Lymphocyte Responses in the Lung in Patients with Respiratory Disease

Thesis presented for the degree of Doctor of Philosophy at the university of London

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Declaration

This work undertaken in this thesis has been undertaken solely by the candidate, Dr Simon Barry.

This thesis has not been submitted or accepted in any previous application for a degree.

Sources of information have been acknowledged in the text.
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  and support I am sure that I could not have completed this work.

Dedication

This thesis is dedicated to my father, who died in a tragic accident soon after I started
working and from whom I learnt the values of enthusiasm and application and with whom
I would have loved to have shared the joys of finishing this project.
Abstract

The initial promise generated by earlier studies of bronchoalveolar lavage (BAL) in the differential diagnosis of lung disease has generally failed to be translated to routine clinical practice. The reasons for this delay stem largely from the fact that the cytospin techniques used to differentiate BAL leukocyte subpopulations are cumbersome, time-consuming and imprecise. Nevertheless, flow cytometry (FCM) offers an alternative technology that is rapid, precise and well suited to document complex changes in cellular phenotype in fresh and cultured specimens.

The patients included in this thesis were all investigated for suspected respiratory disease and FCM was undertaken in addition to routine diagnostic tests on the BAL specimens. Three key findings were observed. First, a simple single four-colour panel has been developed that enables the rapid enumeration of the major clinically relevant leukocyte components in BAL, including the CD4/CD8 lymphocyte ratio. This technology is shown to be superior to cytospin techniques in terms of precision and speed, and should be adopted for routine clinical investigation.

Second, it has been demonstrated that the lung is a distinct immunological site when compared to the blood. CD8 T lymphocytes have been investigated using the discriminatory markers, CD27 and CD45RA, and it has been shown that there is a preferential accumulation of mature memory CD8 cells in the lung.

Lastly, the differences between the lung and the blood have been further evaluated by analysing antigen-specific responses in patients with tuberculosis. It has been shown that powerful CD4 interferon-γ and tumour necrosis factor-α synthetic responses to short term incubation with purified protein derivative (PPD) in BAL, but not blood, can be used for the rapid diagnosis of acute tuberculosis. This test is a candidate for routine clinical application, particularly because patients with extra-pulmonary tuberculosis also respond.

Most importantly, this thesis has demonstrated that the focused investigation of BAL using a powerful tool such as FCM can deliver important immunological information with direct clinical relevance. It therefore highlights the vital link between medicine and laboratory services in order to define optimal diagnostic technologies on the basis of modern research.
Publications arising from this thesis

Papers


2) Barry SM, Johnson MA and Janossy G. Increased proportions of activated and proliferating memory CD8+ T lymphocytes in both lung and blood are associated with blood HIV viral load. *JAIDS* 2003 34(4): 351-7

3) Barry SM. The utility of bronchoalveolar lavage evaluation in patients with respiratory disease. *CPD Bulletin Immunology and Allergy*. 2003, 3: 8-10


Oral presentations

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Poster presentations

1) Barry SM, Johnson MA and Janossy G. Analysis of lung and blood CD8 T lymphocytes in patients with HIV infection. 13th World AIDS conference, Durban, South Africa 2001
Index of Tables

Table 1.1 Advantages and disadvantages of different methods for determining antigen-specific lymphocyte responses ......................................................20
Table 2.1 Fluorochromes available for use between the CytoronAbsolute and FACSCalibur flow cytometers .................................................................46
Table 2.2 Monoclonal antibodies used in this thesis .................................................................51
Table 2.3 Antigens and substances used to stimulate cytokine synthesis .........................61
Table 3.1 Coefficients of variation for BAL lymphocyte, macrophage and granulocyte percentages derived by flow cytometry and cytopsin ............................75
Table 4.1 Demographic details, diagnoses and CD4/CD8 ratios by different FCM methods in the study population ..........................................................88
Table 5.1 Characteristics of BAL from study population ..........................................................101
Table 5.2 Main BAL diagnoses in HIV- and HIV+ patients ................................................102
Table 5.3 Demographic and diagnostic features of patients with sarcoid .........................104
Table 5.4 Demographic, diagnostic and BAL FCM data of patients with TB ..................107
Table 5.5 BAL lymphocyte percentages from radiologically abnormal and normal lung in patients with pulmonary TB ......................................................110
Table 6.1 Demographic, Immunological, Viral and Diagnostic Data of the HIV+ study Population .........................................................................................127
Table 7.1 Demographic and diagnostic data for patients undergoing CD8 phenotypic analysis in blood and BAL .................................................................143
Table 8.1 Demographic and diagnostic results in patients with TB ........................................159
Table 8.2 Demographic and diagnostic results of patients with non-tuberculous respiratory disease .....................................................................................161
Table 8.3 BAL lymphocyte percentages and CD4 type-1 cytokine responses at diagnosis of TB and following completion of TB therapy ..............................167
Table 8.4 BAL IFN-γ responses to PPD from radiologically affected and unaffected lung in patients with TB ........................................................................168
Index of Figures

