groups. Of particular interest are the Th2 cytokines IL-4, IL-5 and IL-13 which are all found at higher levels in the BAL than the serum in both diseases (figures 6.7 and 6.8). IL-2 is already known to be compartmentalised in patients with acute pulmonary sarcoidosis with higher levels in the BAL than the serum with the hypothesis that the IL-2 seen at the site of disease is needed for T-cell proliferation in response to a particular antigen (Konishi et al., 1988). The data here show for the first time that the Th2 cytokines IL-4, IL-5 and IL-13 are also present at higher levels in the BAL than the serum in both sarcoidosis and tuberculosis patients, which contrasts sharply with findings in healthy volunteers.

The indistinguishable cytokine profile identified through the direct comparison of multiple cytokine analysis in the BAL between matched patients with sarcoidosis and tuberculosis lends weight to the hypothesis that there is an identical host immune response in the acute stage at the site of pathology in both diseases. Identical BAL levels indicate that this immune response is similar in both diseases, regardless of the final diagnosis.

7.32 DIFFERENCES IN SERUM CYTOKINES BETWEEN SARCOIDOSIS AND TUBERCULOSIS

The serum data indicate for the first time an identical significantly enhanced Th1 cytokine response (as demonstrated by INFγ and TNFα) in both diseases compared to healthy controls (figure 6.3). There is also the suggestion of a reduced Th2 response in both diseases but this

was only statistically significant for IL-4 in both diseases and IL-13 in tuberculosis (after adjustment for multiple corrections). There is limited published data on serum cytokine profiles in either disease but tuberculosis studies have found an elevated serum INFγ compared to controls (Guntupova et al., 2006, Tsao et al., 2002).

Median serum levels of IL-4, IL-5 and IL-13 were higher in sarcoidosis compared to tuberculosis with adjusted statistical significance achieved for IL-4 (figure 6.4). Significant differences remained when acute pulmonary sarcoidosis only (rather than all cases) were compared with active pulmonary tuberculosis (figure 6.5). Two of the five tuberculosis patients with measurable IL-4 had evidence of extra pulmonary (abdominal) disease but there was nothing else clinically specific about them compared to the majority with undetectable levels. These differences allowed for the creation of a regression analysis model to differentiate between both diseases with 91% accuracy (figure 6.6) which dropped to 73% when cross validation techniques were employed, perhaps representing a more accurate level of differentiation.

The intracellular IL-4 content of peripheral blood lymphocytes has been shown diminished in patients with pulmonary tuberculosis compared to controls (Aktas et al., 2009) and a selective increase in mRNA of the IL-4 antagonist IL-4δ2 in these cells has been identified in latent tuberculosis infection compared to healthy controls (Demissie et al., 2004). Both studies support the finding here of very low levels of serum IL-4 in the majority of patients with pulmonary tuberculosis. IL-4 has been investigated in other infective diseases where levels were found significantly reduced in HIV-1 infection (Biglino et al., 1996) and CMV pneumonia (Chen et al., 2007).

A plausible explanation for the BAL similarities but serum differences is that both sarcoidosis and tuberculosis share similar aetiological triggers leading to identical granulomatous type

host immune responses in the lungs. Alternatively, it may be that similar classes of antigen are presented in the lungs in both diseases. These hypotheses are supported by indistinguishable levels of all cytokines measured in the BAL spanning the spectrum of pathogen response including T-cell stimulation (IL-2, IL-12), macrophage activation (IL-1b) and granuloma formation (TNFα, IL-8). However, despite similar initial responses at the site of disease, the systemic response towards this trigger varies between individuals as evidenced by significantly higher serum of IL-4 in sarcoidosis and the suggestion of higher of IL-5 and IL-13. IL-4 is associated with allergic inflammatory responses as typified by asthma (Lloyd and Saglani, 2010). The increased serum levels in sarcoidosis may therefore reflect a persistent and ongoing systemic inflammatory allergic type response to an antigen not completely cleared by the immune system. This is in contrast with the lower serum levels seen in patients with tuberculosis. The persistence (and perhaps in increase in-table 6.4) of IL-4 seen in chronic pulmonary sarcoidosis supports the theory of ongoing inflammation leading to chronic disease.

A number of diseases of abnormal host immunity such as systemic sclerosis (Boin et al., 2008) and chronic graft vs. host disease (De Wit et al., 1993) are characterized by Th2 cells secreting IL-4 which subsequently plays a role in the activation and recruitment of B cells producing IgE, mast cells and eospinophils. The higher levels found in sarcoidosis may therefore account for the immune complexes and peripheral antibody response which are stronger than that seen in tuberculosis (Selroos et al., 1980). Elevated IL-4 suggests that, similar to trials in asthma, the use of selective IL-4 antagonists or soluble recombinant IL-4 receptors should be investigated for their efficacy to reduce disease activity in sarcoidosis (Ricci et al., 1997). Further investigations of serum cytokine profiles may provide information to strengthen the argument of a systemic allergic type cytokine response seen in sarcoidosis. Of particular interest are serial levels of IL-4 in acute resolving sarcoidosis which may drop, similar to that seen in acute tuberculosis, as the disease comes under control.

Within the sarcoidosis patients is tempting to suggest that the TH1/TH2 profiles are different between acute and chronic disease with a reduction in Th1 cytokines and an increase in Th2 cytokines in the unstimulated sera from patients with chronic disease (table 6.4) but the differences do not achieve statistical significance after corrections for multiple comparisions and so no further speculation can be made.

There are currently no consistently reliable blood tests to either diagnose sarcoidosis or to distinguish between sarcoidosis and tuberculosis. Serum ACE levels measured in sarcoidosis are non-specific as ACE is elevated in a number of diseases including tuberculosis (Brice et al., 1995). Confirmation of the differences found here in other cohorts may result in a test based on serum IL-4 which can rapidly and reliably distinguish acute pulmonary sarcoidosis from active pulmonary tuberculosis.

7.4 LIMITATIONS OF THIS WORK

In general, the limitations of this work have been discussed where appropriate. However, a number of important points need to be reiterated. Firstly, the use of of 1D-SDS-PAGE and 2D-DIGE for proteomic analysis of Kveim reagent and sarcoidosis tissue do not represent an analysis of the entire proteome in sarcodiosis. Such analysis would involve further complementary techniques such as isobaric tagging, chip based analysis or shotgun proteomics, as previously discussed. This limited analysis means that it has not been possible to investigate for all potential antigenic proteins within Kveim reagent and sarcoidosis tissue. However, it is reassuring that a number of the proteins from chapters 4 & 5 have been identified before by other research groups as being of potential antigenic targets in the disease.

Additionally, the ideal control tissue would not have been healthy spleen but that from diseased individuals e.g. those with other granulomatous diseases. In particular when looking at differences in protein abundance (as with DIGE), it is important to identify proteins specific to sarcoidosis rather than simply associated with disease and not found in the healthy state. However, such diseased non-sarcoidosis tissue is difficult to obtain. Furthermore, the identification of Kveim antigens requires normal controls only (as with *in vivo* experiments of the past) and it is hoped that healthy tissue is valid when looking for proteins involved with the Kveim reaction.

Sarcoidosis itself may represent an abnormal response to host proteins which are structurally modified, as in the case of T-cells responding to citrullinated vimentin in the case of rheumatoid arthritis (see section 7.22). The database searches performed for analysis of chapters 4 & 5 do not take into account all potential post-translational protein modifications e.g. it they do not identifiy a modification such as citrullination. In order to do so, a

significantly more detailed database analysis would have been needed which was not possible within the timeframe of this project. Indeed, work from other groups (such as Wahlstrom et al 2007 and the identificiation of mKatG as a potential antigen by Song et al 2005) used analytical techniques that would not have picked up such protein modificiaitons. It is possible that any proteins identified here will not elicit T-cell responses without such modificiations but this will only be known once specific proteins are subjected to ex-vivo analysis with sarcoidosis PBMCs.

A more general but important limitation of this work is that the target antigens may not in fact be protein based. Aim 1 of this project relies heavily on the theory that the Kveim antigens are indeed proteins, as has been investigated and hypothesised previously by researchers in the field. However, the work to remove non-protein extracts and still find a strong Kveim response (see see section 1.42 for full details) has not been replicated (or even attempted) using more modern techniques and it is therefore possible that the antigenic components are other classess of molcules e.g. lipids. The investigation of Kveim reagent for further classes of antigenic component is a large undertaking but could perhaps be undertaken if future efforts to identifiy protein antigens are unsuccessful.

In terms of the Th1 signal identified from sarcoidosis PBMCs incubated with Kveim fractionated proteins (see section 7.14), only a small number of patients were investigated. Furthermore, of the 3 patients investigated in the SS experiment, only 2 produced significantly elevated levels of INFγ and TNFα compared to the controls. This was despite all 3 patients having a similar clinicl picture of biopsy proven pulmonary sarcoidosis within 2 weeks of diagnosis. This difference in response is consitent with the known properties of the Kveim reagent when used in vivo but suggests that a large number of patients with sarcoidosis will need to be investigated with these specific proteins in order to identify those proteis which elicit a response in disease.

In terms of Aim2 of this work, a total of 10 cytokines (which represent the immune spectrum in sarcoidosis and tuberculosis) were investigated in BAL and serum but these 10 represent a small fraction of the entirety of chemokines and cytokines involved in both diseases. For this reason, it cannot be said that the BAL cytokine profile in sarcoidosis and tuberculosis is identical, merely that with the data presented here the profiles are indistinguishable from one another. Furthermore, in terms of the serum analysis, despite the use of reliable cross validation techniques (leave one out analysis) to ascertain the true accuracy of distinguishing between conditions, the gold standard method would have been to repeat the measurements on a new validation set of samples and compare the findings to that seen here.

7.5 NOVEL CONCLUSIONS FROM THIS WORK

The list below is subdivided by aims and represent discrete novel findings from this work.

AIM 1

- Clinical phenotyping of sarcoidosis patients without tuberculosis infections is possible through risk analysis and IGRA testing
- BCG vaccinated sarcoidosis patients show a reduced peripheral blood PPD-specific INFγ T-cell response compared to BCG vaccinated healthy volunteers
- PPD responses may warrant further investigation in sarcoidosis (in terms of BAL responses and measurement of different secreted cytokines)
- Kveim reagent which has been stored at $+4^0$C for a long period of time is able to elicit a specific INFγ response from PBMCs of patients with sarcoidosis and different Kveim reagents elicit different levels of response in keeping with the known qualities of historic *in vivo* validated Kveim reagent

- Kveim reagent can be fractionated by pI to elicit a specific INFγ response from PBMCs of patients with sarcoidosis but fractionation was unable to concentrate the active component, suggesting that the antigens may be large conglomerates of proteins in keeping with previous theories about the active component of Kveim
- The Th1 cytokines INFγ and TNFα are the dominant secreted cytokines when Kveim reagent is incubated with PBMCs from patients with acute pulmonary sarcoidosis whereas IL-2 and the Th2 cytokines IL-4, IL-5 and IL-13 may be less important
- The majority of proteins identified were human host proteins, suggesting that these, not exogenous microbial antigens, are responsible for the Kveim reaction
- Proteins have been identified that are specific to validated *in vivo* and newly created *de novo* Kveim reagent and not found in control spleens
- A number of Kveim reagent specific proteins have served as targets of the immune system in diseases where patients have demonstrated false positive Kveim reactions e.g. Rheumatoid Arthritis, Inflammatory Bowel Disease and Coeliac disease, suggesting a common immune response in all these conditions
- There is a similarity between Kveim-specific proteins and biological pathways in Systemic Lupus Erythematosus
- A number of proteins found specific to Kveim reagent are involved in multiple arms of the host immune response
- Proteins of decreased abundance in sarcoidosis spleen tissue are vital to T-cell co-stimulation during the process of antigen presentation

AIM 2

- Th1/Th2 cytokine profiles in the BAL of matched pulmonary sarcoidosis and pulmonary tuberculosis patients are indistinguishable
- Differences in serum IL-4 (and perhaps IL-13) exist between patients with pulmonary sarcoidosis and pulmonary tuberculosis. Despite the measurement of 10 cytokines in both BAL and serum, only one (serum IL-4) showed differences between sarcoidosis and TB-this could form part of the basis of a larger cytokine profile
- The similarities in BAL profile but differences in serum profile support the theory that the initial host response in the lung is identical in both diseases but the systemic responses are different. This could mean that mycobacteria can cause either tuberculosis or sarcoidosis, depending on this systemic response

In conclusion, a limited number of Kveim- and sarcoidosis-specific proteins have been reproducibly identified through 1D-SDS-PAGE and 2D-DIGE. These proteins represent a small fraction of those found in human spleen tissue. However, of key importance is that of the 78 proteins identified through these methods, 8 of them have been idenfied as possible T-cell antigens through a different technique in a different diseased compartment (Wahlstrom et al 2007). Of these proteins, 3 have structural functions within the cell. The lack of ability to fractionate the antigens by pI also suggest that large proteins, perhaps existing in conglomerates, may be responsible for the Kveim reaction. The underlying fact that the antigenic proteins within Kveim reagent remain stable for long periods of time at near room temperature also supports the theory of one, or several, of these idenfied structural proteins as being responsible for the Kveim reaction.

Additoinally, a number of non-structural human proteins have been identified here as potential self antigens in disease. Of these, superoxide dismutase is of real interest as it was found specifically in 9 out of 14 Kveim reagents but in 0 out of 7 controls. Whilst the protein exists in almost all living cells, it is key that a mycobacterial form of the protein (sodA) has been recently idenfied through PCR from sarcoidosis lung tissue and that peripheral blood IFNγ responses towards peptides from sodA have been specifically identified in patients with the disease (see section 7.21). The similarities in sequence (7.21) between human and mycobacterial forms of the protein, and the fact that the human form only was identified in Kveim reagent despite extensive searches of the data with microbial databases, means that T-cell responses towards this protein need investigating further.

The identification of a limited set of disease-specific proteins, along with the Th1 cytokine signal seen when Kveim fractionated proteins are incubated with sarcoidosis PBMCs (see section 7.14), provides the blueprint for further investigation. The reproducibitlity of this Th1 signal towards one or more of these proteins would represent a rapid, sensitive and specific method to diagnose disease. Such a test would obviate the need for an invasive biopsy of diseased tissue and allow for further insights into the immunopathogenesis of sarcoidosis.

Appendices

APPENDIX 1: STOCK SOLUTIONS

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Colloidal Coomasie: 0.08% w/v Coomassie Brilliant Blue G250, 1.6% v/v orthophosphoric acid, 8% w/v ammonium sulphate, 20% methanol

DIGE lysis buffer: 30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) Chaps, pH 8.5

Elution buffer: 0.1% SDS, 0.05M tris-HCL, 0.1mM EDTA, 5mM DTT, 0.15M NaCl

Freezing mix: 10% DMSO in heat inactivated foetal calf serum

Laemmli buffer: 0.25 M tris-HCl pH 6.8, 20% glycerol, 4% SDS, 2% DTT, trace amount of bromophenol blue

Lysis buffer: 8.75M urea, 2.5M thiourea, 5% w/v Chaps, 2.5% v/v, 2% v/v IPG buffer of appropriate pI, 0.025M tris-HCL

Matrix solution: 10 mg of alpha-cyano-4-hydroxycinnamic acid mixed with 0.1% TFA, 495 µl of acetonitrile and 495 µl of 99.7% ethanol

Q-Tof Capillary buffer A1: 0.1% v/v formic acid in HPLC grade H_20

Q-Tof Capillary buffer B1: 90% Acetonitrile , 0.1% Formic acid in HPLC grade H_20

Q-Tof Capillary buffer A2/B2: 50% v/v methanol in 1 HPLC grade H_20

Q-Tof Nanopump buffer A1: 0.1% Trifluoric acid in LCMS grade H_20

Q-Tof Nanopump buffer B1: 50% methanol in LCMS grade H_20

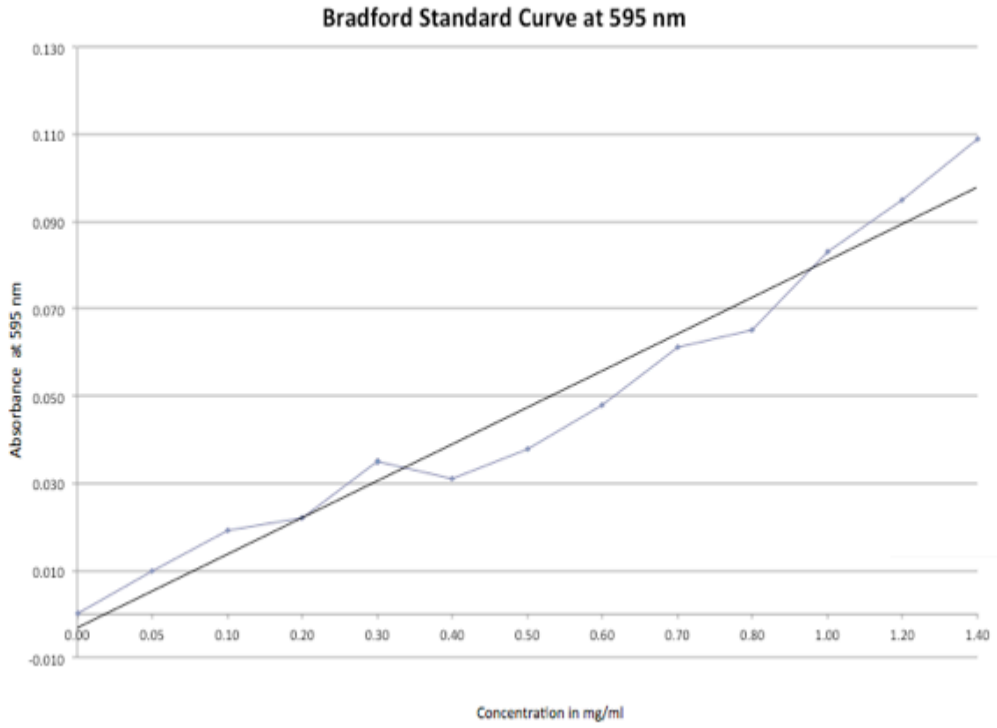
Q-Tof Nanopump buffer A2/B2: 50% methanol, 50% Acetonitrile

R10 solution: RPMI-1640 with 10% heat inactivated foetal calf serum

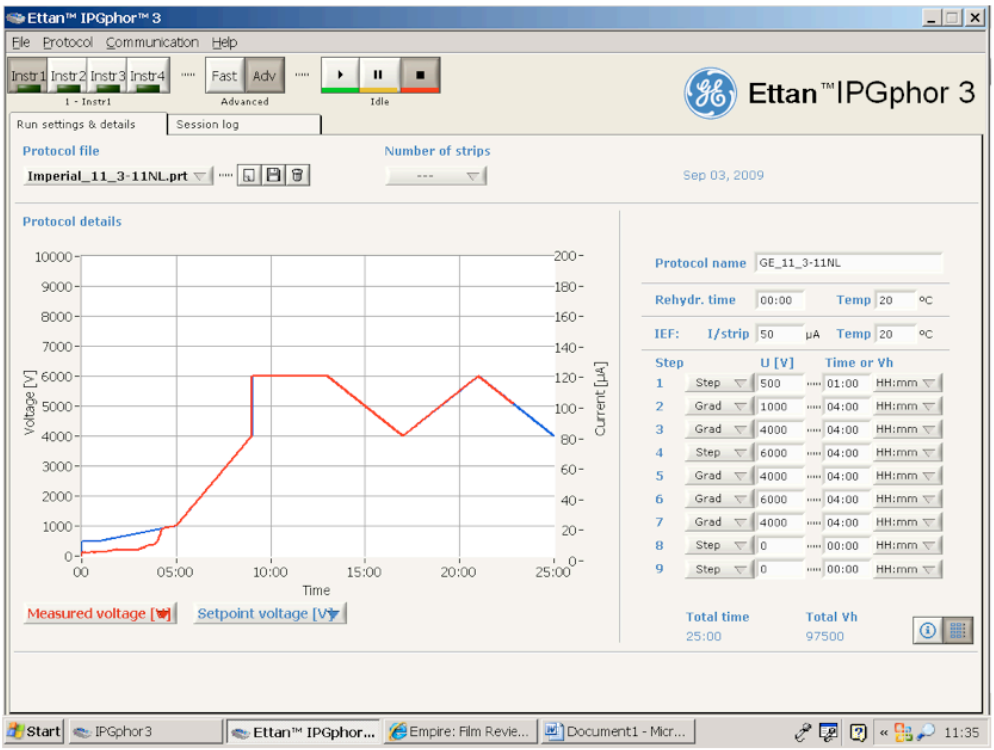
SDS equilibration buffer: 50mM Tris-HCL pH 8.8, 6M Urea, 30% w/v glycerol, 2% w/v SDS, trace amount of bromophenol blue

SDS phastgel buffer: 0.2 M Tricine, 0.2 M Tris, pH 8.1, 0.55% SDS

APPENDIX 2: STANDARD CURVE FOR PROTEIN QUANTIFICATION ASSAY

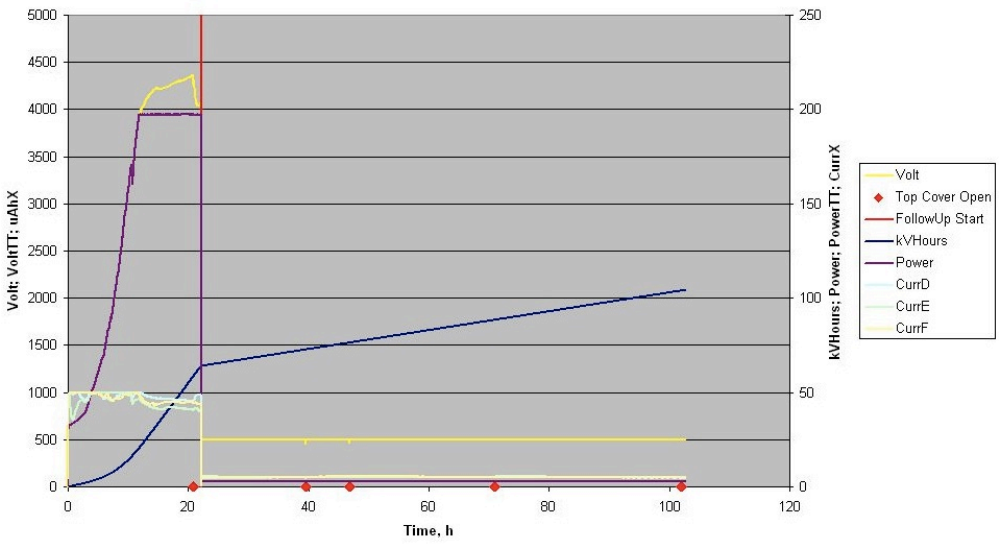


APPENDIX 3: 2D-SDS-PAGE RUN (1ST DIMENSION) EXAMPLE OF 11CM PROTOCOL PRIOR TO OPTIMISATION OF CONDITIONS FOR 24 CM GELS

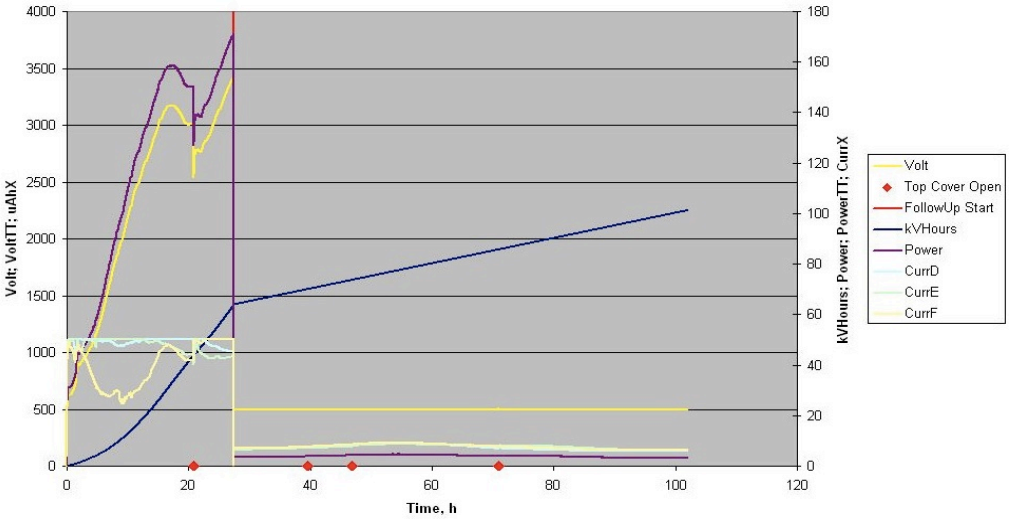


APPENDIX 4: OFFGEL RUN DATA

a) Protein fractionation voltage plot for proteins extracted from *sarcoidosis spleens.* The majority of fractionation is complete after 24 hours but fractionation continues for a total of 100 hours.



b) Protein fractionation voltage plot for proteins extracted from *control spleens.* The majority of fractionation is complete after 24 hours but fractionation continues for a total of 100 hours.

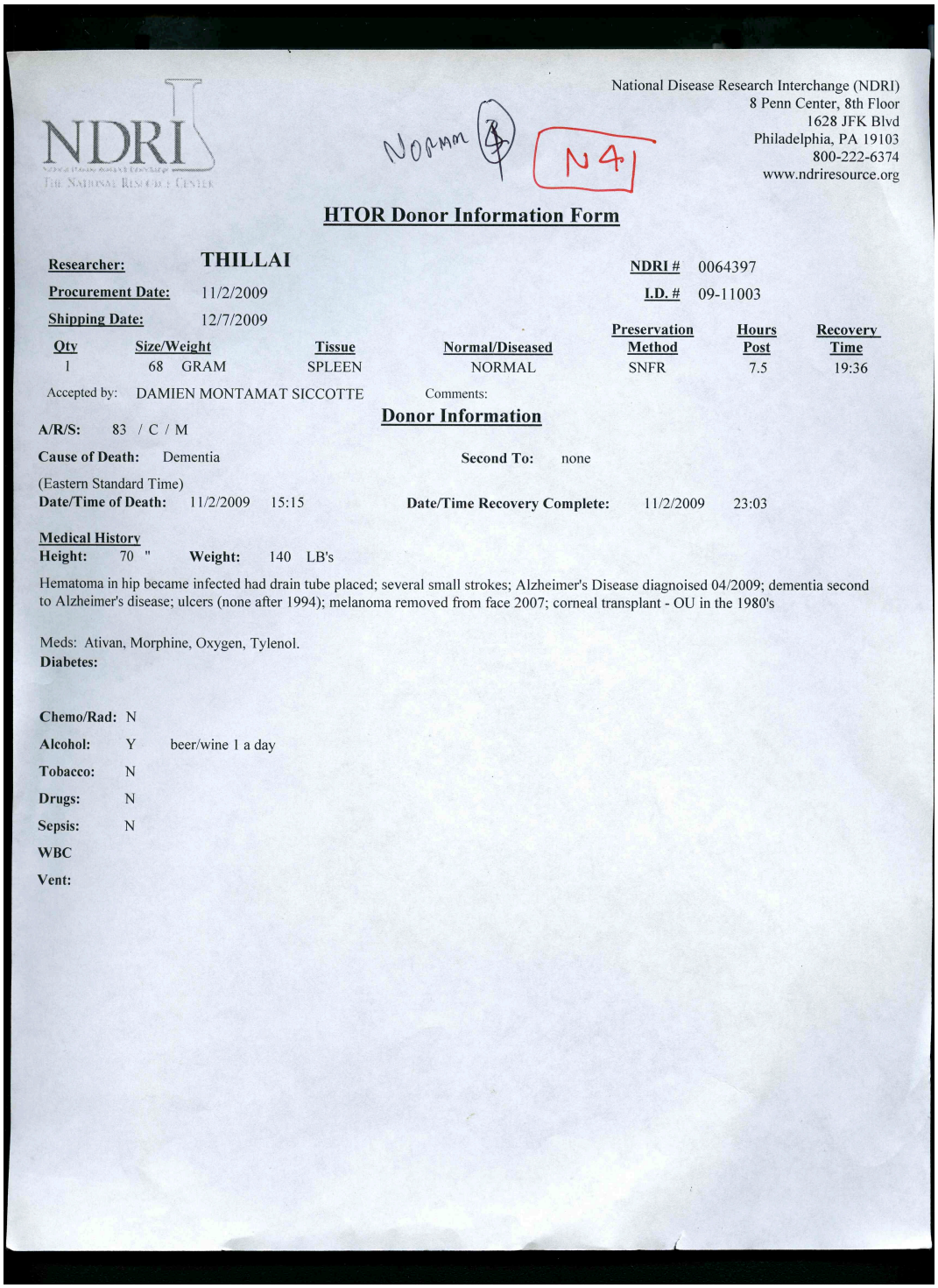


APPENDIX 5: CELL SURVIVAL AFTER INCUBATION WITH OFFGEL PELLETS

This table shows that the cell viability after 20 hours incubation with offgel fractions (with acetone precipitation) is comparable to that seen with ESAT-6 incubation.

Antigen	Number of cells in well after 20 hours	% viability
-	7.6 x 10.5	90
-	1.2 x 10.6	93
PHA	2.1 x 10.5	76
PHA	3 x 10.5	64
Offgel fraction 1 (acetone precipitation)	1.6 x 10.6	90
Offgel fraction 2 (acetone precipitation)	1.4 x 10.6	89
ESAT-6	1.5 x 10.6	92
ESAT-6	1.3 x 10.6	88
Offgel fraction 1 (neat with no clean up)	7.8 x 10.5	69
Offgel fraction 2 (neat with no clean up)	7.5 x 10.5	66

APPENDIX 6: CLINICAL RECORD FORMS FOR ALL SPLEEN SAMPLES USED IN THIS PROJECT



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HTOR Donor Information Form

Researcher: THILLAI
NDRI # 0064397
Procurement Date: 11/2/2009
I.D. # 09-11003
Shipping Date: 12/7/2009

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	68 GRAM	SPLEEN	NORMAL	SNFR	7.5	19:36

Accepted by: DAMIEN MONTAMAT SICCOTTE
Comments:

Donor Information

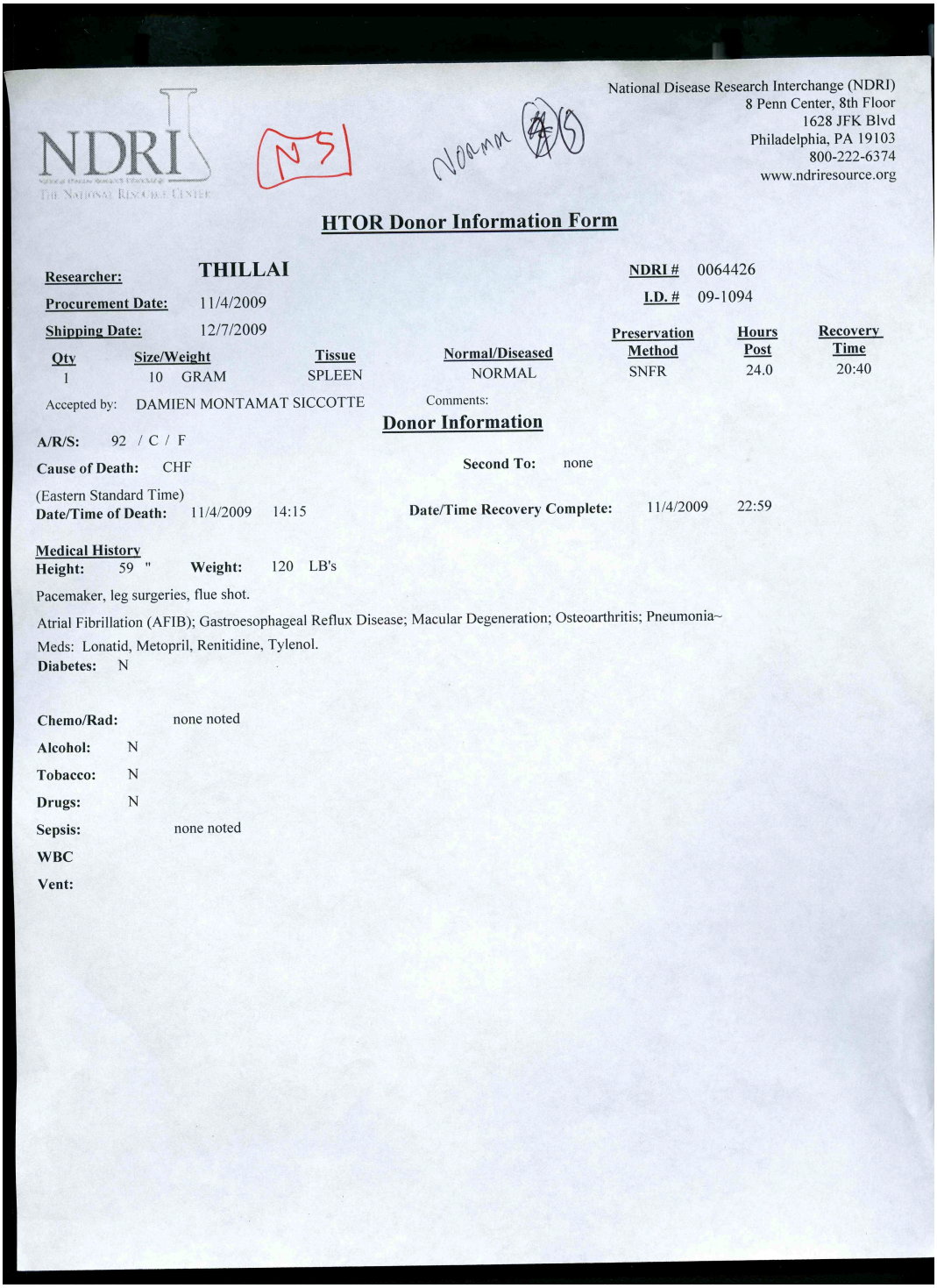
A/R/S: 83 / C / M
Cause of Death: Dementia
Second To: none
(Eastern Standard Time)
Date/Time of Death: 11/2/2009 15:15
Date/Time Recovery Complete: 11/2/2009 23:03

Medical History
Height: 70 " **Weight:** 140 LB's

Hematoma in hip became infected had drain tube placed; several small strokes; Alzheimer's Disease diagnoised 04/2009; dementia second to Alzheimer's disease; ulcers (none after 1994); melanoma removed from face 2007; corneal transplant - OU in the 1980's

Meds: Ativan, Morphine, Oxygen, Tylenol.
Diabetes:

Chemo/Rad: N
Alcohol: Y beer/wine 1 a day
Tobacco: N
Drugs: N
Sepsis: N
WBC
Vent:



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N5

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HTOR Donor Information Form

Researcher: THILLAI

NDRI # 0064426

Procurement Date: 11/4/2009

I.D. # 09-1094

Shipping Date: 12/7/2009

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	NORMAL	SNFR	24.0	20:40

Accepted by: DAMIEN MONTAMAT SICCOTTE

Comments:

Donor Information

A/R/S: 92 / C / F

Cause of Death: CHF

Second To: none

(Eastern Standard Time)
Date/Time of Death: 11/4/2009 14:15

Date/Time Recovery Complete: 11/4/2009 22:59

Medical History
Height: 59 " **Weight:** 120 LB's

Pacemaker, leg surgeries, flue shot.

Atrial Fibrillation (AFIB); Gastroesophageal Reflux Disease; Macular Degeneration; Osteoarthritis; Pneumonia~

Meds: Lonatid, Metopril, Renitidine, Tylenol.

Diabetes: N

Chemo/Rad: none noted

Alcohol: N

Tobacco: N

Drugs: N

Sepsis: none noted

WBC

Vent:

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Normal

NG

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Researcher: THILLAI

NDRI # 0064427

Procurement Date: 11/5/2009

I.D. # 09-1095

Shipping Date: 12/7/2009

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	NORMAL	SNFR	8.0	00:50

Accepted by: MUHUNTHAN THILLAI

Comments: received 2 large pieces and many smaller pieces.

Donor Information

A/R/S: 70 / C / M

Cause of Death: Heart Failure

Second To: N/A

(Eastern Standard Time)

Date/Time of Death: 11/4/2009 16:35

Date/Time Recovery Complete: 11/5/2009 01:30

Medical History

Height: 68 " **Weight:** 76 KG's

Carotid Artery Sx, Triple Bypass, High Cholesterol, Flu Shot, Enlarges Prostate and ALS (5yrs)

Amyotrophic Lateral Sclerosis (ALS)~

Meds: ALS and Prostate Meds

Diabetes: N

Chemo/Rad: N

Alcohol: Y Quit several years ago, Social Drinker

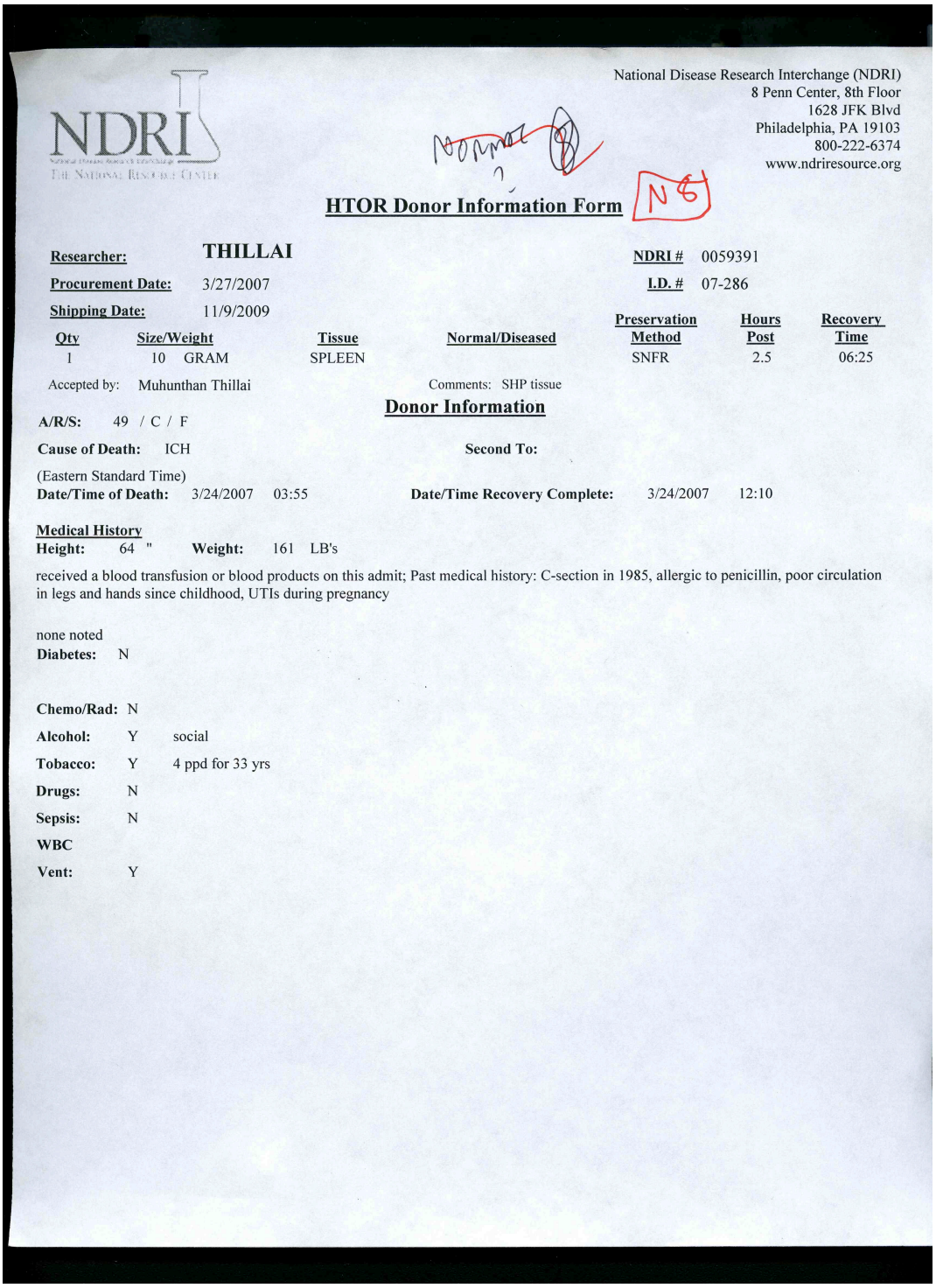
Tobacco: N

Drugs: N

Sepsis: N

WBC

Vent: N



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Researcher: THILLAI
NDRI # 0059391
Procurement Date: 3/27/2007
I.D. # 07-286
Shipping Date: 11/9/2009

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN		SNFR	2.5	06:25

Accepted by: Muhunthan Thillai
Comments: SHP tissue

Donor Information

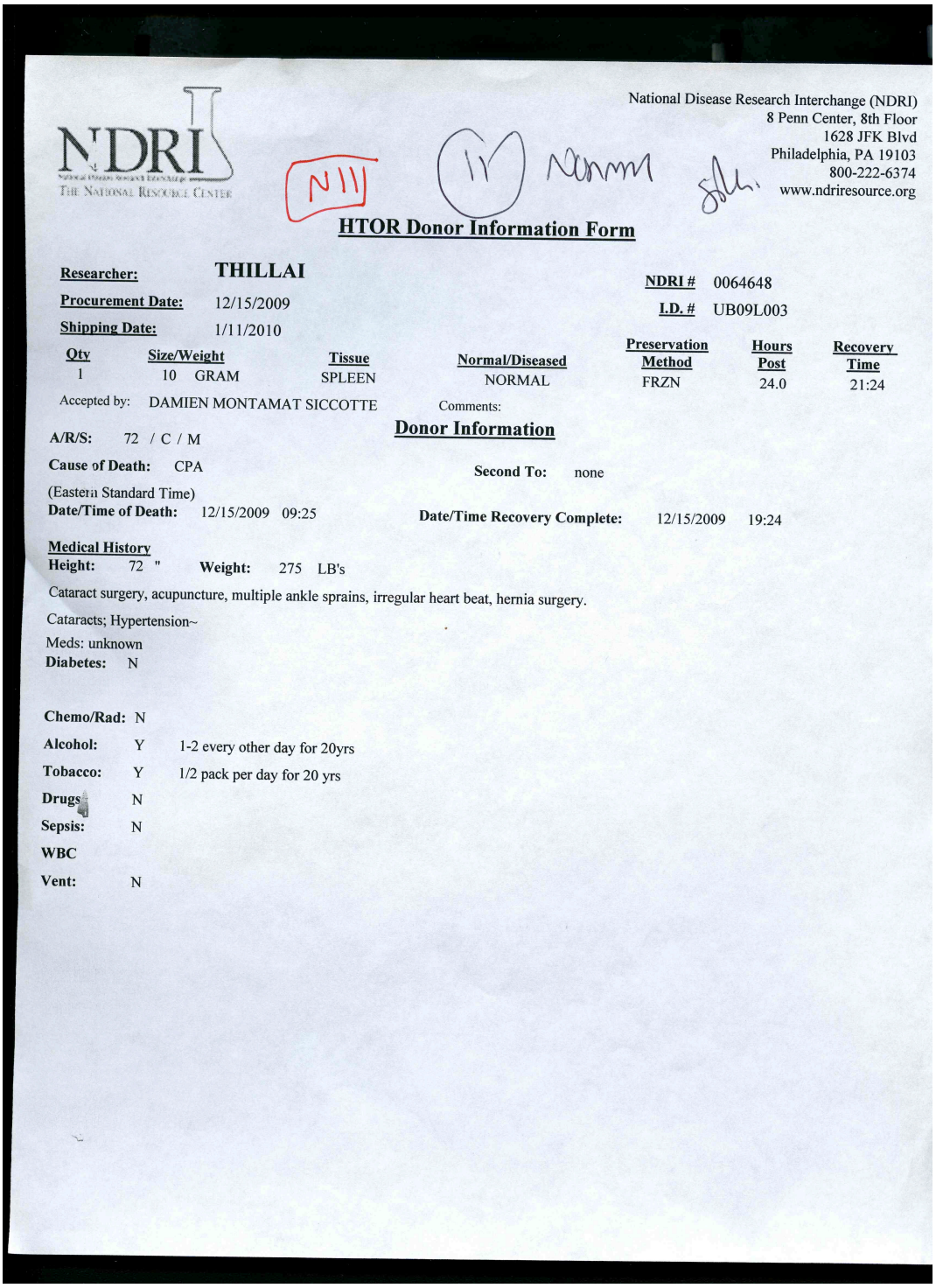
A/R/S: 49 / C / F
Cause of Death: ICH
Second To:
(Eastern Standard Time)
Date/Time of Death: 3/24/2007 03:55
Date/Time Recovery Complete: 3/24/2007 12:10

Medical History
Height: 64 " **Weight:** 161 LB's

received a blood transfusion or blood products on this admit; Past medical history: C-section in 1985, allergic to penicillin, poor circulation in legs and hands since childhood, UTIs during pregnancy

none noted
Diabetes: N

Chemo/Rad: N
Alcohol: Y social
Tobacco: Y 4 ppd for 33 yrs
Drugs: N
Sepsis: N
WBC
Vent: Y



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Researcher: THILLAI

Procurement Date: 12/15/2009

Shipping Date: 1/11/2010

NDRI # 0064648

I.D. # UB09L003

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	NORMAL	FRZN	24.0	21:24

Accepted by: DAMIEN MONTAMAT SICCOTTE

Comments:

Donor Information

A/R/S: 72 / C / M

Cause of Death: CPA

Second To: none

(Eastern Standard Time)

Date/Time of Death: 12/15/2009 09:25

Date/Time Recovery Complete: 12/15/2009 19:24

Medical History

Height: 72 " **Weight:** 275 LB's

Cataract surgery, acupuncture, multiple ankle sprains, irregular heart beat, hernia surgery.

Cataracts; Hypertension~

Meds: unknown

Diabetes: N

Chemo/Rad: N

Alcohol: Y 1-2 every other day for 20yrs

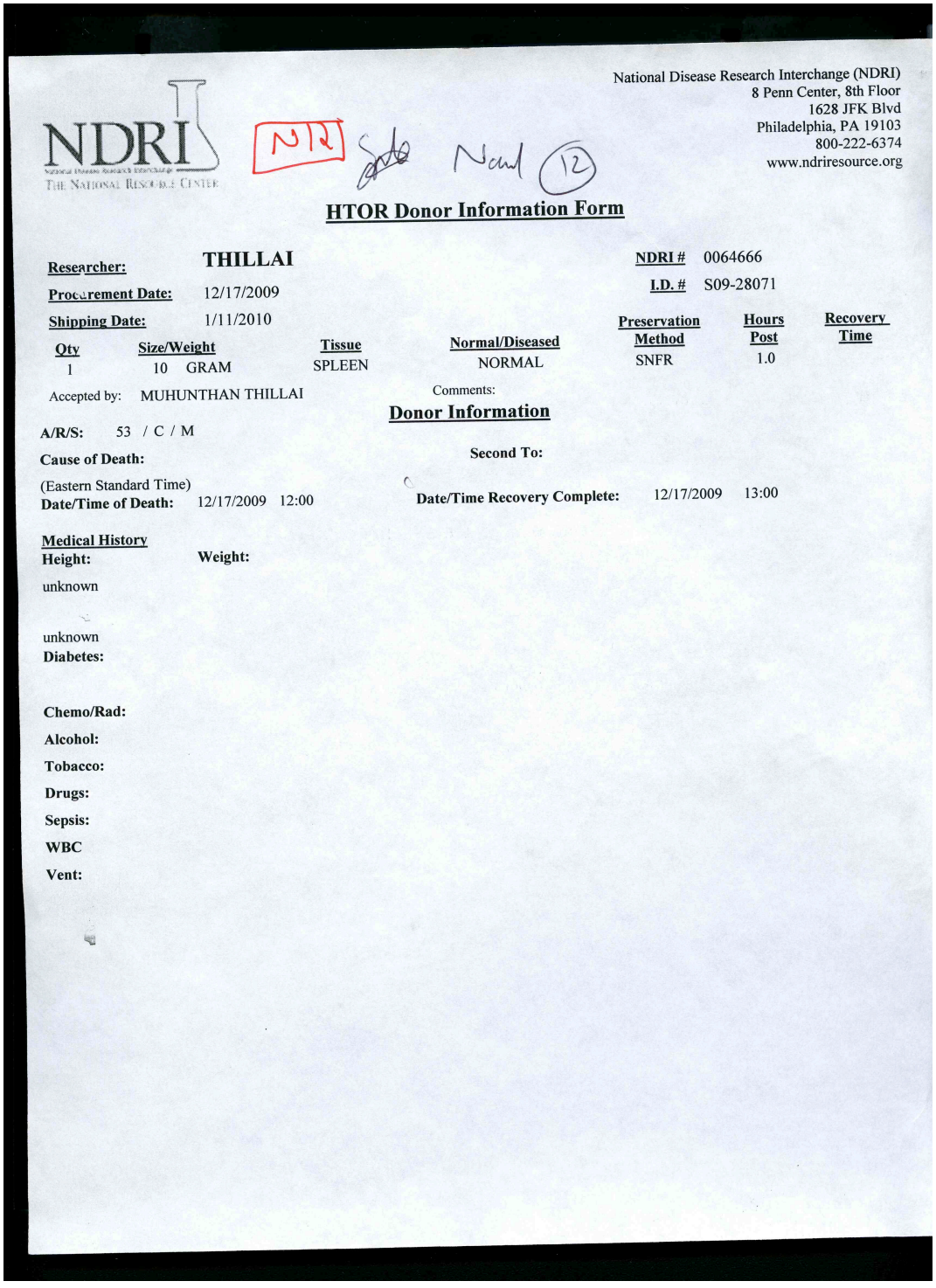
Tobacco: Y 1/2 pack per day for 20 yrs

Drugs N

Sepsis: N

WBC

Vent: N



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Researcher: THILLAI

NDRI # 0064666

Procurement Date: 12/17/2009

I.D. # S09-28071

Shipping Date: 1/11/2010

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	NORMAL	SNFR	1.0	

Accepted by: MUHUNTHAN THILLAI

Comments:

Donor Information

A/R/S: 53 / C / M

Cause of Death:

Second To:

(Eastern Standard Time)
Date/Time of Death: 12/17/2009 12:00

Date/Time Recovery Complete: 12/17/2009 13:00

Medical History

Height: unknown

Weight:

unknown

Diabetes:

Chemo/Rad:

Alcohol:

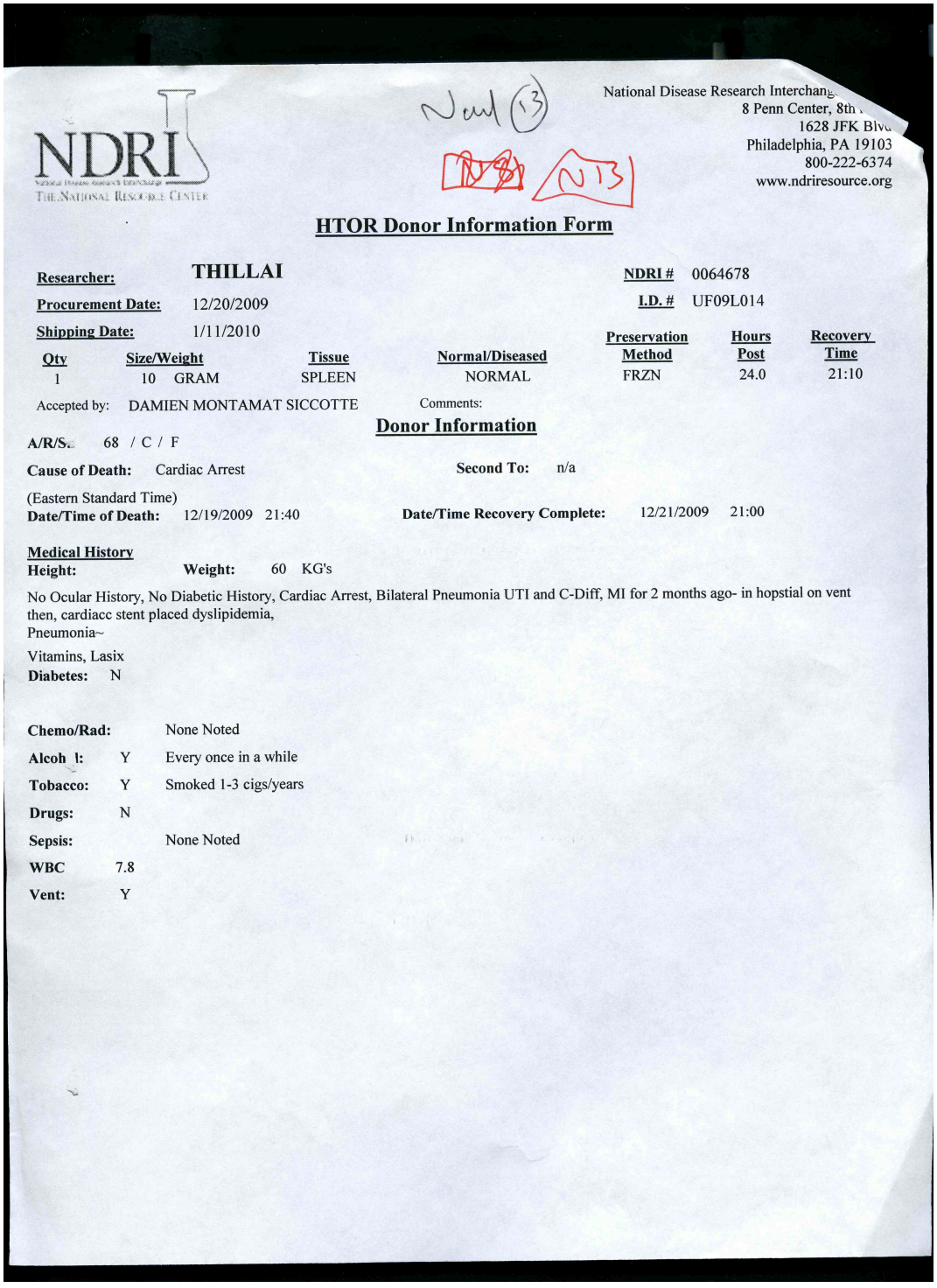
Tobacco:

Drugs:

Sepsis:

WBC

Vent:



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HTOR Donor Information Form

Researcher: THILLAI

NDRI # 0064678

Procurement Date: 12/20/2009

I.D. # UF09L014

Shipping Date: 1/11/2010

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	NORMAL	FRZN	24.0	21:10

Accepted by: DAMIEN MONTAMAT SICCOTTE

Comments:

Donor Information

A/R/S. 68 / C / F

Cause of Death: Cardiac Arrest

Second To: n/a

(Eastern Standard Time)

Date/Time of Death: 12/19/2009 21:40

Date/Time Recovery Complete: 12/21/2009 21:00

Medical History

Height:

Weight: 60 KG's

No Ocular History, No Diabetic History, Cardiac Arrest, Bilateral Pneumonia UTI and C-Diff, MI for 2 months ago- in hopstial on vent then, cardiacc stent placed dyslipidemia,
Pneumonia~

Vitamins, Lasix

Diabetes: N

Chemo/Rad: None Noted

Alcohol: Y Every once in a while

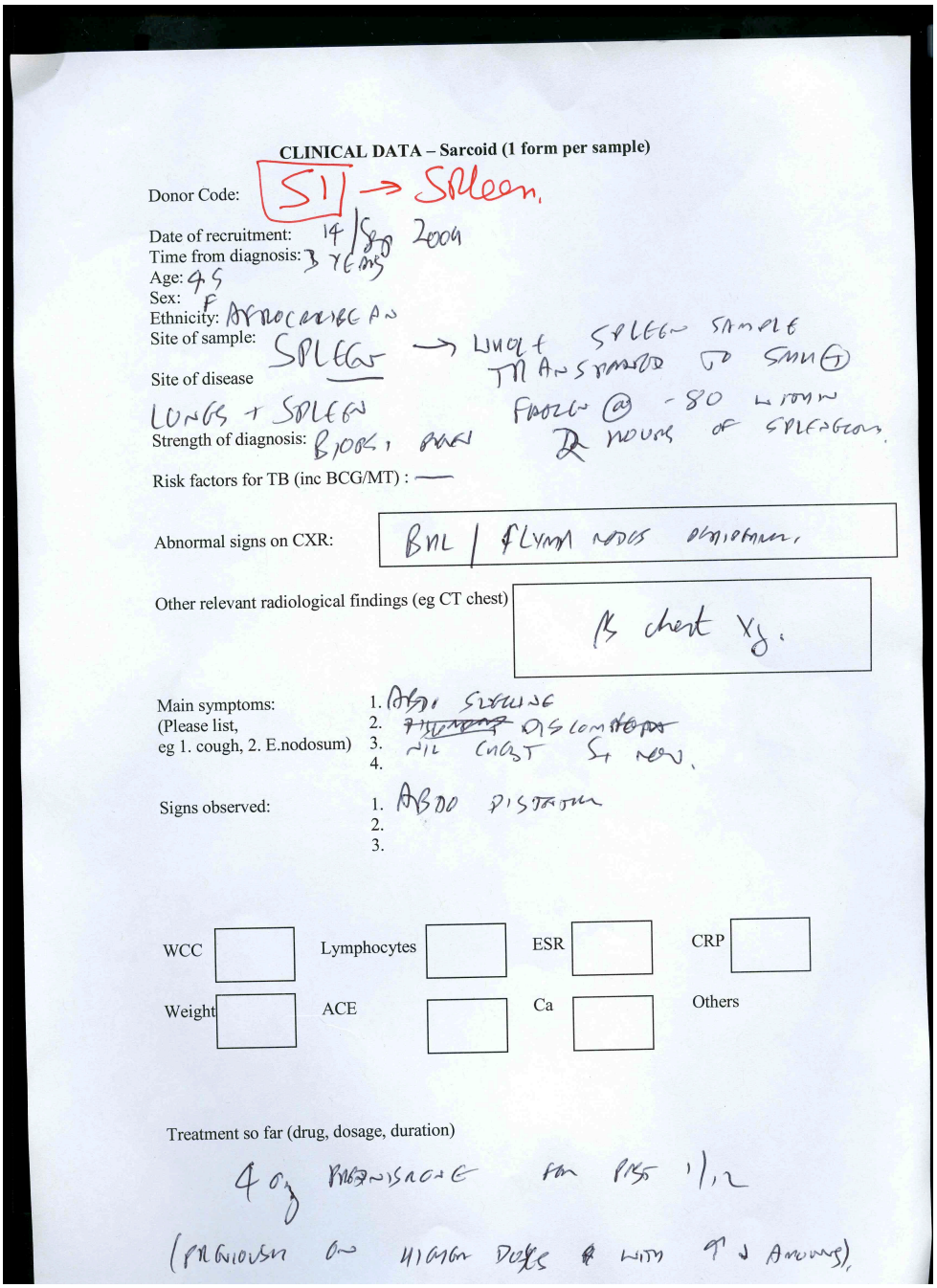
Tobacco: Y Smoked 1-3 cigs/years

Drugs: N

Sepsis: None Noted

WBC 7.8

Vent: Y



CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: S1 → Spleen.