Figure 1.1 FCM dotplots of lysed whole blood and BAL...............................................17
Figure 1.2 Cartoon of immune response to *Mycobacterium tuberculosis* (TB)............25
Figure 2.1 Photomicrographs of stained BAL cytospin preparations............................43
Figure 2.2 FCM dotplot of lysed whole blood...............................................................45
Figure 2.3 FCM dotplot of CD45 panleukogating against side scatter (SSC) to
differentiate the major leukocyte populations in lysed whole blood.............47
Figure 2.4 FCM dotplot from BAL demonstrating the separation of CD45+
leukocytes from CD45- non-leukocyte debris.............................................48
Figure 2.5 Epithelial cell contamination of BAL in FCM dotplots and cytopspins.......49
Figure 2.6 FCM gating strategy for phenotyping of lymphocytes..........................50
Figure 2.7 Mean fluorescence intensity (MFI) of CD4 expression on BAL
lymphocytes with incubation time.................................................................55
Figure 2.8 Time course of BAL CD4 cytokine responses to PPD..................................56
Figure 2.9 CD69 expression on CD4 and CD8 lymphocytes in fresh BAL..................57
Figure 2.10 Dose response curve of CD4 cytokine synthesis to PPD stimulation......59
Figure 2.11 BAL CD4 expression before and after permeabilisation.......................60
Figure 3.1 CD45 panleukogating in BAL......................................................................67
Figure 3.2 BAL eosinophil discrimination by FCM........................................................68
Figure 3.3 BAL lymphocyte determination by CD45 expression and light scatter
compared with lymphosum gating...............................................................69
Figure 3.4 CD45 expression and light side scatter characteristics in BAL cells
expressing the dead cell marker, 7-AAD..........................................................72
Figure 3.5 Correlation plots comparing the enumeration of BAL lymphocytes,
granulocytes and macrophages by flow cytometry and cytopspin...............73
Figure 3.6 Bland-Altman plots comparing the enumeration of BAL lymphocytes,
granulocytes and macrophages by flow cytometry and cytopspin.............74
Figure 3.7 Correlation and Bland Altman plots comparing BAL lymphocyte
percentages by CD45 gating with lymphosum.............................................76
Figure 3.8 Immunofluoresence staining of BAL with an eosinophilia.......................77
Figure 4.1 Optimum gating strategy for determining BAL CD4/CD8 ratios.............85
Figure 4.2 Simplified gating strategy for determining BAL CD4/CD8 ratios.............86
Figure 4.3 Standard gating strategy to determine the BAL CD4/CD8 ratios.............86
Figure 4.4  Correlation between method 1 and method 2 for determining the CD4/CD8 ratio determination.................................................................89
Figure 4.5  Bland Altman comparisons between method 1 and 2 for the determination of CD4/CD8 ratios.................................................................89
Figure 4.6  Correlation between method 1 and method 3 for determining the BAL CD4/CD8 ratios............................................................................................90
Figure 4.7  Bland Altman comparisons between method 1 and 3 for the determination of CD4/CD8 ratios.................................................................91
Figure 5.1  Percentage of BAL lymphocytes by FCM in patients with sarcoïdosis according to the stage of their pulmonary disease.........................105
Figure 5.2  BAL CD4/CD8 ratios by FCM in patients with sarcoïdosis according to the stage of their pulmonary disease...........................................105
Figure 5.3  Percentage of BAL lymphocytes by FCM in patients with tuberculosis, sarcoïdosis, and in healthy controls ..................................................108