Date of recruitment: 14/Sep 2004
Time from diagnosis: 3 YEARS
Age: 45
Sex: F
Ethnicity: AFROCARIBEAN
Site of sample: SPLEEN → WHOLE SPLEEN SAMPLE TRANSPORTED TO SMH (T) FROZEN @ -80 WITHIN 2 HOURS OF SPLENECTOMY.
Site of disease LUNGS + SPLEEN
Strength of diagnosis: BIOPSY PROVEN

Risk factors for TB (inc BCG/MT) : —

Abnormal signs on CXR: BHL / FLYMP NODES OCCIDENTAL.

Other relevant radiological findings (eg CT chest): PS chest Xg.

Main symptoms: (Please list, eg 1. cough, 2. E.nodosum)
1. ABDO SWELLING
2. ~~[illegible]~~ DISCOMFORT
3. NIL CHEST S+ NOV.
4.

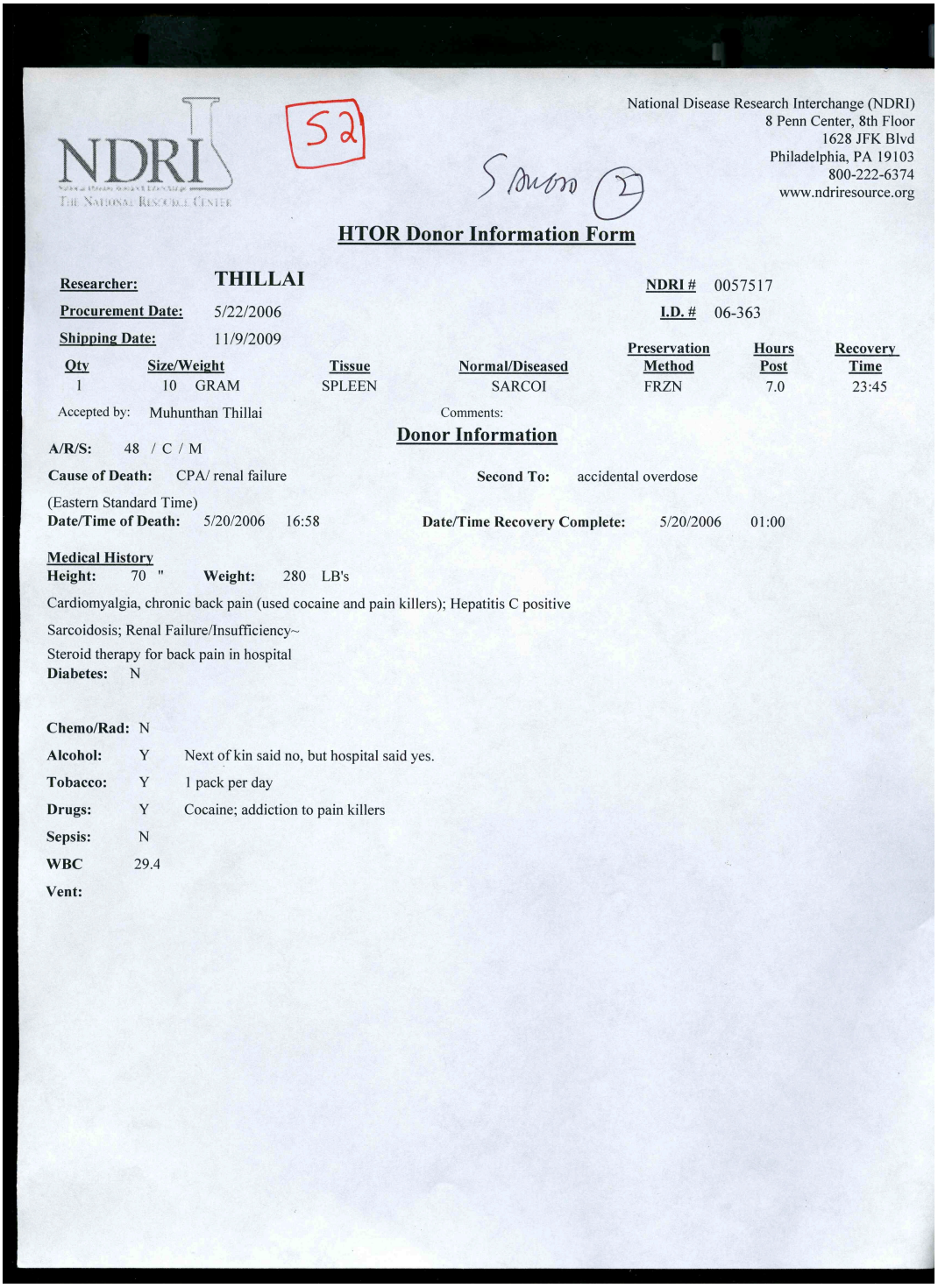
Signs observed:
1. ABDO DISTENTION
2.
3.

WCC		Lymphocytes		ESR		CRP	
Weight		ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

4 oz PREDNISRONE FOR PAST 1/12

(PREVIOUSLY ON HIGHER DOSES WITH ↑ ↓ AMOUNTS).



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52

Spleen (2)

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HTOR Donor Information Form

Researcher: THILLAI **NDRI #** 0057517

Procurement Date: 5/22/2006 **I.D. #** 06-363

Shipping Date: 11/9/2009

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	SARCOI	FRZN	7.0	23:45

Accepted by: Muhunthan Thillai Comments:

Donor Information

A/R/S: 48 / C / M

Cause of Death: CPA/ renal failure **Second To:** accidental overdose

(Eastern Standard Time)

Date/Time of Death: 5/20/2006 16:58 **Date/Time Recovery Complete:** 5/20/2006 01:00

Medical History

Height: 70 " **Weight:** 280 LB's

Cardiomyalgia, chronic back pain (used cocaine and pain killers); Hepatitis C positive

Sarcoidosis; Renal Failure/Insufficiency~

Steroid therapy for back pain in hospital

Diabetes: N

Chemo/Rad: N

Alcohol: Y Next of kin said no, but hospital said yes.

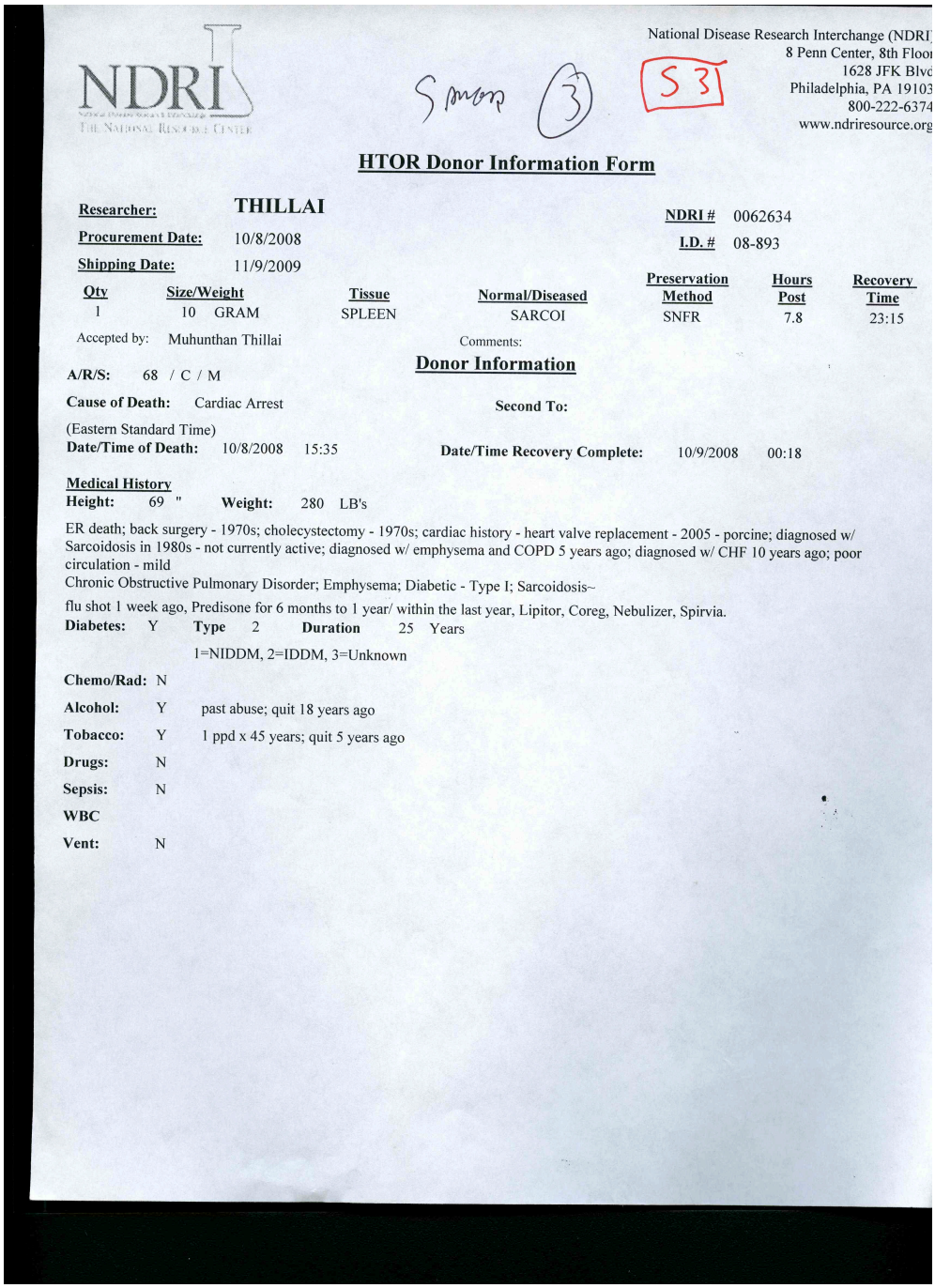
Tobacco: Y 1 pack per day

Drugs: Y Cocaine; addiction to pain killers

Sepsis: N

WBC 29.4

Vent:



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HTOR Donor Information Form

Researcher: THILLAI
Procurement Date: 10/8/2008
Shipping Date: 11/9/2009

NDRI # 0062634
I.D. # 08-893

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	SARCOI	SNFR	7.8	23:15

Accepted by: Muhunthan Thillai

Comments:

Donor Information

A/R/S: 68 / C / M

Cause of Death: Cardiac Arrest

Second To:

(Eastern Standard Time)
Date/Time of Death: 10/8/2008 15:35

Date/Time Recovery Complete: 10/9/2008 00:18

Medical History
Height: 69 " **Weight:** 280 LB's

ER death; back surgery - 1970s; cholecystectomy - 1970s; cardiac history - heart valve replacement - 2005 - porcine; diagnosed w/ Sarcoidosis in 1980s - not currently active; diagnosed w/ emphysema and COPD 5 years ago; diagnosed w/ CHF 10 years ago; poor circulation - mild
Chronic Obstructive Pulmonary Disorder; Emphysema; Diabetic - Type I; Sarcoidosis~

flu shot 1 week ago, Predisone for 6 months to 1 year/ within the last year, Lipitor, Coreg, Nebulizer, Spirvia.

Diabetes: Y **Type** 2 **Duration** 25 Years

1=NIDDM, 2=IDDM, 3=Unknown

Chemo/Rad: N

Alcohol: Y past abuse; quit 18 years ago

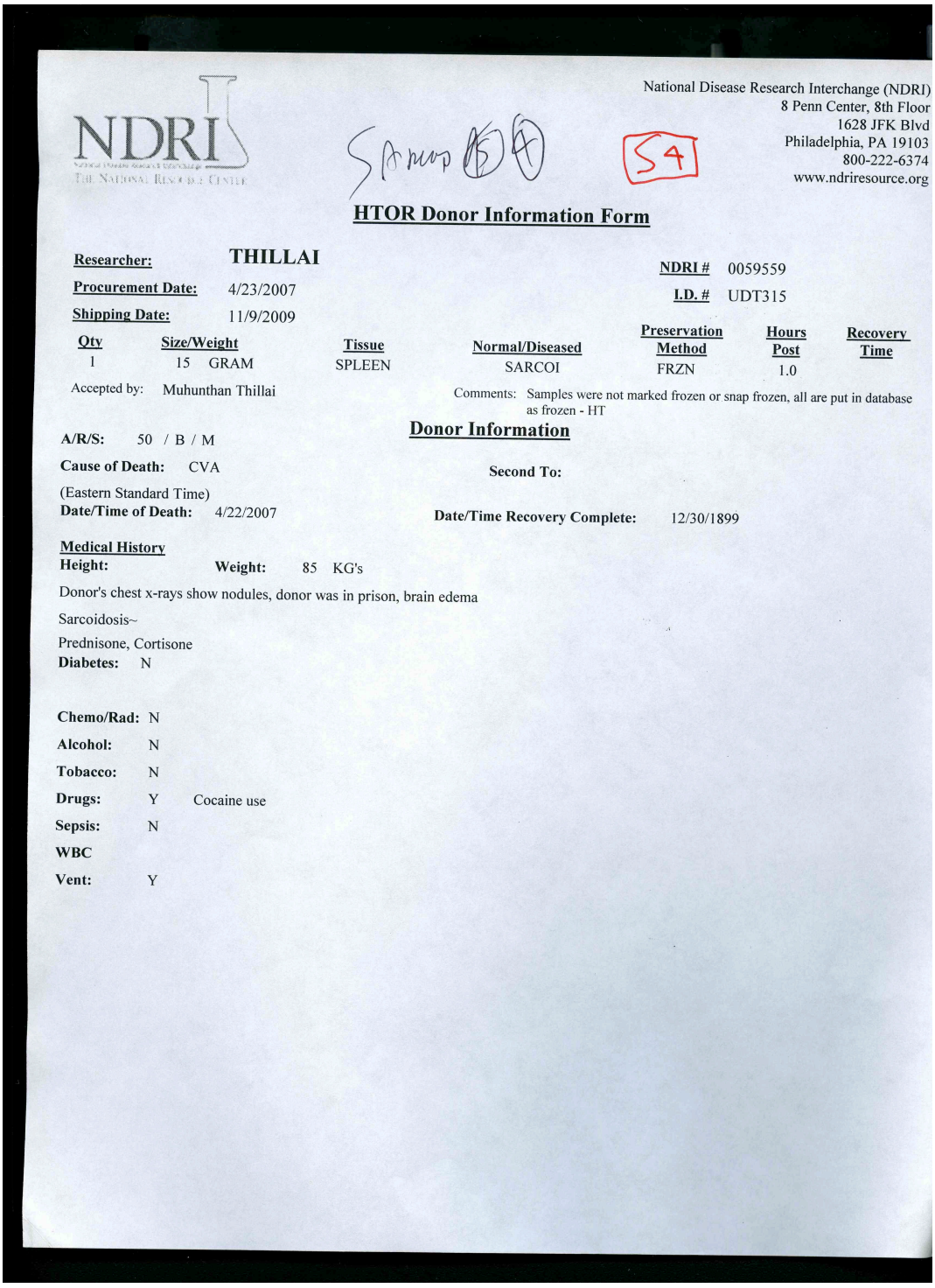
Tobacco: Y 1 ppd x 45 years; quit 5 years ago

Drugs: N

Sepsis: N

WBC

Vent: N



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S4

HTOR Donor Information Form

Researcher: THILLAI
Procurement Date: 4/23/2007
Shipping Date: 11/9/2009

NDRI # 0059559
I.D. # UDT315

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	15 GRAM	SPLEEN	SARCOI	FRZN	1.0	

Accepted by: Muhunthan Thillai

Comments: Samples were not marked frozen or snap frozen, all are put in database as frozen - HT

Donor Information

A/R/S: 50 / B / M
Cause of Death: CVA
Second To:

(Eastern Standard Time)
Date/Time of Death: 4/22/2007
Date/Time Recovery Complete: 12/30/1899

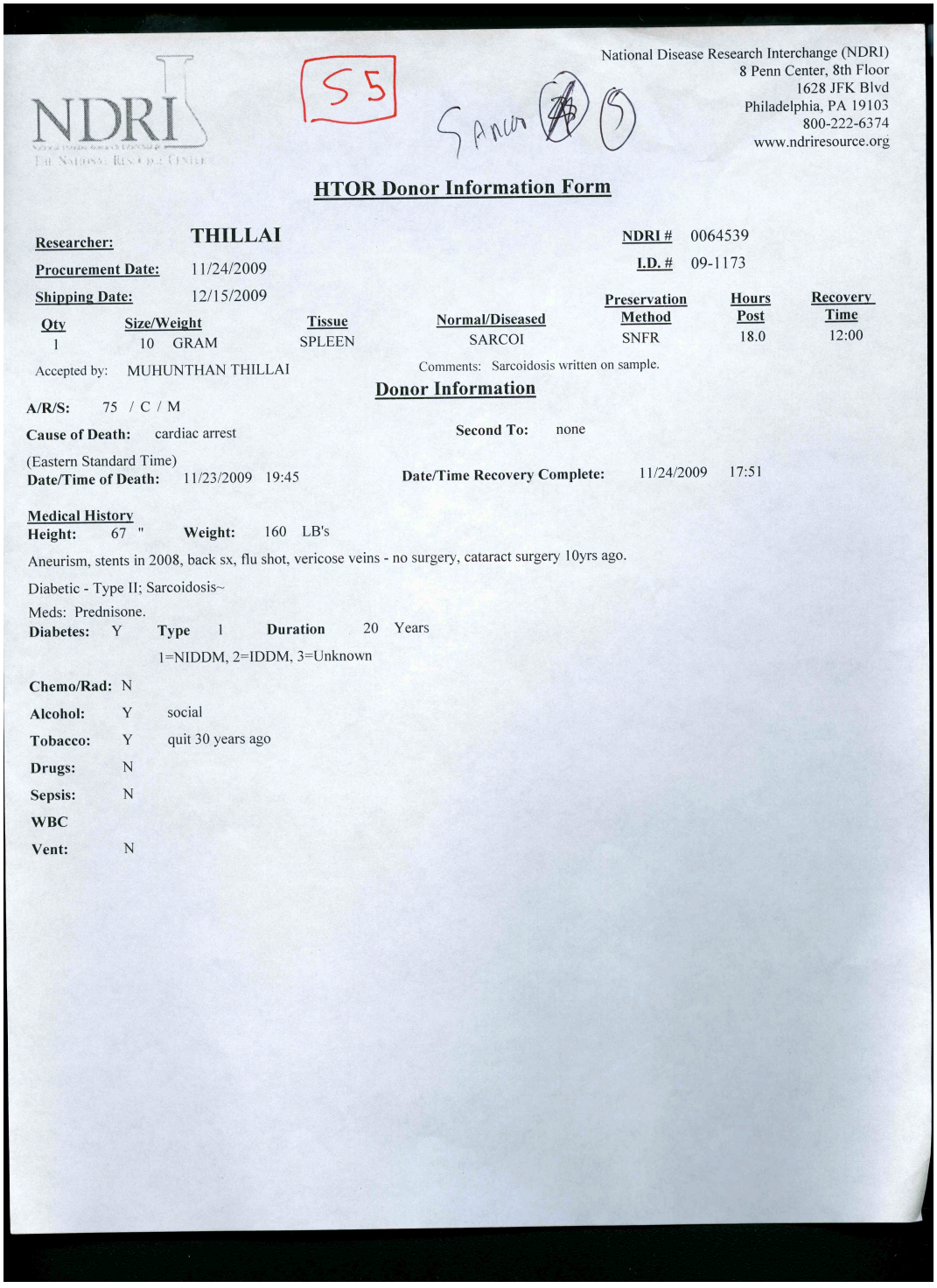
Medical History
Height: **Weight:** 85 KG's

Donor's chest x-rays show nodules, donor was in prison, brain edema

Sarcoidosis~

Prednisone, Cortisone
Diabetes: N

Chemo/Rad: N
Alcohol: N
Tobacco: N
Drugs: Y Cocaine use
Sepsis: N
WBC
Vent: Y



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Sarco (A) (S)

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HTOR Donor Information Form

Researcher: THILLAI

NDRI # 0064539

Procurement Date: 11/24/2009

I.D. # 09-1173

Shipping Date: 12/15/2009

Qty	Size/Weight	Tissue	Normal/Diseased	Preservation Method	Hours Post	Recovery Time
1	10 GRAM	SPLEEN	SARCOI	SNFR	18.0	12:00

Accepted by: MUHUNTHAN THILLAI

Comments: Sarcoidosis written on sample.

Donor Information

A/R/S: 75 / C / M

Cause of Death: cardiac arrest

Second To: none

(Eastern Standard Time)

Date/Time of Death: 11/23/2009 19:45

Date/Time Recovery Complete: 11/24/2009 17:51

Medical History

Height: 67 " **Weight:** 160 LB's

Aneurism, stents in 2008, back sx, flu shot, vericose veins - no surgery, cataract surgery 10yrs ago.

Diabetic - Type II; Sarcoidosis~

Meds: Prednisone.

Diabetes: Y **Type** 1 **Duration** 20 Years

1=NIDDM, 2=IDDM, 3=Unknown

Chemo/Rad: N

Alcohol: Y social

Tobacco: Y quit 30 years ago

Drugs: N

Sepsis: N

WBC

Vent: N

S6

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: S6 - Spleen tissue while.

Date of recruitment: 2 APRIL 2010
Time from diagnosis: 2 YEARS.
Age: 43
Sex: WHITE (CAUCASIAN)
Ethnicity: MALE
Site of sample: SPLEEN

Site of disease ~~WHOLE~~ SPLEEN SAMPLE. (20g) ~~FROZEN (SNAP)~~
~~SPLENECTOMY~~ SPLEEN Bx → FROZEN @ -80°C
WITHIN 2 HOURS
OF POST MORT.

Strength of diagnosis: BIOPSY

Risk factors for TB (inc BCG/MT) : UNKNOWN.

Abnormal signs on CXR: UNKNOWN.

Other relevant radiological findings (eg CT chest) UNKNOWN.

Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)
1.
2.
3.
4.

Signs observed:
1.
2.
3.

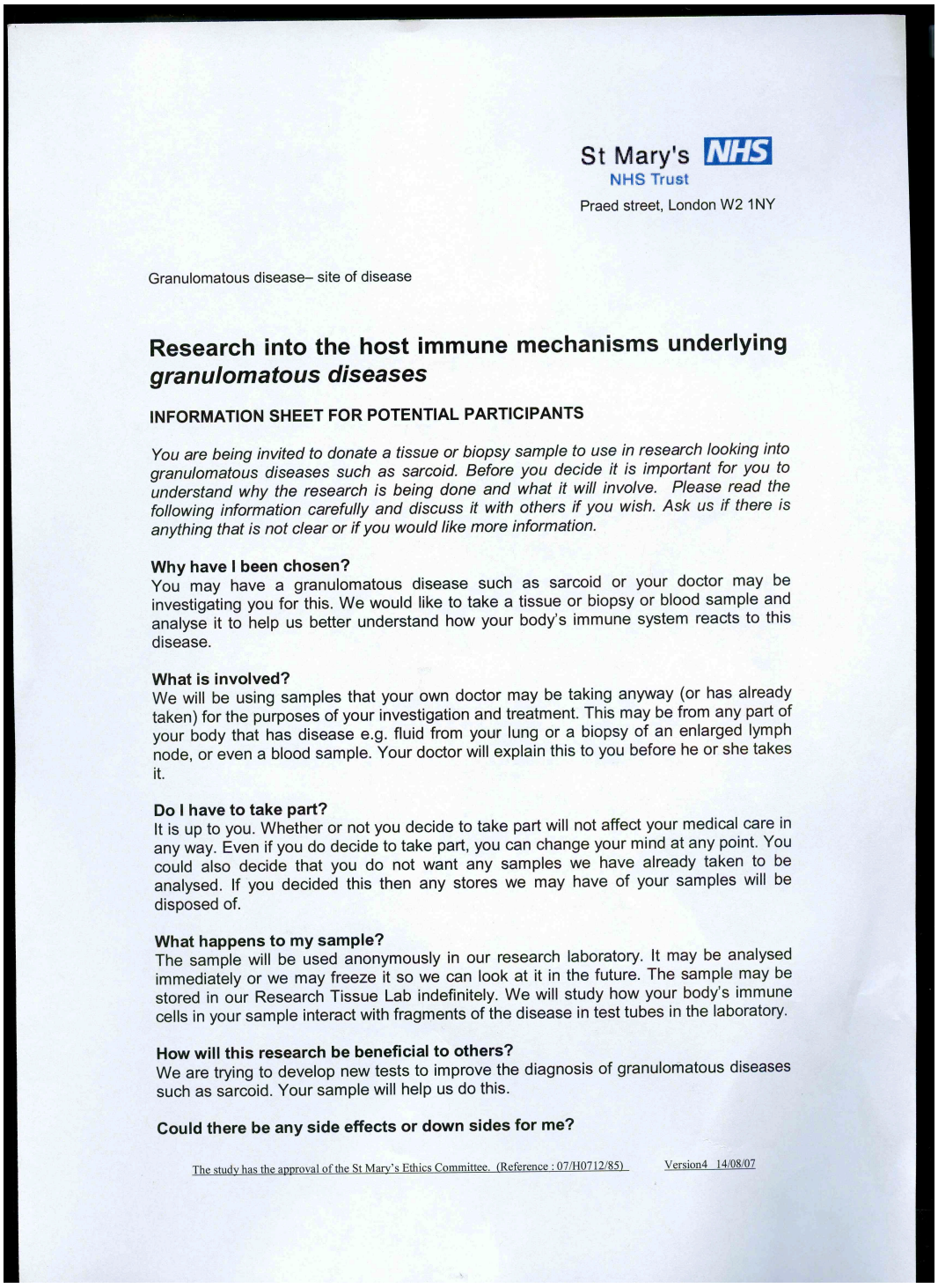
UNKNOWN.

WCC		Lymphocytes		ESR		CRP	
Weight		ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

APPENDIX 7: PROFORMAS OF PATIENT INFORMATION SHEETS, CONSENT FORMS AND CLINICAL RECORD FORMS FOR SELECT PATIENT BLOOD AND BAL SAMPLES USED IN THIS PROJECT (SEE RELEVENT PARTS OF CHAPTERS 4 & 6 FOR FULL INFORMATION)

PATIENT INFORMATION SHEET



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Granulomatous disease– site of disease

Research into the host immune mechanisms underlying *granulomatous diseases*

INFORMATION SHEET FOR POTENTIAL PARTICIPANTS

You are being invited to donate a tissue or biopsy sample to use in research looking into granulomatous diseases such as sarcoid. Before you decide it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Why have I been chosen?
You may have a granulomatous disease such as sarcoid or your doctor may be investigating you for this. We would like to take a tissue or biopsy or blood sample and analyse it to help us better understand how your body's immune system reacts to this disease.

What is involved?
We will be using samples that your own doctor may be taking anyway (or has already taken) for the purposes of your investigation and treatment. This may be from any part of your body that has disease e.g. fluid from your lung or a biopsy of an enlarged lymph node, or even a blood sample. Your doctor will explain this to you before he or she takes it.

Do I have to take part?
It is up to you. Whether or not you decide to take part will not affect your medical care in any way. Even if you do decide to take part, you can change your mind at any point. You could also decide that you do not want any samples we have already taken to be analysed. If you decided this then any stores we may have of your samples will be disposed of.

What happens to my sample?
The sample will be used anonymously in our research laboratory. It may be analysed immediately or we may freeze it so we can look at it in the future. The sample may be stored in our Research Tissue Lab indefinitely. We will study how your body's immune cells in your sample interact with fragments of the disease in test tubes in the laboratory.

How will this research be beneficial to others?
We are trying to develop new tests to improve the diagnosis of granulomatous diseases such as sarcoid. Your sample will help us do this.

Could there be any side effects or down sides for me?

The study has the approval of the St Mary's Ethics Committee. (Reference : 07/H0712/85) Version4 14/08/07

St Mary's NHS
NHS Trust
Praed street, London W2 1NY

The risks involved are those with the procedure and these will be explained to you by your own doctor. You will be asked to sign a specific consent form to say you are happy to donate a sample for our research. This is in addition to any consent form that your own doctor may ask you to sign for the procedure.

Could there be any benefits for me?
The results of the test will only be of use to the research team, and are unlikely to affect any future medical care you may receive.

Who runs this research?
Doctors and researchers from the TB Immunology Group at Imperial College. Our project supervisor is called Professor Ajit Lalvani. We also have collaborating research teams across the world. Our research is funded by the Wellcome Trust, Imperial College London and St. Mary's Hospital NHS Trust.

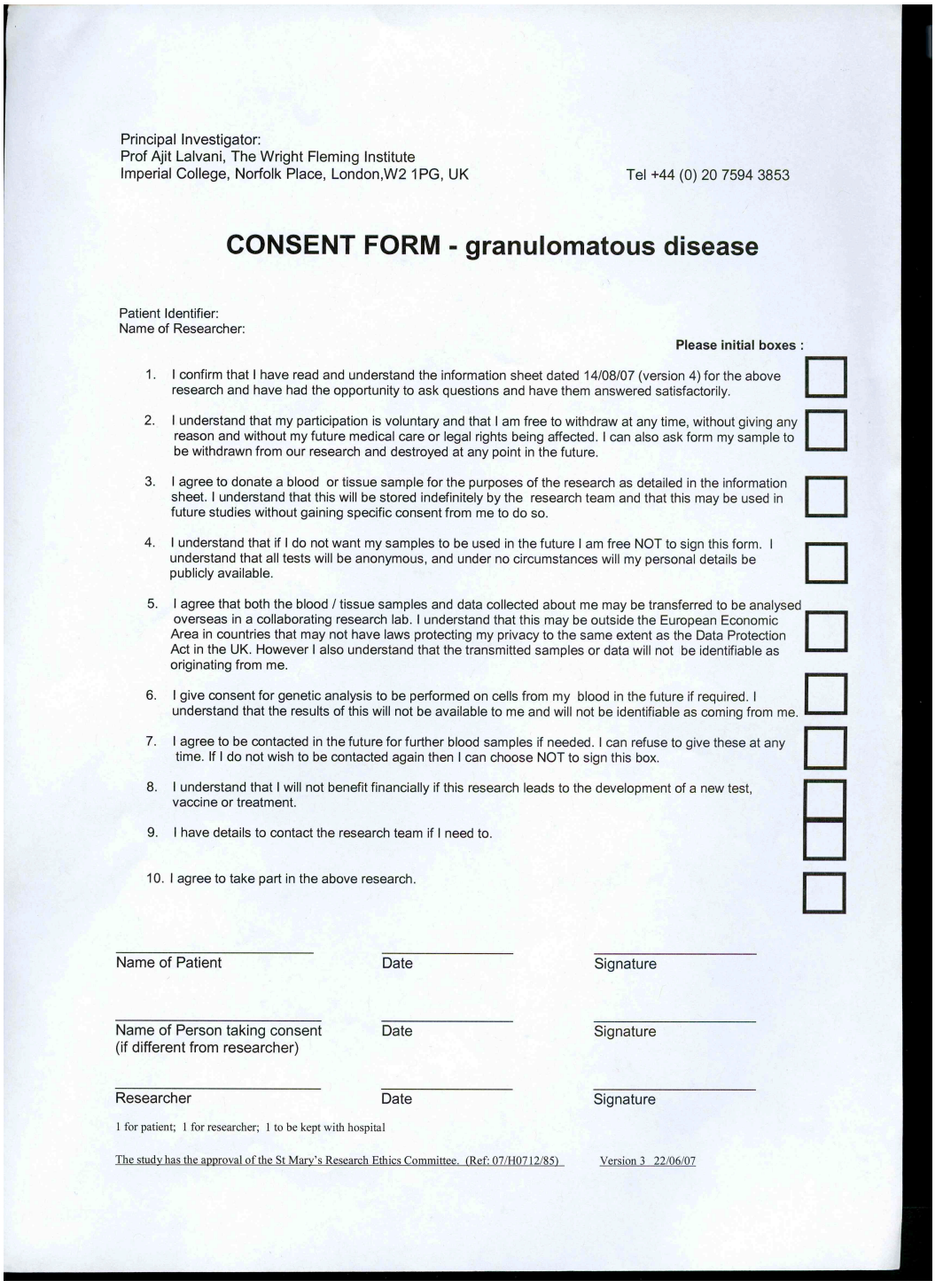
Confidentiality
If you consent to take part in the research your name will not be disclosed outside the research group. Under no circumstances will your personal details be available to the general public. The results of this research are likely to be published, but will contain no identifiable personal information.

Statement of Indemnity from Imperial College London
The creation of the Research Tissue Bank and the research proposal has been discussed, assessed and registered with Imperial College Clinical Research Office and provisional sponsor approval has been granted. The Chief Investigator has undergone a process of scientific critique commensurate with the scale of the project. Research and support are available to the research team to aid delivery of the research as proposed. Indemnity and insurance arrangements have been put in place to cover the project by Imperial College London.

Who to contact for more information
If you have further questions before deciding, please contact Dr Muhunthan Thillai, Wright-Fleming Institute of Imperial College London, Norfolk Place, London, W2 1PG. Tel: 020 7594 0884. He is one of the researchers and would be very pleased to talk to you.

Thank you for reading this information sheet.

PATIENT CONSENT FORM



Principal Investigator:
Prof Ajit Lalvani, The Wright Fleming Institute
Imperial College, Norfolk Place, London,W2 1PG, UK Tel +44 (0) 20 7594 3853

CONSENT FORM - granulomatous disease

Patient Identifier:
Name of Researcher:

Please initial boxes :

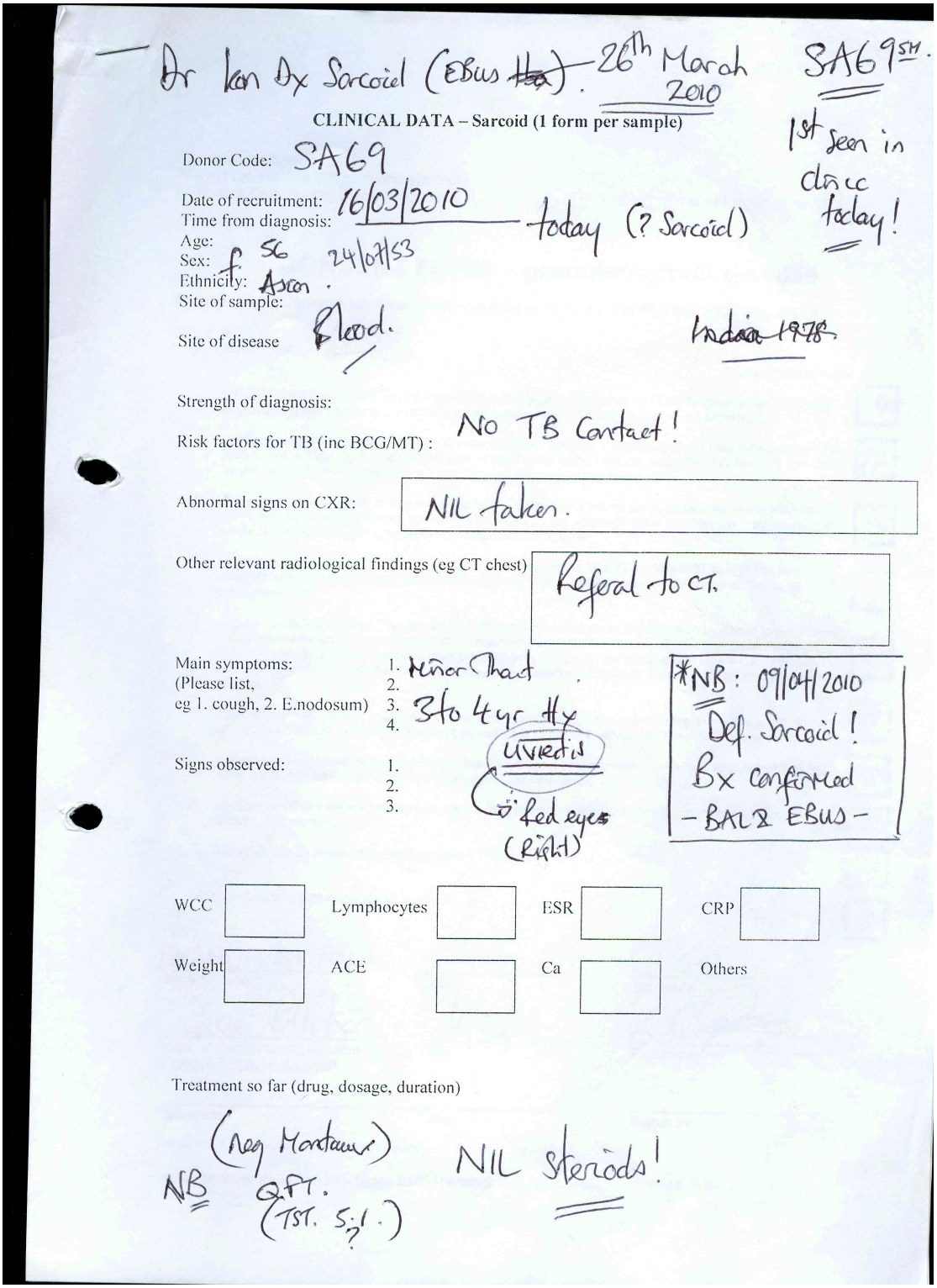
1. I confirm that I have read and understand the information sheet dated 14/08/07 (version 4) for the above research and have had the opportunity to ask questions and have them answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my future medical care or legal rights being affected. I can also ask form my sample to be withdrawn from our research and destroyed at any point in the future. ☐
3. I agree to donate a blood or tissue sample for the purposes of the research as detailed in the information sheet. I understand that this will be stored indefinitely by the research team and that this may be used in future studies without gaining specific consent from me to do so. ☐
4. I understand that if I do not want my samples to be used in the future I am free NOT to sign this form. I understand that all tests will be anonymous, and under no circumstances will my personal details be publicly available. ☐
5. I agree that both the blood / tissue samples and data collected about me may be transferred to be analysed overseas in a collaborating research lab. I understand that this may be outside the European Economic Area in countries that may not have laws protecting my privacy to the same extent as the Data Protection Act in the UK. However I also understand that the transmitted samples or data will not be identifiable as originating from me. ☐
6. I give consent for genetic analysis to be performed on cells from my blood in the future if required. I understand that the results of this will not be available to me and will not be identifiable as coming from me. ☐
7. I agree to be contacted in the future for further blood samples if needed. I can refuse to give these at any time. If I do not wish to be contacted again then I can choose NOT to sign this box. ☐
8. I understand that I will not benefit financially if this research leads to the development of a new test, vaccine or treatment. ☐
9. I have details to contact the research team if I need to. ☐
10. I agree to take part in the above research. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital

The study has the approval of the St Mary's Research Ethics Committee. (Ref: 07/H0712/85) Version 3 22/06/07

CLINICAL RECORD FORMS FOR CHAPTER 3 PATIENTS (TABLE 3.4)



Dr Ken Dx Sarcoid (EBus ~~Hx~~). 26th March 2010 SA69 5M.

CLINICAL DATA – Sarcoid (1 form per sample)

1st seen in clinic today!

Donor Code: SA69

Date of recruitment: 16/03/2010

Time from diagnosis: today (? Sarcoid)

Age: 56

Sex: f 24/07/53

Ethnicity: Asian.

Site of sample:

Site of disease Blood.

~~India 1978.~~

Strength of diagnosis:

Risk factors for TB (inc BCG/MT) : No TB Contact!

Abnormal signs on CXR: NIL taken.

Other relevant radiological findings (eg CT chest) Referal to CT.

Main symptoms: (Please list, eg 1. cough, 2. E.nodosum)
1. Minor Thaut
2.
3. 3 to 4 yr Hx
4.

Signs observed:
1. Uveitis
2.
3. Red eyes (Right)

*NB: 09/04/2010 Def. Sarcoid! Bx confirmed – BAL & EBUS –

WCC		Lymphocytes		ESR		CRP	
Weight		ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

(Neg Mantaux)

NB Q.FT. (TST. 5:1 ?)

NIL steriods!

(SA92)

SM.

SA92

92

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: SA92

Date of recruitment: 28/04/10
Time from diagnosis: 31/01/10
Age: 05/05/1959
Sex: M
Ethnicity: White
Site of sample: Male
Site of disease Head

Seen by Dr David Cornel — 28/04/10.

Mediastinal Lymphadenopathy (?) Sarcoidosis

Strength of diagnosis: CT Consistent with Sarcoid.

Risk factors for TB (inc BCG/MT) :

Abnormal signs on CXR: CXR ?

Other relevant radiological findings (eg CT chest): CT Sept 09 Then 31/01/10

CT to be repeated

Main symptoms (Please list, eg 1. cough, 2. E.nodosum):
1. Pulmonary.
2.
3.
4.

Signs observed:
1.
2. Appears well.
3.

WCC		Lymphocytes		ESR		CRP	
Weight	↑ how osc over last yr. Can't see how much two!	ACE	106(↑)	Ca		Others	Globulin = 40

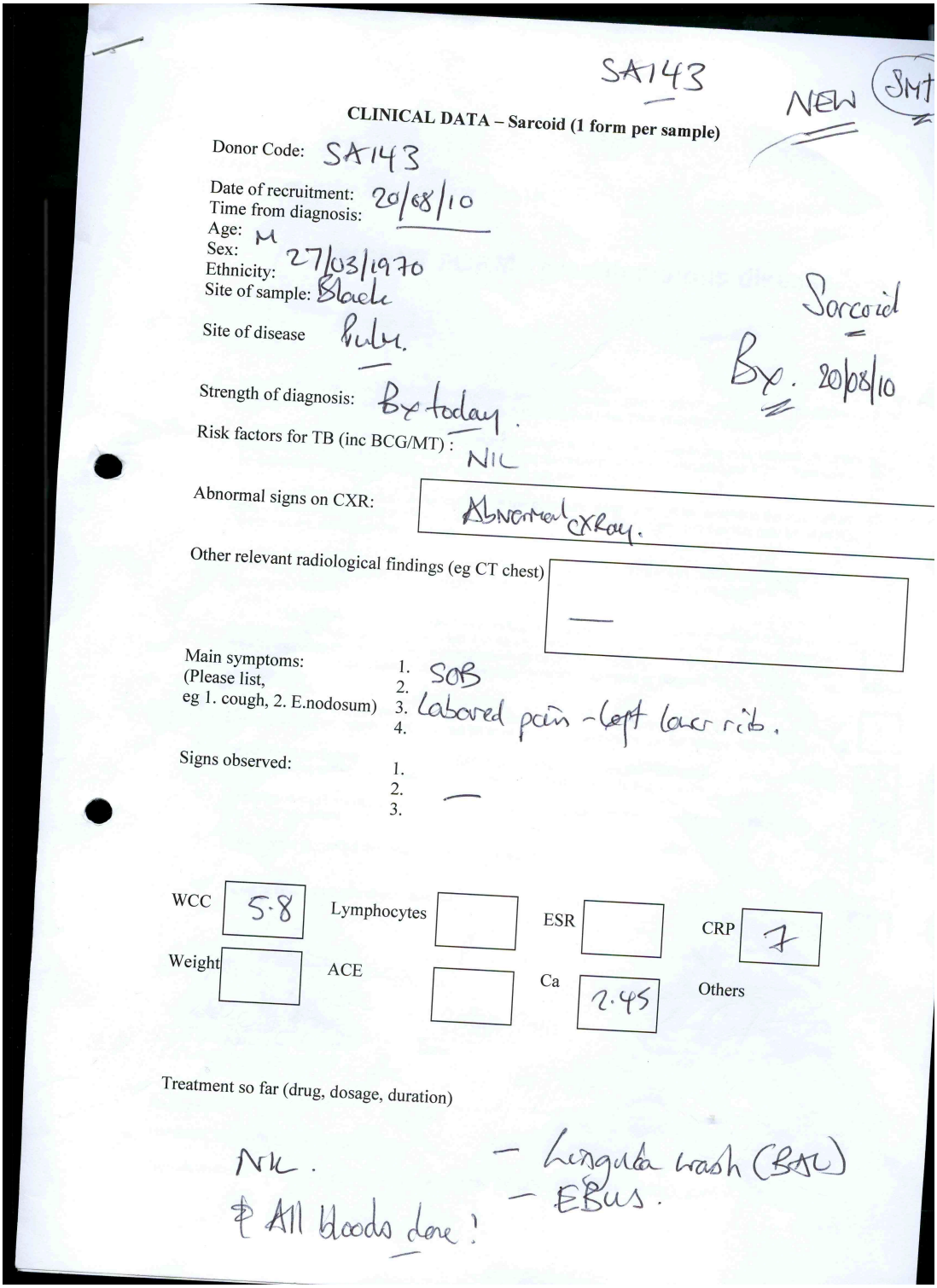
Treatment so far (drug, dosage, duration)

Nil Steroids.

BAL = Fri 30th Nov

Wife = Tracy.

NB Clinic Dr Cornel: 12/05/10 * Minimal Pulm component but Def. S!



SA143

NEW SMT

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: SA143

Date of recruitment: 20/08/10
Time from diagnosis:
Age: M
Sex: 27/03/1970
Ethnicity: Black
Site of sample:

Site of disease Pulm.

Sarcoid
Bx. 20/08/10

Strength of diagnosis: Bx today.

Risk factors for TB (inc BCG/MT): NIL

Abnormal signs on CXR: Abnormal CXRay.

Other relevant radiological findings (eg CT chest) —

Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)
1. SOB
2.
3. Labored pain - left lower rib.
4.

Signs observed:
1.
2. —
3.

WCC	5·8	Lymphocytes		ESR		CRP	7
Weight		ACE		Ca	2.45	Others	

Treatment so far (drug, dosage, duration)

NK.

- Lingula wash (BAL)
- EBUS.

& All bloods done!

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: KP (HEALTHY DONOR).

Date of recruitment: 9/1/2010

Time from diagnosis: N/A

Age: 33

Sex: f

Ethnicity: CAUCASIAN

Site of sample: B 1000 → PBMC → PAX gene → Serum

Site of disease N/A

Strength of diagnosis:

Risk factors for TB (inc BCG/MT) :

Abnormal signs on CXR:

Other relevant radiological findings (eg CT chest)

Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)

1.
2.
3.
4.

Signs observed:

1.
2.
3.

WCC		Lymphocytes		ESR		CRP	
Weight		ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

Nil meds

No respiratory / cardiac comorbidities

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: CE

Date of recruitment: ~~9/9/10~~ 9/9/10
Time from diagnosis: N/A
Age: 33
Sex: M
Ethnicity: C
Site of sample: Blood → SERUM ONLY

Site of disease

Strength of diagnosis:

Risk factors for TB (inc BCG/MT) :

Abnormal signs on CXR:

Other relevant radiological findings (eg CT chest)

Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)
1.
2.
3.
4.

Signs observed:
1.
2.
3.

WCC | Lymphocytes | ESR | CRP

Weight | ACE | Ca | Others

Treatment so far (drug, dosage, duration)

Nil.

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: H2

Date of recruitment: 5/2/2010
Time from diagnosis: → Dx TODAY
Age: 29
Sex: F
Ethnicity: BLACK
Site of sample: LUNG – BAL
BLOOD → SERUM

Dx
HYPERSENSITIVITY
PNEUMONITIS.

Site of disease
LUNG.

Strength of diagnosis: CT + Bx PROVEN.

Risk factors for TB (inc BCG/MT) : —

Abnormal signs on CXR: CT TRAX 25 Jan 2010 – GENERALISED LUNG INFLAMMATION / FIBROSIS.

Other relevant radiological findings (eg CT chest)

Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)
1. COUGH – YELLOW SPUTUM / NO HAEMOPTYSIS
2. SOB
3. 4 kg WEIGHT LOSS
4.

Signs observed:
1. WHEEZE
2. SMOKY.
3.

23/12/9

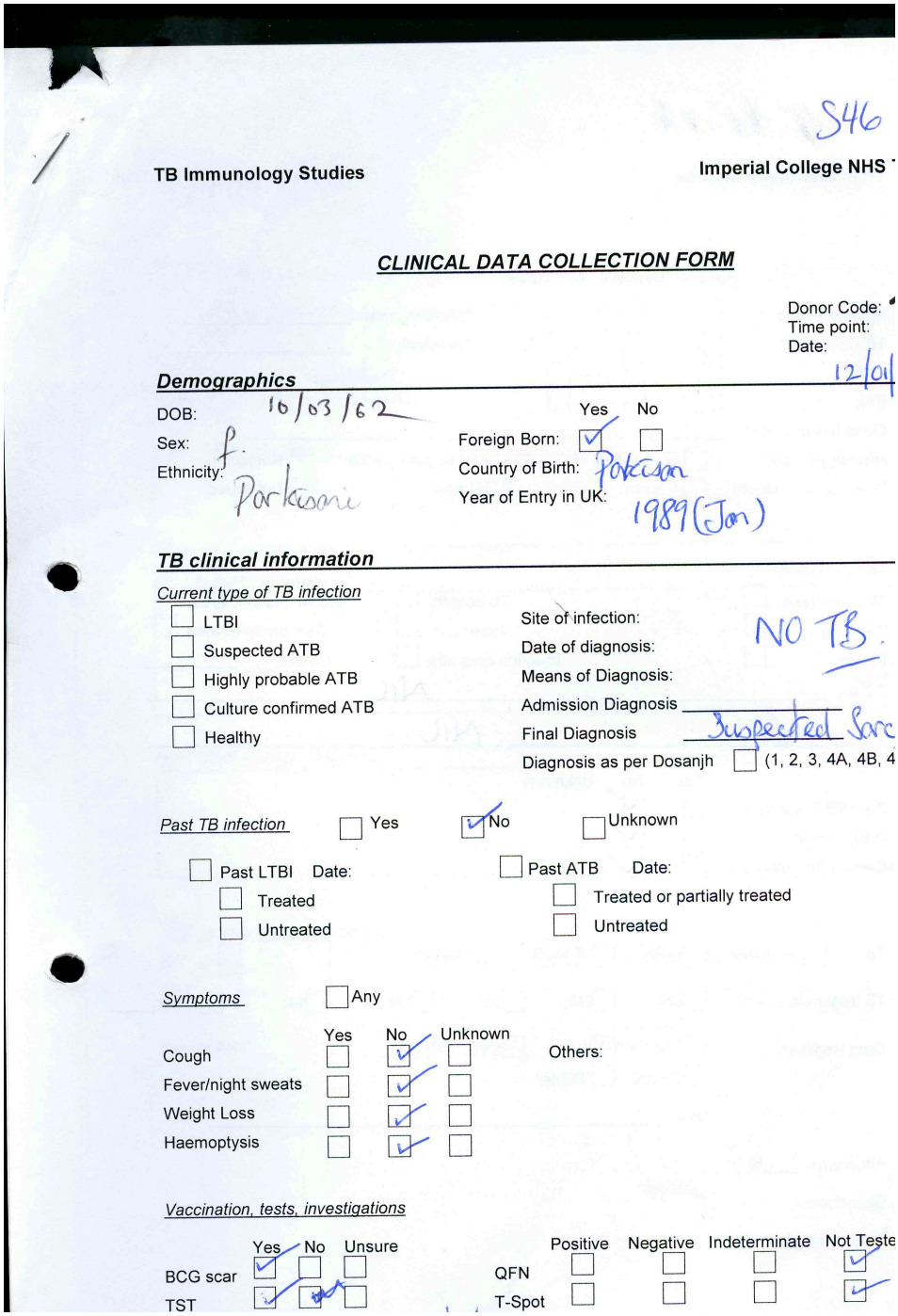
WCC	9.0	Lymphocytes		ESR		CRP	
Weight		ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

Nil steroids
Asthma meds

BM → ① upper lobe
18 cells in
100 ml out.

CLINICAL RECORD FORMS FOR CHAPTER 6 (SELECT SARCOID AND TB PATIENTS



S46

TB Immunology Studies

Imperial College NHS

CLINICAL DATA COLLECTION FORM

Donor Code:
Time point:
Date: 12/01

Demographics

DOB: 10/03/62
Sex: f
Ethnicity: Parkistani

Foreign Born: Yes ☑ No ☐
Country of Birth: Pakistan
Year of Entry in UK: 1989 (Jan)

TB clinical information

Current type of TB infection

☐ LTBI
☐ Suspected ATB
☐ Highly probable ATB
☐ Culture confirmed ATB
☐ Healthy

Site of infection:
Date of diagnosis:
Means of Diagnosis:
Admission Diagnosis NO TB.
Final Diagnosis Suspected Sarc
Diagnosis as per Dosanjh ☐ (1, 2, 3, 4A, 4B, 4

Past TB infection ☐ Yes ☑ No ☐ Unknown

☐ Past LTBI Date:
☐ Treated
☐ Untreated

☐ Past ATB Date:
☐ Treated or partially treated
☐ Untreated

Symptoms ☐ Any

	Yes	No	Unknown
Cough	☐	☑	☐
Fever/night sweats	☐	☑	☐
Weight Loss	☐	☑	☐
Haemoptysis	☐	☑	☐

Others:

Vaccination, tests, investigations

	Yes	No	Unsure
BCG scar	☑	☐	☐
TST	☑	☐	☐

	Positive	Negative	Indeterminate	Not Teste
QFN	☐	☐	☐	☑
T-Spot	☐	☐	☐	☑

SA62

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: SA62

Date of recruitment: 26/02/2010 (2010)
Time from diagnosis:
Age: 24/09/1934
Sex: f
Ethnicity: White
Site of sample:

No BAL – v.
co

Site of disease Blood.