Figure 5.4  Percentage of BAL lymphocytes by FCM in patients with tuberculosis with washings taken from cavities and radiologically normal lung........108
Figure 5.5  BAL lymphocyte percentages in all patients with TB, in symptomatic TB patients without cavities and in healthy controls.........................110
Figure 5.6  BAL CD4/CD8 ratios in patients with TB, sarcoïdosis and in controls ....111
Figure 5.7  Blood CD4 count in HIV+ patients according to pathogens obtained in BAL.............................................................................................................112
Figure 5.8  BAL lymphocyte percentages in HIV+ patients without respiratory pathogens according their blood CD4 counts................................................113
Figure 5.9  CD4/CD8 ratios in blood and BAL in HIV+ patients without respiratory pathogens according their blood CD4 counts..............................114
Figure 5.10  Box and whisker plots comparing the percentage of CD4 lymphocytes in BAL and blood according to different blood CD4 categories in HIV+ Patients without respiratory pathogens.................................115
Figure 5.11  Box and whisker plots comparing the percentage of CD8 lymphocytes in BAL and blood according to different blood CD4 categories in HIV+ Patients without respiratory pathogens.................................115
Figure 5.12  Leukocyte discrimination and CD4/CD8 ratios by FCM in pleural fluid, Ascetic fluid and cerebrospinal fluid..................................................116
Figure 6.1  FCM gating strategy to determine the activation and proliferation status of CD8+ memory lymphocytes ................................................................. 129
Figure 6.2  Determination of CD38 gating strategy by FCM ....................................................... 130
Figure 6.3  Comparison between the percentages of CD38+ CD8+ T lymphocytes in BAL and blood in controls and HIV+ patients ................................. 133
Figure 6.4  Comparison between CD38+ CD8+ T lymphocytes from BAL of HIV+ Patients with and without respiratory pathogens ........................................ 134
Figure 6.5  Box and whisker plots comparing the percentage of Ki67+ CD8+ T Lymphocytes in CD38+ and CD38- populations in BAL and blood ............... 135
Figure 7.1  FCM dotplots demonstrating CD8 naïve and memory subsets in BAL
In HIV infection and sarcoidosis ................................................................... 145
Figure 7.2  Expression of CD8 CD45 isoforms in BAL ............................................................. 146
Figure 7.3  Box and whisker plots comparing memory CD8 lymphocytes in BAL
and blood .................................................................................................... 147
Figure 7.4  Pie charts of CD8 naïve and memory CD8 subpopulations in BAL and
Blood for the whole study population ...................................................... 149
Figure 7.5  Pie charts of CD8 naïve and memory CD8 subpopulations in BAL and
and blood from HIV+ patients and controls ........................................... 151
Figure 8.1  IFN-γ and TNF-α responses in BAL T lymphocytes following incubation
with PPD in a patient with TB .................................................................... 163
Figure 8.2  CD4 IFN-γ responses in BAL in patients with TB and non-TB
respiratory disease .................................................................................. 164
Figure 8.3  CD4 IFN-γ responses in BAL in patients with pulmonary and non-
pulmonary TB ....................................................................................... 165
Figure 8.4  CD4 IFN-γ responses in blood in patients with TB, BCG-vaccinated
healthy controls and non-BCG vaccinated patients without TB ........... 168
# Table of Contents