Strength of diagnosis: Bx today 26/02/10 – EBUS TBNA

Risk factors for TB (inc BCG/MT) : Mantoux -ve

Abnormal signs on CXR: CXR – Sept 09 & Dec 09. Paratracheal Subcarinal ad

Other relevant radiological findings (eg CT chest): Calcified granuloma + Soft grand glass nodes

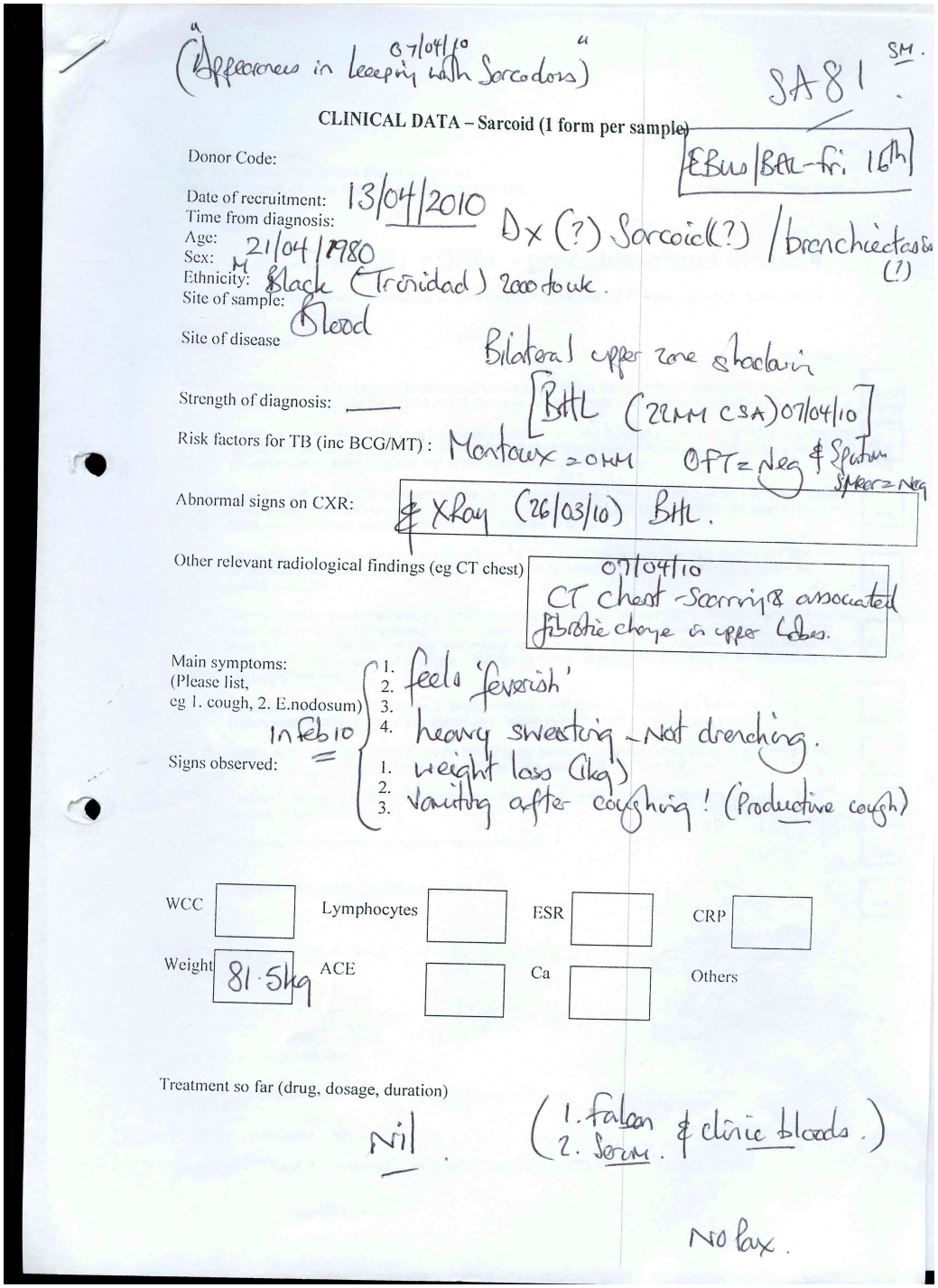
Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)
1. Swallow probs (2yrs)
2. Cough (1yr)
3.
4. CVA – Oct 09.

Signs observed:
1. peripheral neuropathy.
2.
3. Cough

WCC		Lymphocytes		ESR		CRP	
Weight		ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

IV



("Appearances in keeping with Sarcodosis" 07/04/10)

SA81 SM.

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: EBus/BAL-fri 16th

Date of recruitment: 13/04/2010

Time from diagnosis: Dx (?) Sarcoid(?) / bronchiectasis (?)

Age: 21/04/1980

Sex: M

Ethnicity: Black (Trinidad) 2000 to uk.

Site of sample: Blood

Site of disease: Bilateral upper zone shadowing

Strength of diagnosis: — [BHL (22MM CSA) 07/04/10]

Risk factors for TB (inc BCG/MT): Mantoux = 0MM OFT = Neg & Sputum Smear = Neg

Abnormal signs on CXR: & XRay (26/03/10) BHL.

Other relevant radiological findings (eg CT chest): 07/04/10 CT chest - Scarring & associated fibrotic change in upper lobes.

Main symptoms: (Please list, eg 1. cough, 2. E.nodosum)
1. feels 'feverish'
2.
3.
4. heavy sweating - Not drenching.

In Feb 10

Signs observed:
1. weight loss (1kg)
2.
3. vomiting after coughing! (Productive cough)

WCC		Lymphocytes		ESR		CRP	
Weight	81.5kg	ACE		Ca		Others	

Treatment so far (drug, dosage, duration)

Nil. (1. Falcon 2. Serum. & clinic bloods.)

No Pax.

SA133 SM.

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: SA133

Date of recruitment: 23/07/2010
Time from diagnosis: TODAY — Bx.
Age: M
Sex:
Ethnicity: White
Site of sample: Pulmonary ?.
Site of disease Lung.
Strength of diagnosis: Bx - transbronc.
Risk factors for TB (inc BCG/MT) : NIL.

Dr Mels pt.

Abnormal signs on CXR: —

Other relevant radiological findings (eg CT chest) CT Scan - likely to be Sarcoid (Mid 2010)

Main symptoms: (Please list, eg 1. cough, 2. E.nodosum)
1. SOB
2. infection (lung) - 2 doses of abs but did not go away.
3.
4.

Signs observed:
1. Healthy.
2.
3.

WCC		Lymphocytes		ESR		CRP	
Weight		ACE		Ca	Low	Others	

Treatment so far (drug, dosage, duration)

Nil steroids

Bloods-all
+ BAL & Transbronc Bx.

& Back to clinic 2 wks time.

SA 87.

CLINICAL DATA – Sarcoid (1 form per sample)

Donor Code: SA 87

Date of recruitment: 20/04/2010
Time from diagnosis: → 01/04/2010 (weeks) * Dx = Sarcoid.
Age: M 27/10/72
Sex:
Ethnicity: Black African (Nigerian)
Site of sample:

Site of disease Blood.

Bx 29/03/2010

Strength of diagnosis: Instertial Lung markings in Lower zones.

Risk factors for TB (inc BCG/MT) : NO TB. -ve QTF.

Abnormal signs on CXR: BHL = 07/03/2010.

Other relevant radiological findings (eg CT chest) —

Main symptoms:
(Please list,
eg 1. cough, 2. E.nodosum)
1. Weight loss
2. Decreased appetite
3. Night sweats.
4.

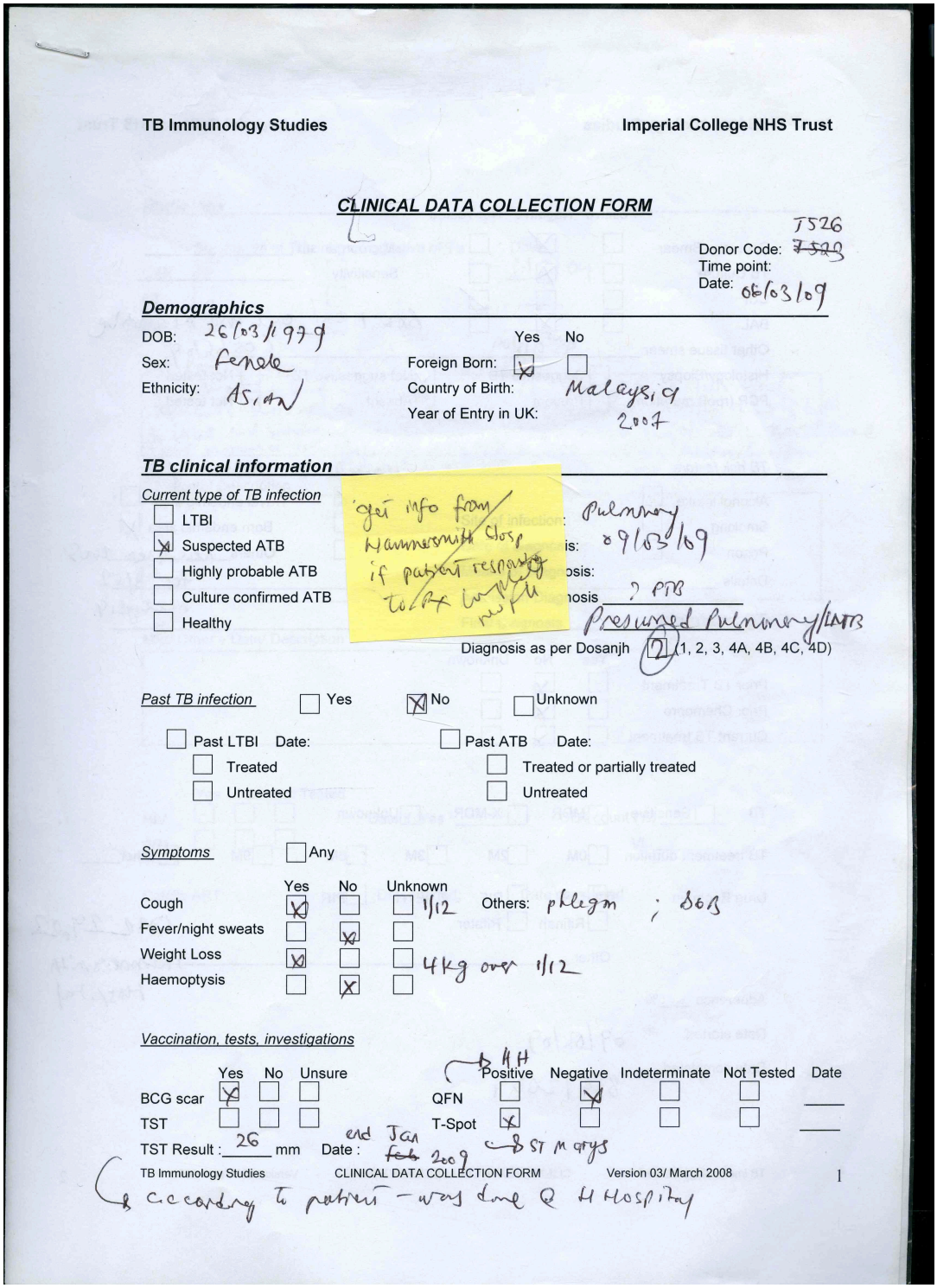
Signs observed:
1.
2.
3.

WCC		Lymphocytes		ESR		CRP	
Weight		ACE	400 NORMAL FOR SARC	Ca		Others	NEW

Treatment so far (drug, dosage, duration)

NIL Rx.

* NB Bloods done by MT. 20/04/10



TB Immunology Studies **Imperial College NHS Trust**

CLINICAL DATA COLLECTION FORM

Donor Code: ~~7523~~ T526
Time point:
Date: 06/03/09

Demographics

DOB: 26/03/1979
Sex: Female
Ethnicity: Asian

	Yes	No
Foreign Born:	☒	☐

Country of Birth: Malaysia
Year of Entry in UK: 2007

TB clinical information

Current type of TB infection

- ☐ LTBI
- ☒ Suspected ATB
- ☐ Highly probable ATB
- ☐ Culture confirmed ATB
- ☐ Healthy

get info from Hammersmith Hosp if patient responding to RX [illegible]

Pulmonary
is: 09/03/09
osis:
nosis ? PTB
Presumed Pulmonary/LATB

Diagnosis as per Dosanjh ☒ (1, 2, 3, 4A, 4B, 4C, 4D)

Past TB infection ☐ Yes ☒ No ☐ Unknown

☐ Past LTBI Date:
- ☐ Treated
- ☐ Untreated

☐ Past ATB Date:
- ☐ Treated or partially treated
- ☐ Untreated

Symptoms ☐ Any

	Yes	No	Unknown	
Cough	☒	☐	☐	1/12
Fever/night sweats	☐	☒	☐	
Weight Loss	☒	☐	☐	4kg over 1/12
Haemoptysis	☐	☒	☐	

Others: phlegm ; DOB

Vaccination, tests, investigations

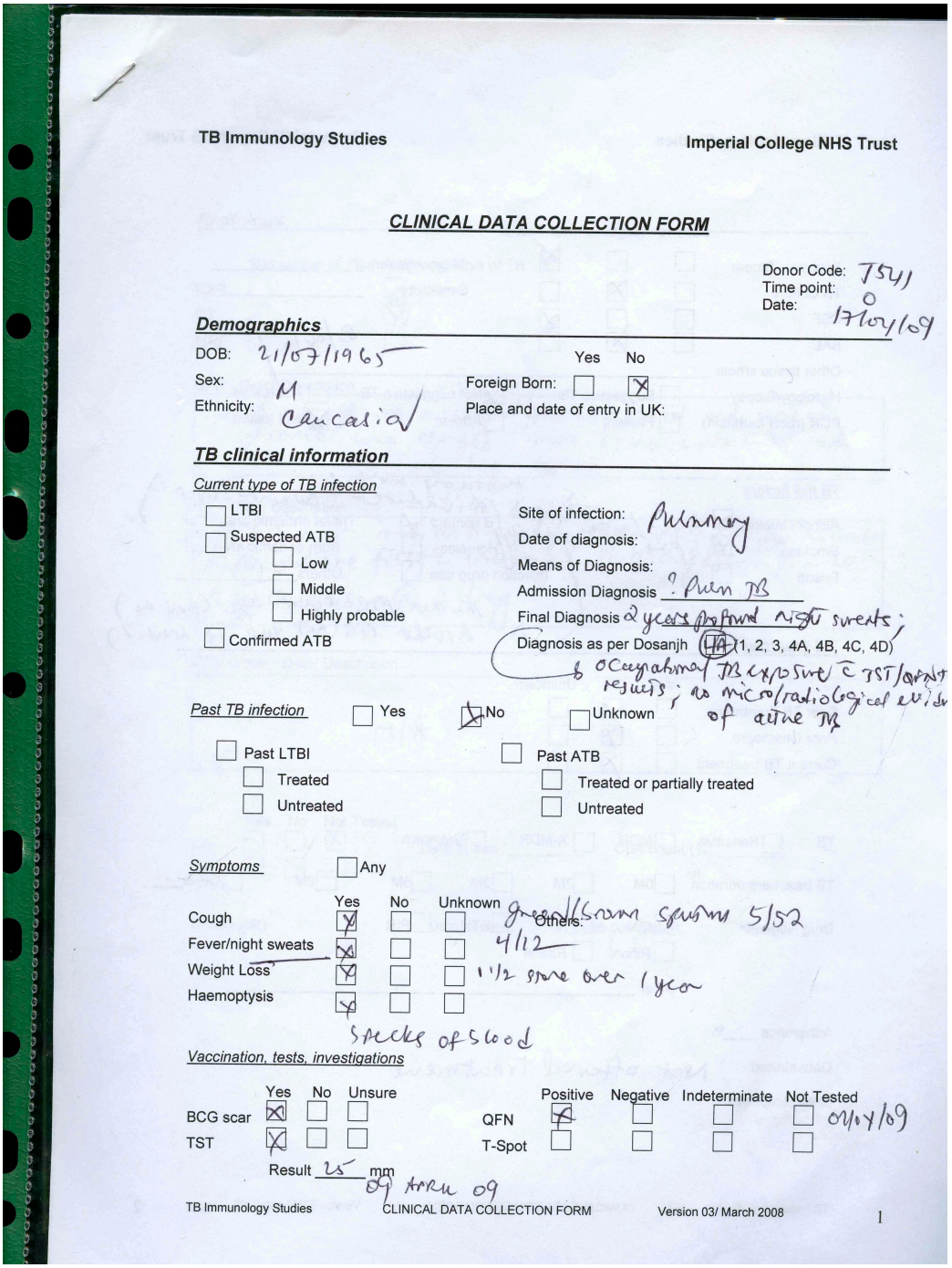
	Yes	No	Unsure
BCG scar	☒	☐	☐
TST	☐	☐	☐

	Positive	Negative	Indeterminate	Not Tested	Date
QFN	☐	☒	☐	☐	
T-Spot	☒	☐	☐	☐	

→ HH

TST Result : 26 mm Date : end Jan ~~Feb~~ 2009 → ST M grys

→ according to patient – was done @ H Hospital



TB Immunology Studies | Imperial College NHS Trust

CLINICAL DATA COLLECTION FORM

Donor Code: T541
Time point: 0
Date: 17/04/09

Demographics

DOB: 21/07/1965
Sex: M
Ethnicity: Caucasia

Foreign Born: Yes ☐ No ☒
Place and date of entry in UK:

TB clinical information

Current type of TB infection

☐ LTBI
☐ Suspected ATB
- ☐ Low
- ☐ Middle
- ☐ Highly probable

☐ Confirmed ATB

Site of infection: Pulmonary
Date of diagnosis:
Means of Diagnosis:
Admission Diagnosis ? Pulm TB
Final Diagnosis 2 years profound night sweats;
Diagnosis as per Dosanjh (4A) (1, 2, 3, 4A, 4B, 4C, 4D)
→ occupational TB exposure c̄ TST/QFNT results; no micro/radiological evidence of active TB

Past TB infection ☐ Yes ☒ No ☐ Unknown

☐ Past LTBI
- ☐ Treated
- ☐ Untreated

☐ Past ATB
- ☐ Treated or partially treated
- ☐ Untreated

Symptoms ☐ Any

	Yes	No	Unknown	Others:
Cough	☒	☐	☐	green/brown sputum 5/52
Fever/night sweats	☒	☐	☐	4/12
Weight Loss	☒	☐	☐	1 1/2 stone over 1 year
Haemoptysis	☒	☐	☐	

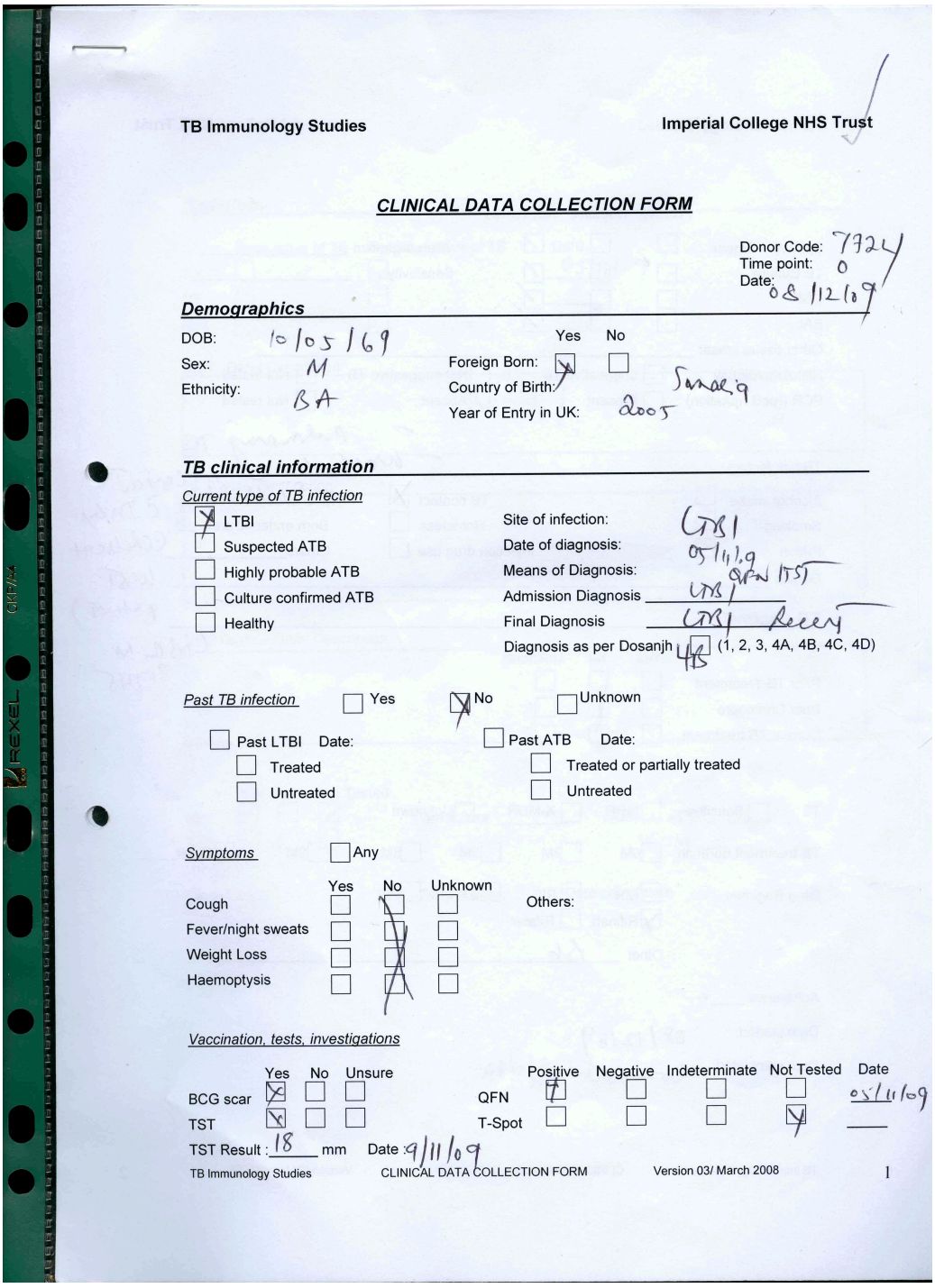
specks of blood

Vaccination, tests, investigations

	Yes	No	Unsure
BCG scar	☒	☐	☐
TST	☒	☐	☐

	Positive	Negative	Indeterminate	Not Tested
QFN	☒	☐	☐	☐ 02/04/09
T-Spot	☐	☐	☐	☐

Result 25 mm 09 April 09



TB Immunology Studies

Imperial College NHS Trust

CLINICAL DATA COLLECTION FORM

Donor Code: 7724
Time point: 0
Date: 08/12/09

Demographics

DOB: 10/05/69
Sex: M
Ethnicity: BA

Foreign Born: Yes [X] No [ ]
Country of Birth: Somalia
Year of Entry in UK: 2005

TB clinical information

Current type of TB infection

- [X] LTBI
- [ ] Suspected ATB
- [ ] Highly probable ATB
- [ ] Culture confirmed ATB
- [ ] Healthy

Site of infection: LTBI
Date of diagnosis: 05/11/09
Means of Diagnosis: QFN ITS
Admission Diagnosis: LTBI
Final Diagnosis: LTBI Recent
Diagnosis as per Dosanjh: [ ] (1, 2, 3, 4A, 4B, 4C, 4D)

Past TB infection [ ] Yes [X] No [ ] Unknown

- [ ] Past LTBI Date:
  - [ ] Treated
  - [ ] Untreated
- [ ] Past ATB Date:
  - [ ] Treated or partially treated
  - [ ] Untreated

Symptoms [ ] Any

	Yes	No	Unknown
Cough	[ ]	[X]	[ ]
Fever/night sweats	[ ]	[X]	[ ]
Weight Loss	[ ]	[X]	[ ]
Haemoptysis	[ ]	[X]	[ ]

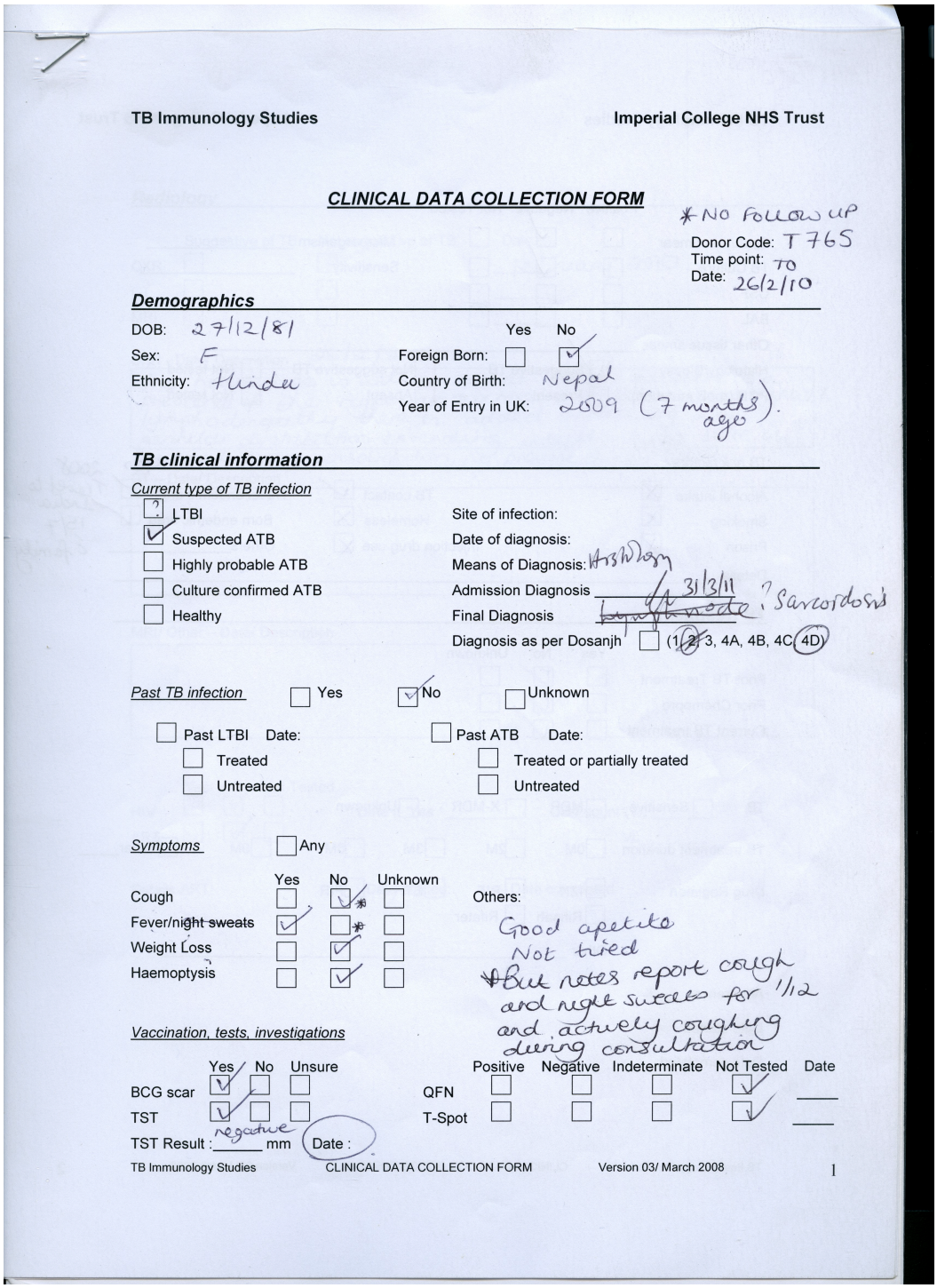
Others:

Vaccination, tests, investigations

	Yes	No	Unsure
BCG scar	[X]	[ ]	[ ]
TST	[X]	[ ]	[ ]

TST Result: 18 mm Date: 9/11/09

	Positive	Negative	Indeterminate	Not Tested	Date
QFN	[X]	[ ]	[ ]	[ ]	05/11/09
T-Spot	[ ]	[ ]	[ ]	[X]	



7

TB Immunology Studies **Imperial College NHS Trust**

CLINICAL DATA COLLECTION FORM

*NO FOLLOW UP

Donor Code: T765
Time point: T0
Date: 26/2/10

Demographics

DOB: 27/12/81
Sex: F
Ethnicity: Hindu

	Yes	No
Foreign Born:	☐	☑

Country of Birth: Nepal
Year of Entry in UK: 2009 (7 months ago).

TB clinical information

Current type of TB infection

- ☐ LTBI
- ☑ Suspected ATB
- ☐ Highly probable ATB
- ☐ Culture confirmed ATB
- ☐ Healthy

Site of infection:
Date of diagnosis:
Means of Diagnosis: Histology
Admission Diagnosis 31/3/11 ? Sarcoidosis
Final Diagnosis ~~Lymphnode~~
Diagnosis as per Dosanjh ☐ (1, 2, 3, 4A, 4B, 4C, 4D)

Past TB infection ☐ Yes ☑ No ☐ Unknown

- ☐ Past LTBI Date:
  - ☐ Treated
  - ☐ Untreated
- ☐ Past ATB Date:
  - ☐ Treated or partially treated
  - ☐ Untreated

Symptoms ☐ Any

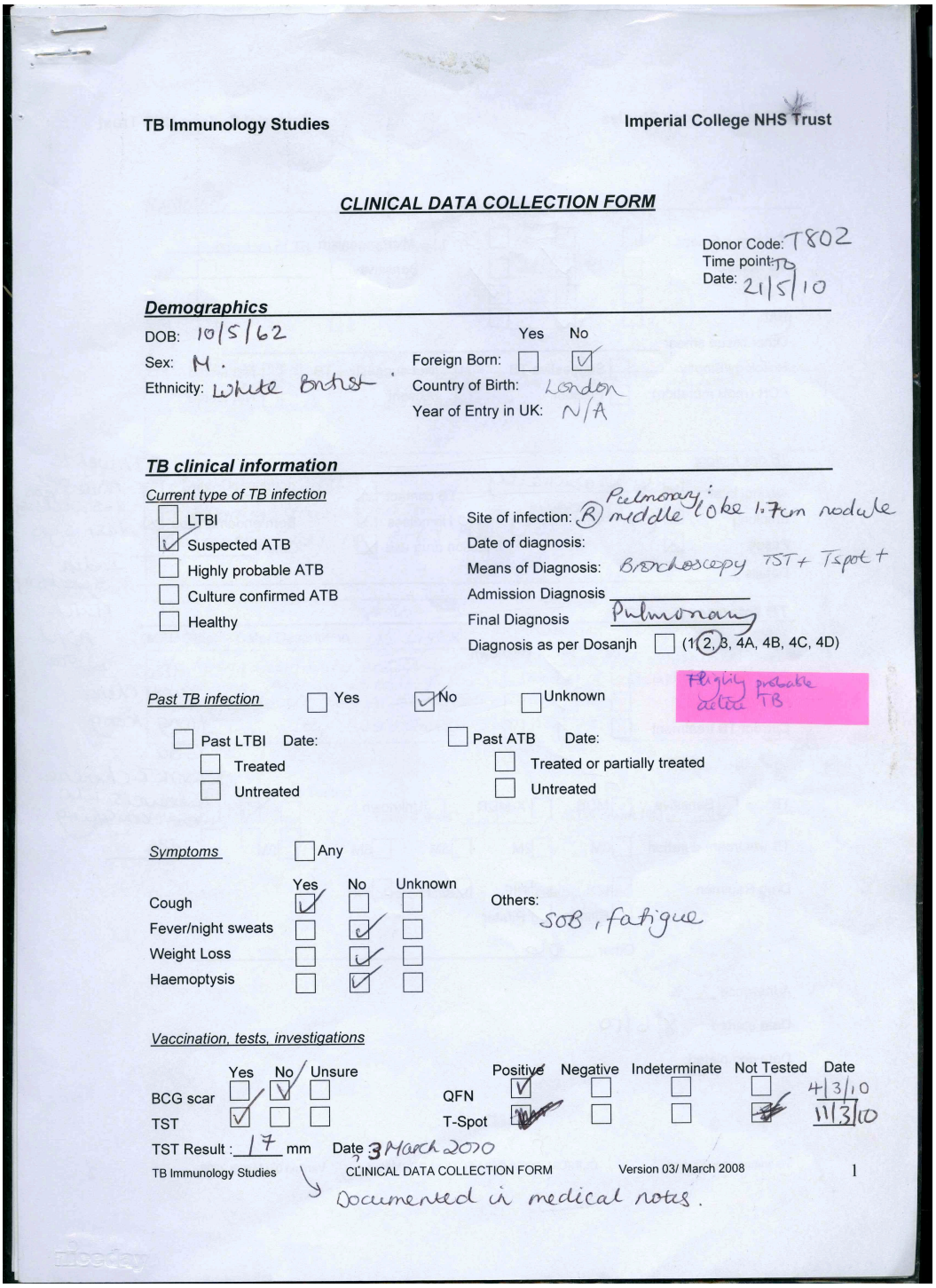
	Yes	No	Unknown
Cough	☐	☑*	☐
Fever/night sweats	☑	☐*	☐
Weight Loss	☐	☑	☐
Haemoptysis	☐	☑	☐

Others:
Good apetite
Not tried
*But notes report cough and night sweates for 1/12 and actively coughing during consultation

Vaccination, tests, investigations

	Yes	No	Unsure		Positive	Negative	Indeterminate	Not Tested	Date
BCG scar	☑	☐	☐	QFN	☐	☐	☐	☑	
TST	☑	☐	☐	T-Spot	☐	☐	☐	☑	

TST Result : negative mm Date :



TB Immunology Studies

Imperial College NHS Trust

CLINICAL DATA COLLECTION FORM

Donor Code: T802
Time point: ~~T0~~
Date: 21/5/10

Demographics

DOB: 10/5/62
Sex: M.
Ethnicity: White British

	Yes	No
Foreign Born:	☐	☑

Country of Birth: London
Year of Entry in UK: N/A

TB clinical information

Current type of TB infection

- ☐ LTBI
- ☑ Suspected ATB
- ☐ Highly probable ATB
- ☐ Culture confirmed ATB
- ☐ Healthy

Site of infection: (R) Pulmonary: middle lobe 1.7cm nodule
Date of diagnosis:
Means of Diagnosis: Bronchoscopy TST+ Tspot+
Admission Diagnosis
Final Diagnosis: Pulmonary
Diagnosis as per Dosanjh ☐ (1, (2), 3, 4A, 4B, 4C, 4D)

Highly probable active TB

Past TB infection ☐ Yes ☑ No ☐ Unknown

- ☐ Past LTBI Date:
  - ☐ Treated
  - ☐ Untreated
- ☐ Past ATB Date:
  - ☐ Treated or partially treated
  - ☐ Untreated

Symptoms ☐ Any

	Yes	No	Unknown
Cough	☑	☐	☐
Fever/night sweats	☐	☑	☐
Weight Loss	☐	☑	☐
Haemoptysis	☐	☑	☐

Others: SOB, fatigue

Vaccination, tests, investigations

	Yes	No	Unsure		Positive	Negative	Indeterminate	Not Tested	Date
BCG scar	☐	☑	☐	QFN	☑	☐	☐	☐	4/3/10
TST	☑	☐	☐	T-Spot	☑	☐	☐	☑	11/3/10

TST Result: 17 mm Date: 3 March 2010

Documented in medical notes.

APPENDIX 8: SELECT PROTEIN IDENTIFICATIONS USING MALDI-TOF

Kveim reagent	**Protein band**	**Protein identification - based on highest MASCOT score. Homo Sapiens unless otherwise indicated (Accession number)**	**MW (Da) Probability based Mowse score**	**Function**
K51	1	exocyst complex component 5, isoform CRA_a (gi|119601103)	62383 53	Component of the exocyst complex involved in the docking of exocystic vesicles with fusion sites on the plasma membrane
K51	2	Chain A, Crystal Structure Of Non-Phosphorylated Ret Tyrosine Kinase Domain (gi|114794789)	35633 62	Receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family of extracellular signalling molecules or ligands
K51	3	hCG1987325, isoform CRA_a (gi|119575168)	27803 54	Molecular function unknown
K51	4	beta globin (gi|221039498)	15988 84	Beta chain of the oxygen carrying metalloprotein in red blood cells
K54	5	No identification made with mascot		
K54	6	MHC class II antigen (gi|1469910)	9933 41	MHC class II antigen
K12	7	No identification made with mascot		
K12	8	unnamed protein product |194378024	41818 51	Molecular function

				unknown
K12	8	unnamed protein product gi|221039498	1426055	Molecular function unknown
K1.6.1	10	17	Chain A, Structure Of Human Serum Albumin With S-Naproxen And The Ga Module gi|168988718	6577862
K1.6.1	11	No identification made with mascot		
K1.6.1	12	No identification made with mascot		
K1.6.1	13	cyclin fold protein 1 variant a gi|18766393	3638255	regulatory subunits of the cyclin-dependent protein kinases
K1.6.1	14	hypothetical protein XP_002344289 gi|239746144	3039949	Hypothetical-molecular function unknown
K1.6.1	15	LONP2 protein gi|38197066	2169443	Probable serine protease which may be involved in peroxisome biogenesis
K55	16	castor homolog 1, zinc finger , isoform CRA_a gi|119592068	14472448	Probable transcription factor
K55	17	host cell factor C1 regulator 1 (XPO1 dependant) isoform 1 gi|8923536	1528147	Regulates HCFC1 activity by modulating its subcellular localization
K55	18	HLA-DRB3	9758	HLA class

		gi|2687823	37	II histocompatibility antigen, DRB3-1 beta chain - It plays a central role in the immune system by presenting peptides derived from extracellular proteins

APPENDIX 9: SPECTRUM MILL IDENTIFICATION DATA FOR PROTEINS IDENTIFIED THROUGH 1D-SDS-PAGE.

Full spectrum mill data will be available for a limited time for public access at: https://rapidshare.com/files/4199811293/sarcoiddata.xlsx

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
6	14	10	155.76	45	4.81E+05	24722.2	HUMAN	P04179	Superoxide dismutase [Mn], mitochondrial OS=Homo sapiens GN=SOD2 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	20.55	20.55	97.1	9.99E+05	(K)GDVTA(	1424.811
2	MTGELSPOT18_5_11	2	19.99	19.99	81.3	4.52E+04	(K)AIWNVII	1743.881
3	MTGELSPOT18_5_11	3	19.85	7.11	88	1.75E+06	(K)GELLEA	1028.61
4	MTGELSPOT18_5_11	3	18.95	18.95	90.1	1.90E+05	(K)GDVTA(	1424.811
5	MTGELSPOT18_5_11	2	16.91	5.93	88	6.76E+05	(K)GELLEA	1028.61
6	MTGELSPOT18_5_11	2	16.52	5.86	76.9	2.49E+05	(R)DFGSFI	1090.52
7	MTGELSPOT18_5_11	4	16.32	16.32	89.7	3.55E+05	(K)HHAAY\	1738.851
8	MTGELSPOT18_5_11	3	16.26	16.26	85.5	4.38E+05	(K)HHAAY\	1738.851
9	MTGELSPOT18_5_11	3	15.92	8.17	83.8	5.25E+05	(R)DFGSFI	1090.52
10	MTGELSPOT18_5_11	4	13.29	13.29	75.9	4.52E+05	(K)HHAAY\	2542.269
11	MTGELSPOT18_5_11	2	13.1	6.45	88.6	1.61E+05	(R)DFGSFI	815.357
12	MTGELSPOT18_5_11	2	12.75	3.12	83.6	3.74E+05	(K)GELLEA	872.509
13	MTGELSPOT18_5_11	4	12.28	5.97	88.7	1.82E+05	(K)FNGGG	2637.259
14	MTGELSPOT18_5_11	2	11.11	3.7	82	3.43E+05	(K)NVRPD'	1004.552

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
5	7	3	40.85	25	3.30E+04	11283.9	HUMAN	P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	kveimgel65	2	16.7	12.38	87.2	2.94E+04	(K)DAVEDI	1161.563
2	kveimgel65	2	16.09	16.09	90.5	4.78E+04	(K)ENAGEI	1128.528
3	kveimgel65	2	14.65	14.65	85.7	4.38E+04	(K)ENAGEI	1128.528
4	kveimgel65	2	12.56	12.56	80.3	3.19E+04	(K)ENAGEI	1128.528
5	kveimgel65	2	11.19	11.19	72.1	3.36E+04	(K)ENAGEI	1128.528
6	kveimgel65	2	10.91	7.27	75.3	1.63E+04	(K)DAVEDI	1161.563
7	kveimgel65	2	8.06	2.22	64.3	2.80E+04	(R)SSLLEK	676.388

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
2	13	12	157	18	1.01E+05	77050.4	HUMAN	P02787	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIM	2	19.64	11.78	96.4	2.25E+05	(K)YLGEEYVK(A)	1000.499
2	NEWKVEIM	2	16.81	16.81	77.4	7.84E+04	(K)IECVSAETTEDCIAK(I)	1725.767
3	NEWKVEIM	2	16.76	16.76	84.3	1.64E+05	(K)DGAGDVAFVK(H)	978.489
4	NEWKVEIM	3	14.88	14.88	78.6	6.31E+04	(K)HSTIFENLANK(A)	1273.653
5	NEWKVEIM	2	14.41	14.41	77.4	1.39E+05	(K)EGYYGYTGAFR(C)	1283.569
6	NEWKVEIM	2	12.32	7.6	69.6	9.94E+04	(K)DSAHGFLK(V)	874.442
7	NEWKVEIM	2	11.41	11.41	64.7	3.77E+04	(K)DSGFQMNQLR(G)	1195.552
8	NEWKVEIM	3	11.38	11.38	74.8	5.69E+04	(K)ADRDQYELLCLDNTR(K)	1881.876
9	NEWKVEIM	2	10.88	10.88	73.4	4.65E+04	(R)DDTVCLAK(L)	921.435
10	NEWKVEIM	2	10.76	2.68	73.9	1.64E+05	(K)GDVAFVK(H)	735.404
11	NEWKVEIM	2	8.91	8.91	68.4	4.79E+04	(K)SASDLTWDNLK(G)	1249.606
12	NEWKVEIM	2	8.84	8.84	64.1	6.91E+04	(R)FDEFFSEGCAPGSK(K)	1577.658
13	NEWKVEIM	2	8.07	8.07	60.2	1.21E+05	(K)HSTIFENLANK(A)	1273.653

Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
11	10	95.29	10	2.13E+05	84622.3	HUMAN	Q16853	Membrane primary amine oxidase OS=Homo sapiens GN=AOC3 PE=1 SV=3

Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
NEWKVEIM	2	16.97	16.97	78.8	5.94E+04	(K)AAALAI	937.521
NEWKVEIM	3	14.17	14.17	84.2	2.92E+05	(K)SPVPP(	2002.055
NEWKVEIM	3	10.07	10.07	64.5	1.70E+05	(R)HGGPL	1033.533
NEWKVEIM	2	9.81	9.81	68.9	1.47E+05	(R)FSVQG	780.4
NEWKVEIM	2	8.74	2.96	82.1	1.31E+05	(K)VFYQGI	769.399
NEWKVEIM	2	8.38	0.55	77.1	1.35E+05	(R)FLTQR(	664.378
NEWKVEIM	2	7.99	2.71	65.2	4.90E+04	(K)SPVPP(	2002.055
NEWKVEIM	2	7.83	0.31	75.1	4.37E+05	(R)LQVTR(	616.378
NEWKVEIM	2	7.1	-0.75	86	3.37E+05	(R)DVTVEI	718.373
NEWKVEIM	2	6.97	3.4	84.2	5.55E+05	(K)ALDPAI	642.357
NEWKVEIM	2	5.25	-0.64	60.1	3.46E+04	(R)GFSWE	781.363

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
5	35	17	171.74	5	7.68E+04	312300.4	HUMAN	P35555	Fibrillin-1 OS=Homo sapiens GN=FBN1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIN	2	14.61	1.56	75.4	3.97E+04	(K)TICIETII	977.534
2	NEWKVEIN	2	14.53	6.2	82.8	2.75E+04	(R)ISPDLC	917.451
3	NEWKVEIN	2	12.72	12.72	77.9	2.77E+05	(R)VGCVD	806.383
4	NEWKVEIN	2	12.66	7.45	78.6	7.00E+04	(R)NTPEYI	1407.621
5	NEWKVEIN	2	12.66	12.66	67.3	1.87E+04	(R)NTPEYI	1407.621
6	NEWKVEIN	3	12.54	12.54	68.1	1.36E+05	(R)SGNCY	1350.658
7	NEWKVEIN	2	11.79	11.79	61.8	1.40E+05	(K)ASCCC	1042.411
8	NEWKVEIN	2	11.78	4.57	83.3	5.44E+04	(R)ISPDLC	917.451
9	NEWKVEIN	2	11.77	4.3	79.8	1.78E+05	(R)GFIPNII	816.473
10	NEWKVEIN	2	11.07	1.8	67.3	4.59E+04	(R)LETCFL	938.476
11	NEWKVEIN	2	11.03	5.18	68.9	3.59E+04	(R)NTPEYI	1407.621
12	NEWKVEIN	3	10.67	10.67	63	2.19E+04	(K)YQCAC	1825.738
13	NEWKVEIN	3	10.16	10.16	64.8	3.68E+04	(R)SGNCY	1350.658
14	NEWKVEIN	2	9.17	2.69	72.8	2.48E+04	(R)ISPDLC	917.451
15	NEWKVEIN	2	8.77	8.77	67.8	2.24E+05	(R)VGCVD	806.383
16	NEWKVEIN	2	8.69	1.84	62.4	2.35E+04	(R)ISPDLC	917.451
17	NEWKVEIN	2	8.64	8.64	65.9	2.95E+05	(R)ATEDFI	824.379
18	NEWKVEIN	2	7.97	7.97	63.9	6.08E+04	(R)GPGFA	677.362
19	NEWKVEIN	2	7.89	2.75	71.8	1.40E+05	(R)GFIPNII	816.473
20	NEWKVEIN	2	7.78	7.78	56.9	3.10E+04	(R)CSNQLI	1275.636
21	NEWKVEIN	2	7.7	7.7	56.4	1.17E+04	(K)NGLCVI	933.457
22	NEWKVEIN	2	7.7	3.42	59	1.51E+04	(R)LETCFL	938.476
23	NEWKVEIN	2	7.67	0.88	71.6	4.65E+04	(R)SLCYR(	698.329
24	NEWKVEIN	2	7.49	7.49	61.1	3.01E+04	(R)YEDEE(	1552.695
25	NEWKVEIN	2	7.4	7.4	60.4	3.00E+04	(R)LETCFL	938.476
26	NEWKVEIN	2	7.21	1.51	79.1	1.75E+05	(R)GFIPNII	816.473
27	NEWKVEIN	2	6.73	-0.32	68.3	2.77E+04	(R)LETCFL	938.476
28	NEWKVEIN	2	6.72	2.14	57.9	1.73E+04	(R)LETCFL	938.476
29	NEWKVEIN	2	6.48	3.31	66.8	2.51E+04	(R)LETCFL	938.476
30	NEWKVEIN	2	6.12	6.12	62.4	1.85E+04	(R)ICLDIR(	789.429
31	NEWKVEIN	2	6.03	1.66	68.2	5.76E+04	(R)LETCFL	938.476
32	NEWKVEIN	2	6.01	6.01	62.5	2.75E+05	(K)LNEVSC	817.441
33	NEWKVEIN	2	5.9	2.87	64.9	3.67E+04	(R)GPGFA	677.362
34	NEWKVEIN	2	5.28	5.28	56.4	4.14E+03	(R)ISPDLC	917.451
35	NEWKVEIN	2	5.23	0	64	3.44E+04	(R)ICLDIR(	789.429

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
23	6	4	42.42	12	6.80E+04	30160.3	HUMAN	P20039	HLA class II histocompatibility antigen, DRB1-11 beta chain OS=Homo sapiens GN=HLA-DRB1 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIN	2	15.14	15.14	86.9	1.12E+05	(R)FDSDV(	1071.474
2	NEWKVEIN	2	12.45	12.45	79.1	1.44E+05	(R)FDSDV(	1071.474
3	NEWKVEIN	2	11.85	11.85	68.4	3.24E+04	(R)YFYNQI	1410.632
4	NEWKVEIN	2	9.54	9.54	66.9	5.36E+04	(R)AAVDT\	955.43
5	NEWKVEIN	2	9.35	9.35	56.1	2.13E+04	(R)AAVDT\	955.43
6	NEWKVEIN	2	7.57	3.85	74.7	1.39E+04	(R)ATPEN\	1295.638
7	NEWKVEIN	2	5.89	5.89	57.5	4.40E+04	(K)VTVYPS	793.445
8	NEWKVEIN	2	5.57	5.57	57.8	2.22E+05	(R)VNVSPS	730.409

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
13	8	6	91.57	28	1.65E+05	22588.2	HUMAN	P80188	Neutrophil gelatinase-associated lipocalin OS=Homo sapiens GN=LCN2 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11_	2	19.6	12.47	95.5	4.07E+05	(K)SYPGLT	1255.668
2	MTGELSPOT18_5_11_	2	19.45	19.45	92.7	1.65E+05	(K)WYVVG	1431.811
3	MTGELSPOT18_5_11_	2	16.81	10.92	93.1	1.07E+05	(K)SYPGLT	1255.668
4	MTGELSPOT18_5_11_	3	15.37	15.37	74.9	1.44E+05	(K)ELTSEL	1478.785
5	MTGELSPOT18_5_11_	2	12.96	3.31	75.2	6.25E+04	(K)ELTSEL	819.446
6	MTGELSPOT18_5_11_	3	12.62	12.62	74.8	1.19E+05	(K)VPLQQI	1790.882
7	MTGELSPOT18_5_11_	2	11.57	4.65	84.2	2.55E+05	(K)ITLYGR	722.42
8	MTGELSPOT18_5_11_	2	10.91	2.54	86.3	5.84E+04	(K)ITLYGR	722.42

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	7	7	106.47	8	2.03E+05	104854.6	O43707	Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK032_8-11_1.1146.1149.2	2	20.71	11.69	93.2	2.55E+05	(R)FAIQDISVEETSAK	1537.774
2	SpotNoK032_8-11_1.1101.1106.2	2	19.83	11.63	96.9	6.08E+05	(K)ALDFIASK(G)	864.482
3	SpotNoK032_8-11_1.0926.0934.3	3	15.1	6.83	89.1	1.89E+05	(R)HRPELIEYDK(L)	1299.669
4	SpotNoK032_8-11_1.1155.1160.3	3	13.81	13.81	77.9	1.06E+05	(R)KDDPVTNLNNAFE	1903.94
5	SpotNoK032_8-11_1.1120.1127.2	2	12.77	12.77	68	1.64E+05	(K)LVSIGAEEIVDGNA	1514.806
6	SpotNoK032_8-11_1.1265.1272.2	2	12.41	2.76	83.8	5.51E+04	(K)EGLLLWCQR(K)	1174.604
7	SpotNoK032_8-11_1.1211.1218.2	2	11.84	11.84	74.5	4.21E+04	(K)DDPVTNLNNAFEV	1775.845

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
6	16	13	148.54	25	1.08E+05	59756.5	HUMAN	P04040	Catalase OS=Homo sapiens GN=CAT PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIM	2	18.66	18.66	88.5	9.34E+04	(K)LVNAN(	1337.652
2	NEWKVEIM	2	16.49	10.26	87.8	6.89E+04	(R)DLFNAI	1049.563
3	NEWKVEIM	3	14.76	14.76	83.1	1.64E+05	(R)LFAYPI	1119.558
4	NEWKVEIM	3	14.34	14.34	74.3	1.03E+05	(K)ADVLTT	2364.288
5	NEWKVEIM	2	14.29	14.29	69.6	1.22E+05	(R)FNTANI	1493.698
6	NEWKVEIM	2	14.18	14.18	63.8	1.00E+05	(R)LSQEDI	1292.612
7	NEWKVEIM	3	12.5	12.5	64.3	4.13E+04	(K)ADVLTT	2364.288
8	NEWKVEIM	2	11.97	11.97	67.1	4.59E+04	(R)LSQEDI	1292.612
9	NEWKVEIM	2	11.71	11.71	69.8	4.12E+04	(K)ADVLTT	1414.717
10	NEWKVEIM	3	9.13	9.13	72.7	6.81E+04	(R)DPILFP	1528.816
11	NEWKVEIM	2	7.98	0.79	84.4	1.64E+05	(K)LVLNR(	614.398
12	NEWKVEIM	2	7.91	3.08	77.2	1.85E+05	(K)NLSVEI	974.49
13	NEWKVEIM	3	6.6	6.6	60.4	5.77E+04	(R)FSTVA(	1851.883
14	NEWKVEIM	2	6.58	6.58	81	2.31E+05	(R)VANYQ	750.389
15	NEWKVEIM	2	5.91	5.91	59.5	1.88E+05	(K)TPIAVR	656.409
16	NEWKVEIM	2	5.11	2.05	65.5	5.57E+04	(K)NLSVEI	974.49

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
4	16	12	165.09	19	8.42E+04	53651.9	HUMAN	P08670	Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	kveimgel66	3	18.98	6.1	89.8	1.84E+05	(K)ILLAELE	1539.911
2	kveimgel66	3	17.9	17.9	91.8	2.19E+05	(R)VEVERI	1688.827
3	kveimgel66	2	17.34	7.39	88.4	7.68E+04	(K)ILLAELE	1169.714
4	kveimgel66	2	16.48	16.48	81.9	6.71E+04	(R)EEAEN	1323.618
5	kveimgel66	3	15.98	15.98	81.4	3.21E+04	(R)VEVERI	1688.827
6	kveimgel66	2	15.36	15.36	86.3	9.78E+04	(R)QDVDN	1088.533
7	kveimgel66	2	14.87	14.87	85.8	3.23E+04	(K)LLEGEE	1005.467
8	kveimgel66	2	13.54	3.87	88.8	2.66E+05	(R)FLEQQI	906.468
9	kveimgel66	2	13.37	13.37	83.1	6.15E+04	(R)EYQDLI	1121.584
10	kveimgel66	2	12.69	3.83	72.3	6.78E+04	(K)LQEEml	1046.53
11	kveimgel66	2	12.03	12.03	77.2	4.63E+04	(R)DNLAEI	1076.504
12	kveimgel66	2	11.37	1.87	90.4	9.97E+04	(R)VEVER(	631.341
13	kveimgel66	2	11.26	11.26	71.2	1.28E+04	(R)QDVDN	1088.533
14	kveimgel66	2	10.1	10.1	69.9	1.61E+04	(R)EYQDLI	1121.584
15	kveimgel66	2	9.89	9.89	69.1	1.87E+04	(K)LLEGEE	1005.467
16	kveimgel66	2	8.87	8.87	63.3	4.11E+04	(R)QVDQL	1060.527
17	kveimgel66	2	8.71	2.46	73.3	3.94E+04	(R)FLEQQI	906.468
18	kveimgel66	2	7.16	7.16	64	1.89E+04	(K)LLEGEE	932.468

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
12	19	9	104.81	1	1.46E+05	468800.7	HUMAN	P98160	Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens GN=HSPG2 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIN	2	15.76	15.76	83.1	2.20E+05	(R)ASYAQ(	1207.57
2	NEWKVEIN	2	14.6	14.6	84.3	7.44E+04	(R)ASYAQ(	1207.57
3	NEWKVEIN	3	13.65	6.94	74.4	1.30E+05	(R)FLGDK\	1541.796
4	NEWKVEIN	2	13.47	13.47	85.2	3.87E+05	(R)VTVTSE	905.469
5	NEWKVEIN	2	12.01	12.01	63.8	1.85E+05	(R)TPSGL\	1353.647
6	NEWKVEIN	3	11.88	5.78	60.5	5.35E+04	(R)FLGDK\	1541.796
7	NEWKVEIN	2	11.81	3.66	81.9	3.82E+05	(R)ESIQAS	790.405
8	NEWKVEIN	2	10.61	3.57	75.9	8.63E+04	(K)VTSYG(	981.5
9	NEWKVEIN	3	10.47	10.47	69.4	5.51E+04	(R)EQAWC	1639.794
10	NEWKVEIN	2	10.37	10.37	81.4	2.30E+05	(R)VTVTSE	905.469
11	NEWKVEIN	2	9.49	9.49	73.5	1.08E+05	(R)VTVTSE	905.469
12	NEWKVEIN	2	9.34	9.34	65.1	8.76E+04	(R)VTVTSE	905.469
13	NEWKVEIN	2	8.83	8.83	61.7	5.92E+04	(R)VTVTSE	905.469
14	NEWKVEIN	2	8.75	8.75	67.1	2.05E+05	(R)FTVTQF	751.41
15	NEWKVEIN	2	8.58	8.58	65.9	7.23E+04	(R)FTVTQF	751.41
16	NEWKVEIN	2	8.28	0	59.4	1.25E+05	(R)GTLIIR(	672.44
17	NEWKVEIN	2	8.03	8.03	62	3.86E+04	(K)VTSYG(	981.5
18	NEWKVEIN	2	6.74	3.61	56.9	1.58E+05	(R)ESIQAS	790.405
19	NEWKVEIN	2	6.73	6.73	64.5	1.24E+05	(R)VTVTSE	905.469

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
16	6	5	44.23	2	2.52E+04	262625.9	HUMAN	P02751	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIN	3	9.78	9.78	69	1.81E+04	(K)LGVRP\	1323.713
2	NEWKVEIN	2	8.8	8.8	62.8	2.18E+04	(K)IYLYTL\	1355.695
3	NEWKVEIN	3	8.76	8.76	61	2.79E+04	(R)VDVIPV	1629.871
4	NEWKVEIN	2	8.65	3.39	60.5	2.10E+04	(R)SYTITG	1543.764
5	NEWKVEIN	3	8.35	8.35	63.7	3.15E+04	(K)LGVRP\	1323.713
6	NEWKVEIN	2	8.24	2.31	70.4	3.07E+04	(R)LTVGLT	759.472

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
2	3	3	39.22	19	1.24E+05	13989.3	Q99879	Histone H2B type 1-M OS=Homo sapiens GN=HIST1H2BM PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK027_5-11_93.1162.1166.2	2	14.71	5.25	94.8	1.86E+05	(R)LLLPGELAK(H)	953.603
2	SpotNoK027_5-11_93.0820.0853.2	2	12.71	4	77.7	1.56E+05	(R)EIQTAVR(L)	816.457
3	SpotNoK027_5-11_93.1033.1073.2	2	11.8	11.8	66.2	2.98E+04	(K)ESYSVYVYK(V)	1137.546

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
28	5	5	59.84	5	5.87E+04	88279.1	HUMAN	P27487	Dipeptidyl peptidase 4 OS=Homo sapiens GN=DPP4 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	15.39	15.39	83.7	8.81E+04	(K)AGAVN\	856.489
2	MTGELSPOT18_5_11	2	11.49	2.9	76	4.14E+04	(K)IISNEE(	1080.532
3	MTGELSPOT18_5_11	2	11.48	2.77	90	6.97E+04	(K)IEPNLP\	1088.574
4	MTGELSPOT18_5_11	2	10.92	2.25	75.1	1.91E+04	(R)ISLQWL	915.541
5	MTGELSPOT18_5_11	2	10.56	5.75	81.9	7.53E+04	(R)VLEDN\	1103.558

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
34	4	3	45.2	9	3.17E+05	28518.2	HUMAN	P08246	Neutrophil elastase OS=Homo sapiens GN=ELANE PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	3	20.68	20.68	89.8	6.51E+05	(R)VVLGAI	1065.616
2	MTGELSPOT18_5_11	2	13.59	13.59	84.4	1.54E+05	(R)SNVCTI	948.493
3	MTGELSPOT18_5_11	2	10.93	3.09	71.4	3.33E+05	(R)QVFAV(	847.479
4	MTGELSPOT18_5_11	2	9.77	3.5	69.8	1.29E+05	(R)VVLGAI	1065.616

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
16	8	8	98.99	34	6.42E+04	22110.5	HUMAN	Q06830	Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	3	18.22	12.03	88.3	4.83E+04	(R)GLFIIDI	1359.8
2	MTGELSPOT18_5_11	2	15.29	15.29	84.6	5.87E+04	(R)QITVNI	1211.674
3	MTGELSPOT18_5_11	2	13.63	13.63	72.8	4.81E+04	(K)ADEGIS	894.432
4	MTGELSPOT18_5_11	2	13.04	6.78	82.6	1.33E+05	(R)SVDETI	819.421
5	MTGELSPOT18_5_11	3	12.45	12.45	72.7	1.05E+05	(R)IGKPAP	972.551
6	MTGELSPOT18_5_11	2	11.57	8.09	77.5	2.81E+04	(R)GLFIIDI	920.509
7	MTGELSPOT18_5_11	2	9.17	9.17	62.2	3.22E+04	(K)IGHPAP	980.531
8	MTGELSPOT18_5_11	2	9.16	9.16	65.2	5.35E+04	(K)ATAVmI	1164.572
9	MTGELSPOT18_5_11	2	8.91	3.47	72.6	1.11E+05	(R)TIAQDY	1107.604

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
4	5	4	67.43	49	3.84E+05	11236.6	P01842	Ig lambda chain C regions OS=Homo sapiens GN=IGLC1 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK032_8-11_1.0726.0741.2	2	20.62	15.65	97.1	8.10E+05	(K)AGVETTTPSK(Q)	990.51
2	SpotNoK032_8-11_1.1215.1249.3	3	18.55	13.65	80.9	4.77E+05	(K)AAPSVTLFPPSSE	1986.018
3	SpotNoK032_8-11_1.0830.0836.3	3	16.98	13.67	86.5	3.10E+05	(R)SYSCQVTHEGST\	1711.759
4	SpotNoK032_8-11_1.1186.1252.2	2	15.2	9.09	90.6	2.53E+05	(K)AAPSVTLFPPSSE	1986.018
5	SpotNoK032_8-11_1.0765.0770.2	2	11.28	11.28	75.1	6.77E+04	(K)TVAPTECS(-)	864.377

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
7	6	4	62.14	59	3.39E+05	11608.9	P01834	Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK032_8-11_1.0874.0900.2	2	19.81	19.81	89.2	2.23E+05	(K)VDNALQSGNSQE	2135.969
2	SpotNoK032_8-11_1.0872.0954.3	3	18.56	18.56	95.7	5.36E+05	(K)VDNALQSGNSQE	2135.969
3	SpotNoK032_8-11_1.1291.1345.2	2	17.73	17.73	84.7	3.64E+05	(-)TVAAPSVFIFPPSD	1946.027
4	SpotNoK032_8-11_1.1294.1344.3	3	15.91	12.58	94.3	3.52E+05	(-)TVAAPSVFIFPPSD	1946.027
5	SpotNoK032_8-11_1.0904.0908.4	4	15.35	15.35	77.7	2.09E+05	(K)HKVYACEVTHQGI	2141.081
6	SpotNoK032_8-11_1.0670.0744.2	2	9.25	9.25	69.2	3.53E+05	(K)SFNRGEC(-)	869.357

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
17	3	3	33.95	12	3.13E+04	27692.8	HUMAN	P30048	Thioredoxin-dependent peroxide reductase, mitochondrial OS=Homo sapiens GN=PRDX3 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	kveimgel68	2	14.2	14.2	82.7	4.58E+04	(R)GLFIIDI	1285.751
2	kveimgel68	2	11.69	11.69	79.5	1.95E+04	(R)DYGVLI	1462.79
3	kveimgel68	2	8.06	8.06	66.3	2.87E+04	(R)SVEETI	833.436

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
35	3	3	35.87	4	5.67E+04	74681.3	HUMAN	Q15582	Transforming growth factor-beta-induced protein ig-h3 OS=Homo sapiens GN=TGFBI PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11_	2	18.36	18.36	96.9	1.12E+05	(R)LTLLAP	1315.798
2	MTGELSPOT18_5_11_	2	8.94	8.94	71.3	3.55E+04	(R)EGVYT	1700.828
3	MTGELSPOT18_5_11_	2	8.57	8.57	60.9	2.26E+04	(K)ELANILI	800.488

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
17	7	5	41.36	2	2.49E+04	187149.1	HUMAN	P01024	Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIN	2	12.5	7.71	75.3	2.44E+04	(K)TIYTPG	1370.731
2	NEWKVEIN	2	11.2	11.2	83.1	3.58E+04	(K)TIYTPG	1370.731
3	NEWKVEIN	2	9.54	9.54	70.2	1.92E+04	(R)ISLPES	886.524
4	NEWKVEIN	3	8.03	8.03	65.7	4.19E+04	(K)VTIKPA	1212.683
5	NEWKVEIN	2	5.78	1.94	63.7	1.32E+04	(R)FLYGK(	627.35
6	NEWKVEIN	2	5.51	5.51	62.9	1.72E+04	(R)TFISPIK	805.482
7	NEWKVEIN	2	5.06	5.06	62.7	2.29E+04	(R)ISLPES	886.524

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
42	3	3	34.87	9	5.69E+04	38604.2	HUMAN	P07355	Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11_	2	12.86	9.39	80.9	4.14E+04	(K)GVDEV	1542.849
2	MTGELSPOT18_5_11_	2	12.42	12.42	66.4	4.82E+04	(K)SLYYY(	1421.695
3	MTGELSPOT18_5_11_	3	9.59	5.03	71.6	8.12E+04	(R)SVPHL(	808.468

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
22	6	5	75.49	19	2.86E+05	28837.4	HUMAN	P08311	Cathepsin G OS=Homo sapiens GN=CTSG PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11_	2	22.17	15.43	93.6	1.14E+05	(R)TIQNDI	1544.847
2	MTGELSPOT18_5_11_	2	16.69	8.47	87.1	3.25E+05	(R)VSSFLF	1104.62
3	MTGELSPOT18_5_11_	2	14.04	14.04	89.4	2.13E+05	(R)IFGSYD	954.468
4	MTGELSPOT18_5_11_	2	13.13	8.15	73.6	4.07E+04	(R)VSSFLF	1104.62
5	MTGELSPOT18_5_11_	2	11.82	3.77	90.1	4.45E+05	(K)SSGVPI	1175.605
6	MTGELSPOT18_5_11_	2	10.77	6.24	72.5	5.76E+05	(R)NVNPV	979.568

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
31	3	3	47.08	11	1.14E+05	27807.2	HUMAN	P24158	Myeloblastin OS=Homo sapiens GN=PRTN3 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11_	2	17.57	10.04	90.6	4.78E+04	(R)VALYVI	1134.631
2	MTGELSPOT18_5_11_	3	17.17	6.86	87.9	2.55E+05	(R)LVNVVL	1290.764
3	MTGELSPOT18_5_11_	2	12.34	5.46	82.6	3.88E+04	(R)LFPDFF	1042.536

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Accession #	Protein Name
2	4	4	56.94	9	2.85E+04	49961	Q9Y6N5	Sulfide:quinone oxidoreductase, mitochondrial OS=Homo sapiens GN=SQRDL PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK018_5-11_84.1060.1065.2	2	19.81	19.81	92	3.75E+04	(K)TAAAVAAQSGILD	1343.728
2	SpotNoK018_5-11_84.1262.1264.2	2	15.89	10.34	77.3	2.05E+04	(K)YADALQEIIQER(N	1448.738
3	SpotNoK018_5-11_84.0889.0893.2	2	12.35	4.93	85.4	3.55E+04	(K)IGSNYSVK(T)	867.457
4	SpotNoK018_5-11_84.1205.1210.2	2	8.89	4.81	72.7	2.06E+04	(R)VILAEFDYK(A)	1097.588

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
15	2	2	30.79	20	2.66E+04	11737.6	HUMAN	P10599	Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	kveimgel67	2	16.51	12.85	78	1.96E+04	(K)TAFQEA	1336.638
2	kveimgel67	2	14.28	14.28	84.9	3.37E+04	(K)VGEFS(	908.447

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
5	2	2	23.43	5	7.80E+04	30772.7	P21796	Voltage-dependent anion-selective channel protein 1 OS=Homo sapiens GN=VDAC1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK015_5-11_81.1232.1237.2	2	14.04	6.95	79.3	3.90E+04	(K)LTLSALLDGK(N)	1030.614
2	SpotNoK015_5-11_81.0926.0929.2	2	9.39	9.39	63.3	1.17E+05	(R)FGIAAK(Y)	606.361

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
54	2	2	24.17	3	8.14E+04	72333.3	HUMAN	P11021	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	3	14.21	6.72	89.6	9.75E+04	(R)IINEPTA	1815.996
2	MTGELSPOT18_5_11	2	9.96	4.75	73.5	6.53E+04	(K)ITITNDC	1074.554

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
55	2	2	21.35	6	5.30E+04	29484	HUMAN	P25789	Proteasome subunit alpha type-4 OS=Homo sapiens GN=PSMA4 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	13.79	6.68	78.9	6.24E+04	(K)SALALA	786.508
2	MTGELSPOT18_5_11	2	7.56	3.42	61.2	4.37E+04	(K)LLDEVF	1226.63

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
26	5	4	39.01	6	3.93E+04	57768.1	HUMAN	P04745	Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	12.93	12.93	78.5	4.99E+04	(R)LSGLLL	1099.672
2	MTGELSPOT18_5_11	2	10.54	6.74	78.6	4.53E+04	(K)IYVSDD	896.436
3	MTGELSPOT18_5_11	2	7.9	7.9	66.4	3.02E+04	(R)SGNED	953.396
4	MTGELSPOT18_5_11	2	7.64	2.15	68.1	4.44E+04	(R)YLAPK(	591.35
5	MTGELSPOT18_5_11	2	7.64	2.22	68	2.64E+04	(R)YLAPK(	591.35

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
36	2	2	25.04	5	1.02E+05	32575.2	HUMAN	P06748	Nucleophosmin OS=Homo sapiens GN=NPM1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	13.53	4.63	86	1.48E+05	(K)VTLATL	745.482
2	MTGELSPOT18_5_11	2	11.51	4.21	84.4	5.47E+04	(R)TVSLGA	803.462

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
3	2	2	25.11	13	4.29E+04	27095.4	C3K5E6	30S ribosomal protein S2 OS=Pseudomonas fluorescens (strain SBW25) GN=rpsB PE=3 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK066_5-11_68.1036.1041.2	2	14.66	9.38	79.6	3.78E+04	(R)DLEVQAEDGTFA	1422.675
2	SpotNoK066_5-11_68.1070.1078.2	2	10.45	2.92	74.4	4.79E+04	(R)NHVAGGTEQFVE	2025.951

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
39	6	3	38.31	5	2.77E+04	75873.7	HUMAN	P08133	Annexin A6 OS=Homo sapiens GN=ANXA6 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	15.42	6.44	85.5	3.65E+04	(K)ALIEILA	999.62
2	MTGELSPOT18_5_11	2	15.23	5.71	86.1	2.00E+04	(K)ALIEILA	999.62
3	MTGELSPOT18_5_11	3	13.99	8.7	75.7	3.88E+04	(K)GLGTDI	1768.908
4	MTGELSPOT18_5_11	2	13.89	8.02	79	2.11E+04	(K)ALIEILA	999.62
5	MTGELSPOT18_5_11	2	8.9	8.9	62.7	2.95E+04	(R)DAFVAI	1176.662
6	MTGELSPOT18_5_11	2	7.21	7.21	61.9	2.05E+04	(R)DAFVAI	1176.662

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
18	11	7	95.41	20	6.62E+04	35936.9	HUMAN	P08758	Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	16.72	12.5	83.1	1.41E+05	(K)VLTEIIA	1001.599
2	MTGELSPOT18_5_11	3	14.63	7.85	83.6	5.61E+04	(R)SEIDLF	1234.679
3	MTGELSPOT18_5_11	3	14.6	8.49	79.6	1.04E+05	(K)GAGTD	1155.575
4	MTGELSPOT18_5_11	2	14.36	14.36	71.1	4.02E+04	(R)GTVTDI	1340.612
5	MTGELSPOT18_5_11	3	12.8	7.18	75	6.65E+04	(K)GAGTD	1155.575
6	MTGELSPOT18_5_11	2	12.57	8.79	79	1.13E+05	(K)VLTEIIA	1001.599
7	MTGELSPOT18_5_11	2	11.82	6.84	77.1	4.23E+04	(R)SEIDLF	1106.584
8	MTGELSPOT18_5_11	2	11.79	11.79	69	3.10E+04	(K)GLGTDI	1704.901
9	MTGELSPOT18_5_11	2	11.74	11.74	68.8	3.20E+04	(K)GLGTDI	1704.901
10	MTGELSPOT18_5_11	2	11.49	11.49	79.9	7.38E+04	(R)LYDAYE	1014.514
11	MTGELSPOT18_5_11	2	8.7	3.02	72.5	2.90E+04	(R)SEIDLF	1106.584

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
20	2	2	14.09	9	3.09E+04	19301.5	HUMAN	P49913	Cathelicidin antimicrobial peptide OS=Homo sapiens GN=CAMP PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MEWCEKVEIM29.1633.1633.2	2	8.16	8.16	60.6	2.48E+04	(R)SSDANI	925.437
2	MEWCEKVEIM29.1694.1694.2	2	5.93	2.12	65.2	3.71E+04	(R)AIDGIN	886.474

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
50	2	2	14.26	0	4.25E+04	188306.3	HUMAN	P01031	Complement C5 OS=Homo sapiens GN=C5 PE=1 SV=4

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIM	2	8.69	8.69	67.9	4.75E+04	(R)VFQFLE	910.503
2	NEWKVEIM	2	5.57	1.66	63.8	3.75E+04	(R)VVPEG	727.435

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
42	2	2	16.66	20	8.43E+04	11748.8	HUMAN	P99999	Cytochrome c OS=Homo sapiens GN=CYCS PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIN	2	8.45	2.81	71.3	6.51E+04	(K)TGQAP(	1428.675
2	NEWKVEIN	2	8.21	2.05	72.9	1.03E+05	(R)ADLIAY	906.529

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
6	2	2	20.98	0	2.53E+04	331775.6	HUMAN	P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	oldsamples	2	12.77	12.77	67.3	2.76E+04	(R)ETQSQ	1220.575
2	oldsamples	2	8.21	8.21	68.1	2.30E+04	(K)IEVLEE	1129.61

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	4	4	61.92	14	3.67E+04	43496	A5VXN3	Elongation factor Tu OS=Pseudomonas putida (strain F1 / ATCC 700007) GN=tuf PE=3 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK066_5-11_68.1298.1306.2	2	21.06	16.16	83.9	2.51E+04	(R)DLLSTYDFPGDDT	2266.124
2	SpotNoK066_5-11_68.1267.1269.2	2	18.74	6.8	91.4	5.78E+04	(R)VQDPLEIVGLR(D)	1238.71
3	SpotNoK066_5-11_68.0995.1001.2	2	11.56	11.56	66.7	3.36E+04	(R)AGENCGVLLR(G)	1088.552
4	SpotNoK066_5-11_68.1222.1229.2	2	10.56	10.56	61.3	3.02E+04	(K)LVETLDSYIPEPVR	1630.869

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
22	5	4	66.31	5	1.12E+05	106667.6	HUMAN	Q9Y6C2	EMILIN-1 OS=Homo sapiens GN=EMILIN1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	19.78	19.78	84.7	5.82E+04	(R)VLLNDC	2425.203
2	MTGELSPOT18_5_11	3	19.43	19.43	81.2	1.07E+05	(R)YLLSAV	1229.7
3	MTGELSPOT18_5_11	2	15.98	7.85	93.1	2.32E+05	(K)VEAVLS	773.452
4	MTGELSPOT18_5_11	3	14.59	14.59	78.1	9.02E+04	(R)VLLNDC	2425.203
5	MTGELSPOT18_5_11	2	11.12	4.03	74.6	7.21E+04	(K)LVGGQ	927.537

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
18	4	3	32.69	3	3.12E+04	74139.8	HUMAN	P02545	Lamin-A/C OS=Homo sapiens GN=LMNA PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	kveimgel66	2	12.51	12.51	72.1	4.37E+04	(R)LADALC	1028.574
2	kveimgel66	2	11.11	11.11	79	3.74E+04	(K)LLEGEE	974.479
3	kveimgel66	2	9.07	9.07	63.8	2.49E+04	(K)LEAALC	901.499
4	kveimgel66	2	7.55	7.55	75.4	1.88E+04	(K)LLEGEE	974.479

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
33	5	3	27.33	5	5.60E+04	56257.4	HUMAN	P00390	Glutathione reductase, mitochondrial OS=Homo sapiens GN=GSR PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIM	2	12.65	12.65	63.6	3.59E+04	(K)ALLTPV	1152.71
2	NEWKVEIM	2	11.98	7.9	64.7	7.70E+04	(K)ALLTPV	1152.71
3	NEWKVEIM	2	8.73	8.73	58.6	3.22E+04	(K)GIYAVG	1138.556
4	NEWKVEIM	2	5.95	5.95	58.5	1.21E+05	(R)DAYVSI	710.347
5	NEWKVEIM	2	5.94	5.94	62	1.42E+04	(K)ALLTPV	1152.71

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
35	3	3	23.45	7	4.20E+04	37540.3	HUMAN	P37837	Transaldolase OS=Homo sapiens GN=TALDO1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIM	2	11.86	11.86	69.1	4.84E+04	(K)LLGELL	1213.679
2	NEWKVEIM	2	6.18	0.25	69.7	5.50E+04	(K)LVPVLS	826.54
3	NEWKVEIM	2	5.41	1.63	62.7	2.25E+04	(R)LIELYK(	778.471

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
31	3	2	30.15	9	3.73E+05	18354.3	HUMAN	P10153	Non-secretory ribonuclease OS=Homo sapiens GN=RNASE2 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	NEWKVEIM	4	16.02	16.02	78.1	4.86E+05	(R)RDPPQ	1787.955
2	NEWKVEIM	3	14.13	14.13	64.5	4.68E+05	(R)DPPQY	1631.854
3	NEWKVEIM	3	13.16	13.16	74.4	1.65E+05	(R)RDPPQ	1787.955

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
25	6	6	71.44	26	1.12E+05	31731	HUMAN	P27105	Erythrocyte band 7 integral membrane protein OS=Homo sapiens GN=STOM PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	16.86	10.68	81.4	1.46E+05	(R)YLQTLT	1351.747
2	MTGELSPOT18_5_11	2	14.48	7.51	83.4	2.80E+05	(R)TISFDIF	1601.879
3	MTGELSPOT18_5_11	2	11.52	3.28	73.2	5.33E+04	(K)NLSQIL	1045.564
4	MTGELSPOT18_5_11	2	11.07	3.07	74.9	1.05E+05	(R)NVLGT	631.377
5	MTGELSPOT18_5_11	2	10.99	10.99	65.3	6.08E+04	(R)VQNATI	1929.999
6	MTGELSPOT18_5_11	2	6.52	6.52	68.6	2.88E+04	(K)GPGLF	1811.94

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
38	3	3	34.09	13	5.80E+04	23742.7	HUMAN	P23284	Peptidyl-prolyl cis-trans isomerase B OS=Homo sapiens GN=PPIB PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	17.21	17.21	92.7	9.26E+04	(K)TVDNF\	1364.706
2	MTGELSPOT18_5_11	2	9.61	4	62.7	4.25E+04	(R)VIFGLF	880.529
3	MTGELSPOT18_5_11	2	7.27	7.27	70.3	3.90E+04	(R)IGDED\	860.411

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
5	2	2	26.05	7	5.15E+04	25405.7	Q9Y2Y8	Proteoglycan 3 OS=Homo sapiens GN=PRG3 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoK019_5-11_85.0919.0923.2	2	14.4	4.69	79.9	7.38E+04	(K)GGYWR(R)	638.304
2	SpotNoK019_5-11_85.0912.0912.2	2	11.65	11.65	67.7	2.92E+04	(K)TFAEAQNVCSR(C	1282.584

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
27	6	6	61.97	8	3.63E+04	78411.3	HUMAN	Q92542	Nicastrin OS=Homo sapiens GN=NCSTN PE=1 SV=2

#	Filename	z	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	12.33	12.33	77	4.62E+04	(K)ALADVA 1085.631
2	MTGELSPOT18_5_11	2	11.9	11.9	71.6	2.50E+04	(R)NQVED 1372.732
3	MTGELSPOT18_5_11	2	10.26	3.65	80.5	5.27E+04	(R)LLYGFL 966.602
4	MTGELSPOT18_5_11	2	10.06	6.66	82.3	3.27E+04	(K)SGAGV 1039.626
5	MTGELSPOT18_5_11	2	9.19	3.6	68.5	3.04E+04	(K)APDVT 969.536
6	MTGELSPOT18_5_11	2	8.23	3.6	68.8	3.08E+04	(R)VVVAA 715.446

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
30	5	4	55.15	3	3.38E+04	127179.2	HUMAN	P11215	Integrin alpha-M OS=Homo sapiens GN=ITGAM PE=1 SV=2

#	Filename	z	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	17.99	10.94	91.5	3.45E+04	(R)SLPISL 1439.898
2	MTGELSPOT18_5_11	2	15.7	15.7	80.5	4.01E+04	(R)YVIGVC 1096.579
3	MTGELSPOT18_5_11	2	11.98	6.37	75.7	3.59E+04	(R)LFTALF 1212.666
4	MTGELSPOT18_5_11	2	9.48	4.14	77.4	3.72E+04	(K)ILVVITD 1086.641
5	MTGELSPOT18_5_11	2	8.5	8.5	71.6	2.12E+04	(K)ILVVITD 1086.641

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
35	3	3	42.8	8	8.57E+04	44659.9	HUMAN	Q13510	Acid ceramidase OS=Homo sapiens GN=ASAH1 PE=1 SV=5

#	Filename	z	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	17.29	17.29	87	8.62E+04	(K)ESLDVY 1281.621
2	MTGELSPOT18_5_11	2	15.25	10.78	86.9	1.24E+05	(K)LTVYTT 1366.783
3	MTGELSPOT18_5_11	2	10.26	4.4	75.5	4.69E+04	(R)WYVVQ 1343.638

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
37	4	4	42.05	4	4.19E+04	92469.3	HUMAN	P14625	Endoplasmin OS=Homo sapiens GN=HSP90B1 PE=1 SV=1

#	Filename	z	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	12.56	4.67	82	4.11E+04	(K)SILFVP 1187.678
2	MTGELSPOT18_5_11	2	11.52	4.12	79.2	4.96E+04	(K)LIINSLY 963.587
3	MTGELSPOT18_5_11	2	10.67	10.67	66.7	4.29E+04	(K)GVVDSI 1485.754
4	MTGELSPOT18_5_11	2	7.3	2.43	64.2	3.38E+04	(K)YLNFVK 783.44

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
38	3	3	38.67	1	1.23E+05	125437.5	HUMAN	Q86VB7	Scavenger receptor cysteine-rich type 1 protein M130 OS=Homo sapiens GN=CD163 PE=1 SV=1

#	Filename	z	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	15.69	3.41	93.1	1.45E+05	(K)GADLSL 731.405
2	MTGELSPOT18_5_11	2	14.36	6.08	87	1.92E+05	(K)INPASLI 857.473
3	MTGELSPOT18_5_11	2	8.62	2.92	71	3.12E+04	(R)DVGVVI 891.435

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
43	4	3	32.63	2	4.24E+04	122205.8	HUMAN	P00450	Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	15.94	10.49	85.8	5.95E+04	(K)GAYPLS	1371.763
2	MTGELSPOT18_5_11	2	11.96	7.25	77.8	3.04E+04	(K)GAYPLS	1371.763
3	MTGELSPOT18_5_11	2	10.54	10.54	69.7	2.68E+04	(K)ALYLQY	1519.743
4	MTGELSPOT18_5_11	2	6.15	6.15	69.8	5.32E+04	(K)IIFK(N)	520.349

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
47	3	2	28	8	7.79E+04	36053.4	HUMAN	P04406	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	14.51	14.51	69.3	3.37E+04	(K)LVINGN	1613.901
2	MTGELSPOT18_5_11	2	13.49	6.91	89.4	1.43E+05	(R)GALQNI	1411.79
3	MTGELSPOT18_5_11	2	10.76	10.76	79.4	5.69E+04	(R)GALQNI	1411.79

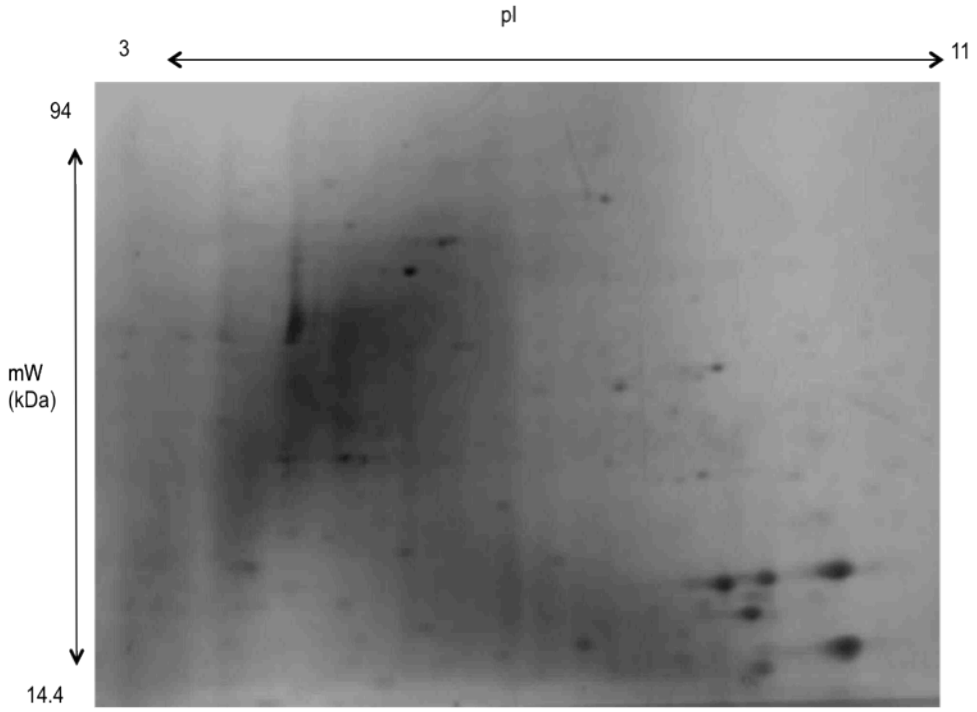
Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
53	2	2	24.47	1	1.12E+05	74732.3	HUMAN	P08236	Beta-glucuronidase OS=Homo sapiens GN=GUSB PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	2	12.39	6.04	69.3	1.60E+05	(K)SAAFLL	777.462
2	MTGELSPOT18_5_11	2	12.08	12.08	67.5	6.42E+04	(K)GSNLFI	665.362

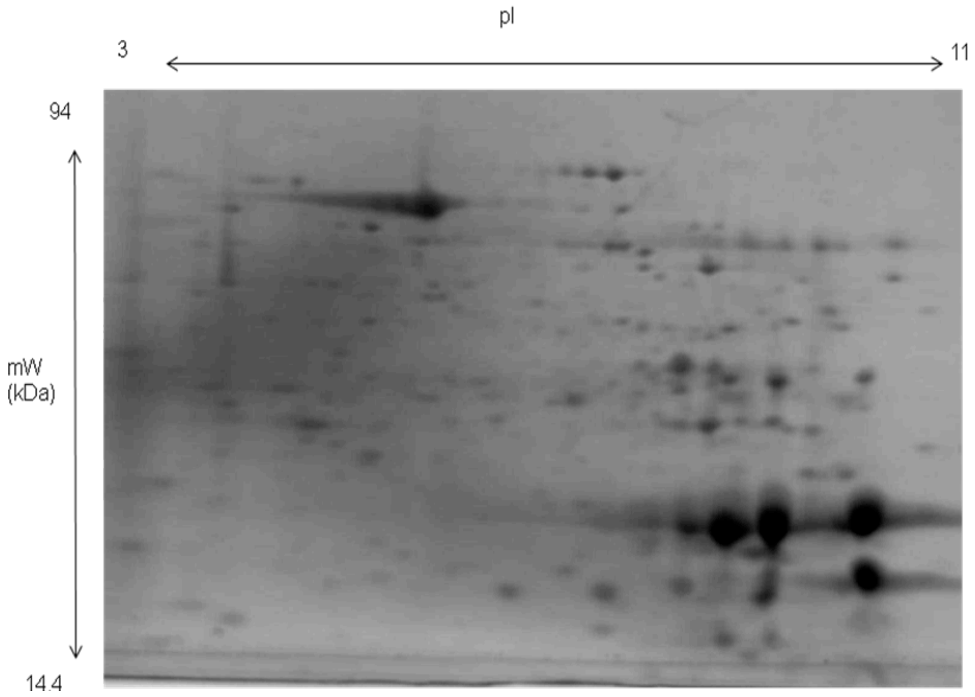
Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
31	3	2	32.03	13	8.18E+04	16214.5	HUMAN	P01036	Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	MTGELSPOT18_5_11	3	16.8	16.8	89.3	1.58E+05	(R)ALHFAI	1292.663
2	MTGELSPOT18_5_11	2	15.23	15.23	81.2	5.00E+04	(K)ATEDEY	1046.443
3	MTGELSPOT18_5_11	2	14.66	14.66	81	3.80E+04	(K)ATEDEY	1046.443

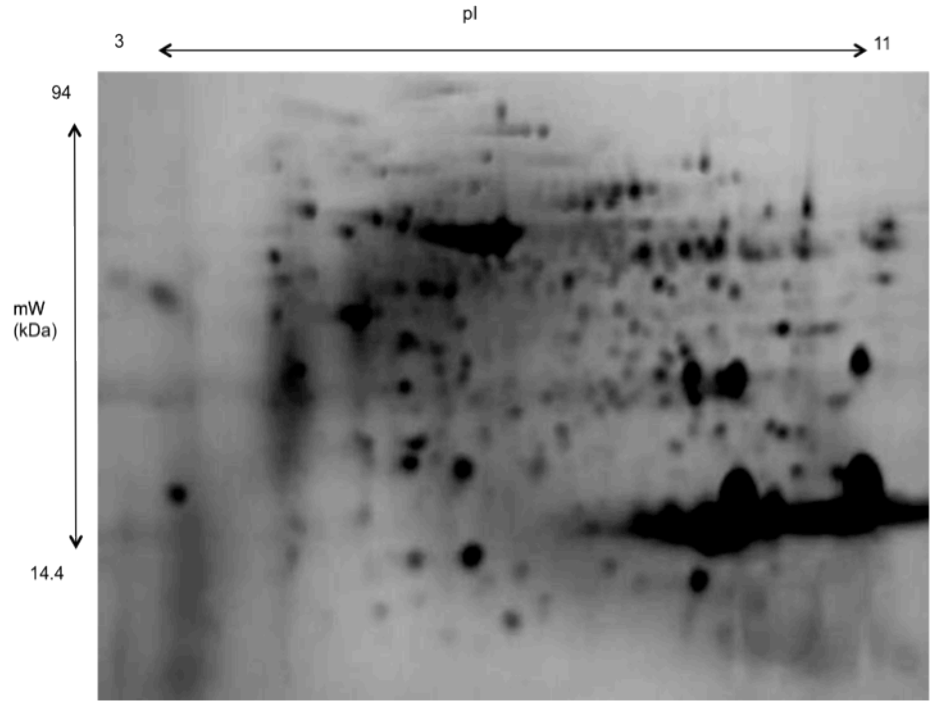
APPENDIX 10: GEL IMAGES SHOWING OPTIMISATION OF 2D-DIGE



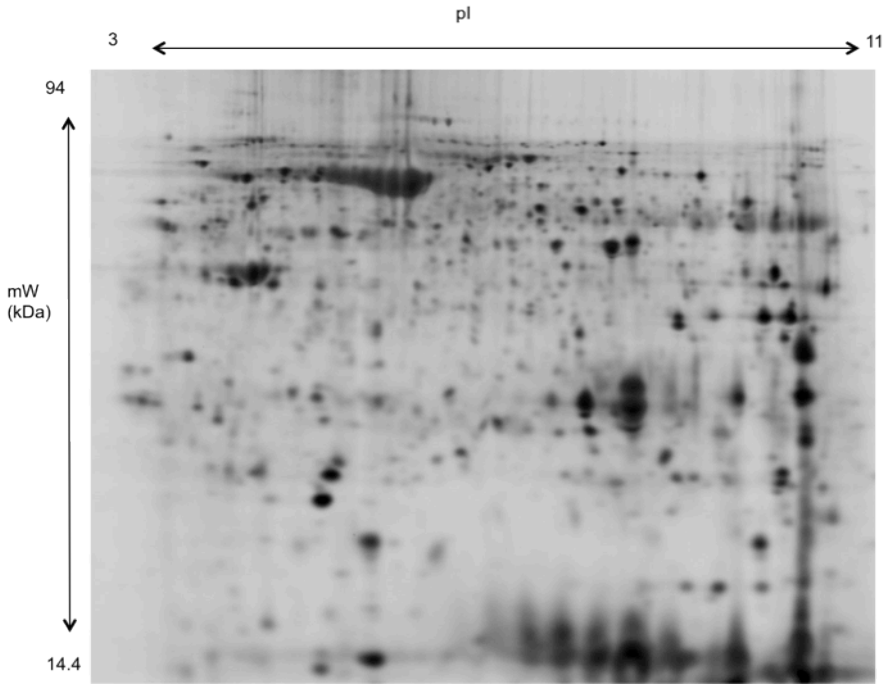
8.1 Sample grinding and protein extraction (coomassie stain)



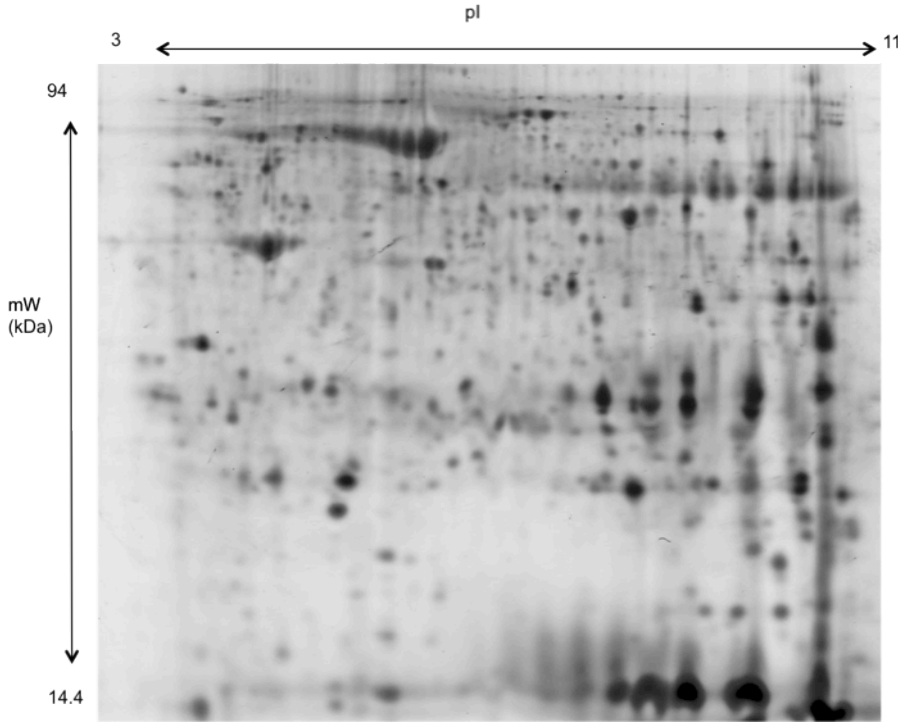
8.2 Detergent R3, sample grinding and protein extraction (coomassie stain)



8.3 Protein extraction and 2D clean up kit (coomassie stain)



8.4 Protein extraction, 2D clean up, destreak, DIGE lysis buffer (typhoon laser stain)



8.5 Protein extraction, DIGE lysis buffer (typhoon laser stain)

APPENDIX 11: SPECTRUM MILL IDENTIFICATION DATA FOR PROTEINS IDENTIFIED THROUGH 2D-DIGE

Full spectrum mill data will be available for a limited time for public access at: https://rapidshare.com/files/4199811293/sarcoiddata.xlsx

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	10	8	106.9	18	8.74E+04	41793.1	HUMAN	P63261	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoA665	2	20.96	20.96	85.1	7.70E+04	(R)GYSFTTT/	1132.527
2	SpotNoA665	2	19.42	19.42	88.2	2.16E+04	(K)AGFAGDD	976.448
3	SpotNoA665	2	16.79	8.73	96.2	2.86E+05	(K)IIAPPER(K	795.472
4	SpotNoA665	2	14.63	14.63	89	9.40E+04	(K)EITALAPS	1161.618
5	SpotNoA665	2	14.07	14.07	89.3	1.34E+05	(K)EITALAPS	1161.618
6	SpotNoA665	2	11.91	11.91	80.3	7.35E+04	(K)SYELPDG	1790.892
7	SpotNoA665	2	8.29	2.42	68.2	1.35E+04	(R)DLTDYLml	998.486
8	SpotNoA665	2	7.54	7.54	69	3.36E+04	(K)IIAPPERK(	923.567
9	SpotNoA665	2	7.36	3.02	77.9	1.13E+05	(R)LDLAGR(I	644.373
10	SpotNoA665	2	6.78	2.3	62.9	2.70E+04	(R)DLTDYLMI	998.486

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	3	3	50.2	23	7.16E+04	15998.5	P68871	Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	tNoB43	2	19.82	9.41	92.9	9.43E+04	(K)EFTPPVQAAYQK(V)	1378.7
2	tNoB43	2	18.13	18.13	84.9	7.32E+04	(K)VNVDEVGGEALGR(L)	1314.665
3	tNoB43	2	12.25	6.52	84.2	4.74E+04	(K)SAVTALWGK(V)	932.52

(#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	8	8	117.51	16	4.76E+04	59756.5	4040	Catalase OS=Homo sapiens GN=CAT PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 300	2	21.64	21.64	95	3.52E+04	(R)LSQEDPD	1292.612
2	Spo 300	2	17.98	17.98	80.7	2.56E+04	(K)LVNANGE	1337.652
3	Spo 300	2	17.03	17.03	82.7	2.48E+04	(R)FNTANDD	1493.698
4	Spo 300	2	15.03	9.81	77.1	3.20E+04	(R)DLFNAIAT	1049.563
5	Spo 300	3	14.87	14.87	78.7	2.62E+04	(R)FSTVAGE	1851.883
6	Spo 300	2	13.05	5.41	83.3	3.57E+04	(K)NLSVEDA	974.49
7	Spo 300	2	10.51	3.08	75.3	1.83E+05	(K)TPIAVR(F)	656.409
8	Spo 300	2	7.4	7.4	61.3	1.78E+04	(K)LNVITVGP	968.589

4	2	2	21.82	3	2.71E+04	52391.3	3228	Selenium-binding protein 1 OS=Homo sapiens GN=SELENBP1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 354	2	12.46	12.46	81.8	2.71E+04	(R)IYVVDVGS	1233.647
2	Spo 354	2	9.36	3.99	68.3	2.70E+04	(K)VIQVPPK(I	780.498

Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
6	6	62.92	15	2.77E+04	46588.6	1310	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Homo sapiens GN=ACADM PE=1 SV=1

Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
Spo 452	2	14.64	9.39	83.3	3.69E+04	(K)ENVLIGDC	1219.632
Spo 452	2	11.57	11.57	65.8	1.97E+04	(K)IYQIYEGT	1598.817
Spo 452	3	10.14	10.14	70.2	3.40E+04	(K)TRPVVAA	1465.86
Spo 452	2	9.43	9.43	76.3	2.48E+04	(K)TGEYPVPI	1144.636
Spo 452	2	9.14	2.53	70.7	2.11E+04	(R)NTYYASIA	1030.52
Spo 452	2	8	3.2	68.7	2.97E+04	(K)YALER(K)	651.346

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
2	3	3	35.49	10	1.91E+04	28870.3	0915	Carbonic anhydrase 1 OS=Homo sapiens GN=CA1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 452	2	16.2	3.21	86.5	2.75E+04	(K)VLDALQAI	970.593
2	Spo 452	2	10.97	4.65	65.6	1.68E+04	(K)YSSLAEA	1026.51
3	Spo 452	2	8.32	8.32	63.7	1.29E+04	(K)GGPFSDS	985.437

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	9	9	148.1	31	2.42E+05	25387.2	HUMAN	P02743	Serum amyloid P-component OS=Homo sapiens GN=APCS PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoA674	2	22.31	22.31	97.6	2.91E+05	(R)VGEYSLY(	1156.6
2	SpotNoA674	2	22.22	17.12	96	1.67E+05	(R)AYSLFSY(	1406.67
3	SpotNoA674	2	19.71	13.61	94.3	1.86E+05	(K)IVLGQEQ(	1393.696
4	SpotNoA674	2	19.56	19.56	93.5	1.38E+05	(R)QGYFVEA	1166.584
5	SpotNoA674	2	17.52	8.12	93.9	4.12E+05	(R)AYSDLSR(	811.394
6	SpotNoA674	3	15.24	5.05	84.4	9.71E+04	(K)IVLGQEQ(	1811.892
7	SpotNoA674	2	13.19	5.65	93.3	5.98E+05	(K)VFVFPR(E	764.445
8	SpotNoA674	2	11.47	11.47	81.9	2.54E+05	(R)DNELLVY(	993.525
9	SpotNoA674	2	6.88	6.88	64.2	3.19E+04	(R)DNELLVY(	1278.669

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	2	2	23.01	15	3.40E+04	18502.6	3528	Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 3530	2	13.23	4.3	81.2	3.67E+04	(R)YALYDAT\	1337.626
2	Spo 3530	2	9.78	9.78	71.2	3.14E+04	(K)LGGSAVIS	1340.778

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
2	7	7	76.39	10	2.52E+04	70289.7	3796	Plastin-2 OS=Homo sapiens GN=LCP1 PE=1 SV=5

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 4280	2	13.54	13.54	63	1.52E+04	(K)LENCNYA\	1409.673
2	Spo 4280	2	11.81	3.91	77.2	3.40E+04	(K)LSPEELL	1069.625
3	Spo 4280	2	11.57	11.57	72	2.62E+04	(K)ALENDPD(	1089.463
4	Spo 4280	2	11.41	11.41	77.1	1.70E+04	(K)IGNFSTDI(	994.52
5	Spo 4280	2	10.34	10.34	73.7	1.82E+04	(R)QFVTATD\	1135.611
6	Spo 4280	2	9.58	9.58	78.3	3.30E+04	(R)NEALIALL	1012.615
7	Spo 4280	2	8.14	2.46	67.1	3.29E+04	(K)AACLPLP(	1117.582

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	6	6	77.52	9	4.36E+04	61055	0809	60 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPD1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 4280	2	18.43	5.84	94	6.51E+04	(R)VTDALNA\	960.511
2	Spo 4280	2	15.61	7.95	79	3.21E+04	(K)VGLQVVA\	912.588
3	Spo 4280	2	14.19	6.58	85.8	5.88E+04	(K)LSDGVAVI	901.535
4	Spo 4280	2	11.01	5.58	71.7	1.23E+04	(R)TVIIEQSW	1344.716
5	Spo 4280	2	10.8	4.91	69.1	5.78E+04	(K)GIIDPTK(V	743.43
6	Spo 4280	2	7.48	3.73	74.1	3.57E+04	(K)APGFGDN	833.39

Group	Spectra	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1		5	69.94	10	9E+04	62294	6555	Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 4305	2	16.86	16.86	90.5	2.62E+04	(R)GSPLVVIS	1084.636
2	Spo 4305	2	14.87	14.87	88.6	3.66E+04	(K)SSAEVIAC	1031.548
3	Spo 4305	2	14.16	14.16	72.6	1.85E+04	(R)GLYDGPV	1620.794
4	Spo 4305	2	12.79	8.96	82.7	2.38E+04	(K)VFNLYPR(	908.499
5	Spo 4305	2	11.26	11.26	61.7	1.43E+04	(R)AITIANQTI	1820.958

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	14	14	214.26	26	1.25E+05	53651.9	8670	Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 406	2	17.43	17.43	89.3	1.14E+05	(R)QQYESVA	1023.511
2	Spo 406	2	17.22	17.22	91.5	1.78E+05	(R)EYQDLLN	1121.584
3	Spo 406	2	16.87	16.87	84	8.57E+04	(R)EEAENTL(	1323.618
4	Spo 406	2	16.72	16.72	88.5	1.73E+05	(R)QDVDNAS	1088.533
5	Spo 406	2	16.43	8.83	91.7	1.16E+05	(K)LLEGEESI	932.468
6	Spo 406	3	16.42	6.87	88.2	1.55E+05	(K)ILLAELEQI	1539.911
7	Spo 406	3	15.9	15.9	80.8	6.87E+04	(R)QVDQLTN	1287.665
8	Spo 406	2	15.51	6.85	86.2	6.18E+04	(K)VELQELNI	1115.569
9	Spo 406	2	15.34	7.42	78.9	8.40E+04	(K)NLQEAEE'	1309.606
10	Spo 406	2	15.22	8.42	86.3	1.37E+05	(K)ILLAELEQI	1169.714
11	Spo 406	2	14.07	14.07	77.5	2.81E+05	(K)FADLSEA/	1093.527
12	Spo 406	2	13.57	13.57	68.8	4.79E+04	(R)QVQSLTC	1490.752
13	Spo 406	3	12.57	12.57	84	7.73E+04	(R)TNEKVEL(	1587.797
14	Spo 406	2	10.99	3.32	76.2	1.71E+05	(R)FLEQQNK	906.468

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
2	4	4	38.88	9	3.22E+04	46402.5	14240	Eukaryotic initiation factor 4A-II OS=Homo sapiens GN=EIF4A2 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	tNoA41	2	11.54	11.54	74.2	1.93E+04	(K)GYDVIA	1394.691
2	tNoA41	2	9.53	4.45	76.9	2.25E+04	(R)AIIPCIK	814.486
3	tNoA41	2	9.24	9.24	68.5	2.23E+04	(R)ELAQQI	957.536
4	tNoA41	2	8.57	2.61	77.1	6.45E+04	(R)VLITTDI	1114.683

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	2	2	24.68	5	2.27E+04	35936.9	8758	Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 541	2	14.11	8.02	82.5	2.81E+04	(K)VLTEIIASR	1001.599
2	Spo 541	2	10.57	10.57	69.3	1.72E+04	(R)LYDAYELI	1014.514

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
2	4	4	38.52	18	2.40E+04	21892	HUMAN	P32119	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoA753	2	12.15	3.92	77.9	3.62E+04	(R)SVDEALR	789.41
2	SpotNoA753	2	9.97	9.97	68.5	2.71E+04	(R)QITVNDLF	1211.674
3	SpotNoA753	2	9.85	9.85	69.9	1.83E+04	(K)ATAVVDG	978.525
4	SpotNoA753	2	6.55	6.55	62.2	1.43E+04	(K)TDEGIAYF	924.442

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
2	5	5	61.31	13	4.13E+04	37540.3	7837	Transaldolase OS=Homo sapiens GN=TALDO1 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 535	2	15.44	15.44	85.7	3.42E+04	(K)SYEPLEDI	1233.6
2	Spo 535	2	13	7.17	79.6	3.25E+04	(K)LLGELLQI	1213.679
3	Spo 535	2	12.9	12.9	73.6	5.45E+04	(R)VSTEVDAI	876.442
4	Spo 535	2	10.2	10.2	68.2	3.78E+04	(K)AAQASDLI	932.468
5	Spo 535	2	9.77	3.38	74.5	4.76E+04	(K)LVPVLSAK	826.54

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
3	4	4	51.47	5	2.66E+04	104854.6	HUMAN	O43707	Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoA056	2	15.92	15.92	76.4	2.65E+04	(R)TINEVENC	1429.765
2	SpotNoA056	2	14.22	14.22	70.6	1.63E+04	(R)FAIQDISVI	1537.774
3	SpotNoA056	2	13.16	13.16	67.2	2.32E+04	(R)ETTDTDTA	1741.813
4	SpotNoA056	2	8.17	8.17	71.2	4.05E+04	(K)ALDFIASK	864.482

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	5	4	60.62	9	4.19E+04	49831.3	P68371	Tubulin beta-2C chain OS=Homo sapiens GN=TUBB2C PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	tNoA36	2	18.41	18.41	88.2	4.77E+04	(K)TAVCDI	1028.519
2	tNoA36	2	15.12	15.12	81.3	2.57E+04	(K)LAVNm\	1143.634
3	tNoA36	2	13.58	13.58	84.1	2.59E+04	(R)INVYYN	1328.648
4	tNoA36	2	13.51	6.05	76.7	6.69E+04	(R)FPGQLI	1130.595
5	tNoA36	2	12.28	7.23	68.7	4.07E+04	(R)ISVYYN	1301.637
6	tNoA36	2	8.53	8.53	70.1	4.31E+04	(K)TAVCDI	1028.519

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
4	3	3	40.84	10	3.32E+04	37765.7	P96C23	Aldose 1-epimerase OS=Homo sapiens GN=GALM PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	tNoA50	2	16.29	9.73	87.5	4.41E+04	(R)AVFGEI	1447.743
2	tNoA50	2	13.4	7.26	75.7	2.56E+04	(R)ISPDGE	1490.701
3	tNoA50	2	11.15	2.14	82.7	3.01E+04	(K)VLWTPI	771.451

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	13	12	174.38	40	1.28E+05	36814.7	P09467	Fructose-1,6-bisphosphatase 1 OS=Homo sapiens GN=FBP1 PE=1 SV=4

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	tNoA50	2	21.21	21.21	94.2	1.71E+05	(K)DFDPA\	1453.696
2	tNoA50	2	18.28	10.28	94.1	2.00E+05	(K)IYSLNE	1157.584
3	tNoA50	3	16.4	16.4	82.6	1.02E+05	(K)EAVLD\	1605.86
4	tNoA50	3	15.18	15.18	81.5	8.41E+04	(K)STDEPS	1629.772
5	tNoA50	3	14.73	14.73	79.2	4.54E+04	(K)AGIAHL	2072.077
6	tNoA50	3	14.4	14.4	85.7	8.88E+04	(K)KFPPDI	1419.701
7	tNoA50	3	14.33	10.33	78.3	7.45E+04	(R)YVGSm	1233.604
8	tNoA50	2	14.09	14.09	71.8	4.81E+04	(R)LLYECN	1760.806
9	tNoA50	2	12.9	12.9	81.3	5.85E+04	(R)TLVYG(	1555.852
10	tNoA50	2	11.35	11.35	68.1	5.01E+04	(R)FVmEE(	867.403
11	tNoA50	2	10.8	1.32	93.6	3.41E+05	(K)AISSAV	703.41
12	tNoA50	2	10.75	2.21	78.3	1.98E+04	(R)FVMEE(	867.403
13	tNoA50	2	10.71	5.1	76.2	3.75E+05	(K)DALQP(	756.4

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	3	3	41.64	9	2.71E+04	39834.7	HUMAN	Q9Y5P6	Mannose-1-phosphate guanyltransferase beta OS=Homo sapiens GN=GMPPB PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoA472	2	16.98	16.98	91.9	2.47E+04	(K)YGVVVCE	1325.615
2	SpotNoA472	2	12.68	7.28	80	3.25E+04	(K)SIGESVPE	1070.548
3	SpotNoA472	2	11.98	11.98	78.2	2.40E+04	(K)ALILVGGY	1119.652

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	3	3	35.24	14	4.11E+04	23264.4	HUMAN	P28065	Proteasome subunit beta type-9 OS=Homo sapiens GN=PSMB9 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	SpotNoA765	2	14.9	14.9	81.3	6.85E+04	(R)VSAGEAV	1001.537
2	SpotNoA765	2	11.83	6.1	85.2	2.92E+04	(R)VILGNELP	982.593
3	SpotNoA765	2	8.51	8.51	68.8	2.56E+04	(R)FTTDAIAL	1296.662

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	4	4	53.19	17	6.26E+04	22588.2	0188	Neutrophil gelatinase-associated lipocalin OS=Homo sapiens GN=LCN2 PE=1 SV=2

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 493	2	13.75	4.88	87.8	1.35E+05	(K)ITLYGR(T)	722.42
2	Spo 493	2	13.58	2.74	76	3.62E+04	(K)ELTSELK(I	819.446
3	Spo 493	3	13.19	13.19	79.4	4.45E+04	(K)VPLQQNF	1790.882
4	Spo 493	2	12.67	12.67	74.1	3.49E+04	(K)CDYWIR(T	912.403

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	4	4	57.14	12	3.79E+04	34793.7	HC38	Glyoxalase domain-containing protein 4 OS=Homo sapiens GN=GLOD4 PE=1 SV=1

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	Spo 593	2	17.61	13.04	91.1	7.48E+04	(K)VTLAVSDL	1073.62
2	Spo 593	2	15.51	6.09	90.3	2.91E+04	(R)SLPQSDP'	1083.604
3	Spo 593	2	12.05	12.05	71.3	2.22E+04	(R)ALLGYADI	1252.599
4	Spo 593	2	11.97	3.28	77.4	2.55E+04	(R)IAFSCPQK	950.476

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	3	3	35.66	13	3.12E+04	21056.9	P30086	Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3

#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	tNoB47	2	13.43	5.69	88.2	4.29E+04	(K)VLTPTQVK(N)	885.54
2	tNoB47	2	12.14	5.82	84	3.23E+04	(K)LYEQLSGK(-)	937.499
3	tNoB47	2	10.09	10.09	65.7	1.85E+04	(K)CDEPILSNR(S)	1103.515

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