Acknowledgements .......................................................................................................1  
Dedication.........................................................................................................................1  
Publications arising from this thesis ........................................................................3  
   Papers.........................................................................................................................3  
   Oral presentations.......................................................................................................4  
   Poster presentations...................................................................................................4  
Index of Tables..............................................................................................................5  
Index of Figures.............................................................................................................6  
Table of Contents............................................................................................................9  

1. Chapter 1 .............................................................................................................13  
   1.1 Background .......................................................................................................14  
   1.2 Flow cytometry and CD45 panleukogating ......................................................17  
   1.3 Techniques for detecting antigen-specific T lymphocytes ................................19  
   1.4 Overview of recent developments in understanding lung immune responses ...23  
   1.5 Summary of rationale and aims ........................................................................28  
   1.6 References........................................................................................................29  

2. Chapter 2 .............................................................................................................40  
   2.1 Introduction .......................................................................................................41  
   2.2 Fibreoptic bronchoscopy and bronchoalveolar lavage ........................................41  
   2.3 Preparation of BAL ..........................................................................................41  
   2.4 Cytospins...........................................................................................................42  
   2.5 Immunofluorescence staining ..........................................................................42  
   2.6 Flow cytometry: general introduction and gating strategies .........................44  
      2.6.1 General characteristics of the flow cytometers used ..................................44  
      2.6.2 Mechanisms of analyte discrimination by FCM ........................................44  
      2.6.3 CD45 directed panleukogating in blood and BAL .........................................46  
      2.6.4 Gating strategy to identify bronchial epithelial and squamous cells in BAL by FCM 48  
      2.6.5 General gating strategy for lymphocyte phenotypic analysis: primary immunological  
          gating................................................................................................................49  
   2.7 Flow cytometry: Reagents, panels and protocols ............................................50  
      2.7.1 Reagents and panels for three and four colour FCM ..................................50  
      2.7.2 Protocols for staining of fresh whole blood and BAL .................................52  
      2.7.3 Intracellular staining by FCM: fixation and permeabilisation of cells ..........52  
   2.8 Measurement of antigen-specific responses: cytokine synthesis assay ..........53  
      2.8.1 General introduction to the method...............................................................53  
      2.8.2 Time course experiment for cytokine synthesis following incubation with PPD 54  
      2.8.3 Use of CD69 in BAL ..................................................................................57  
      2.8.5 Dose response curve for purified protein derivative ......................................58  
      2.8.6 Optimisation of antibody surface staining sequence ....................................60  
      2.8.7 Method for the detection of intracellular cytokine synthesis in whole blood and BAL  60  
      2.8.8 Antigens used for the cytokine synthesis assay .............................................61  
   2.9 Statistics............................................................................................................61
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1 Introduction</td>
<td>158</td>
</tr>
<tr>
<td>8.2 Methods</td>
<td>158</td>
</tr>
<tr>
<td>8.2.1 Patients</td>
<td>158</td>
</tr>
<tr>
<td>8.2.2 Bronchoalveolar lavage</td>
<td>161</td>
</tr>
<tr>
<td>8.2.3 Sample preparation</td>
<td>162</td>
</tr>
<tr>
<td>8.2.4 PPD stimulation and FCM analysis</td>
<td>162</td>
</tr>
<tr>
<td>8.2.5 Statistics</td>
<td>163</td>
</tr>
<tr>
<td>8.3.1 Comparison of IFN-γ and TNF-α responses to PPD in BAL between TB-infected and uninfected individuals</td>
<td>164</td>
</tr>
<tr>
<td>8.3.3 Type-1 cytokine responses in PPD-stimulated CD4 lymphocytes in BAL in patients with pulmonary and non-pulmonary TB</td>
<td>165</td>
</tr>
<tr>
<td>8.3.4 Type-1 cytokine synthetic responses to PPD in BAL CD4 and CD8 lymphocytes in patients with TB</td>
<td>166</td>
</tr>
<tr>
<td>8.3.5 Persistence of type-1 cytokine synthetic responses to PPD in BAL following initiation of treatment for TB</td>
<td>166</td>
</tr>
<tr>
<td>8.3.6 Type-1 cytokine synthetic responses to PPD in BAL from radiologically normal and abnormal areas of lung in patients with TB</td>
<td>167</td>
</tr>
<tr>
<td>8.3.7 Comparison of IFN-γ and TNF-α responses in the blood of TB patients with BCG-vaccinated controls</td>
<td>167</td>
</tr>
<tr>
<td>8.4 Discussion</td>
<td>169</td>
</tr>
<tr>
<td>8.5 References</td>
<td>172</td>
</tr>
</tbody>
</table>

............................................................................................................................ Chapter 9
............................................................................................................................ 176

9.1 Discussion                                                          | 177  |
9.2 References                                                          | 182  |

Glossary of Abreviations.............................................................................. 184

Appendix 1................................................................................................. 185

Appendix 2................................................................................................. 186
1. Chapter 1

Introduction, Rationale and Aims
1.1 Background

The burden of respiratory infections worldwide is enormous. The broad spectrum of these diseases encompasses upper and lower respiratory tract infections through to community and hospital acquired pneumonia and tuberculosis. Tuberculosis (TB) is estimated to infect one third of the world’s population and causes eight million new infections and nearly two million deaths each year [1]. Untreated, the mortality rate of clinical disease has been estimated at 40-60% [2]. One of the terrible tragedies of this disease is that effective chemotherapeutic regimes exist [3], although 95% of cases and deaths occur in resource-poor countries [1] that often cannot afford the drugs and do not have the health infrastructure to cope. Despite a commitment to reduce the death rate from tuberculosis by 50% by the year 2010, the leaders of the world’s most powerful countries have seemingly set themselves an impossible task. The rising TB pandemic in Sub-Saharan Africa is fuelled by a number of factors including HIV co-infection [4, 5], poor health infrastructures, famine, poverty and war. Nevertheless, sensible directly observed therapy (DOTS) treatment programmes adapted to local situations have proved highly effective [6-8] and have led some observers to be cautiously optimistic about TB control [9].

Even more disastrous than TB in terms of mortality rates is pneumonia which is the most frequent cause of death worldwide in children under five [10] and also carries a high mortality rate in both resource-rich and poor settings in adults [11]. Viral respiratory tract infections are generally less severe in the immunocompetent host, but they are a very significant factor in exacerbations in patients with underlying asthma [12, 13] and chronic obstructive pulmonary disease [14].

The burden of respiratory disease in patients who are immunocompromised either due to HIV infection, organ transplantation, or immunosuppressive therapy is even greater than in the immunocompetent patient. Worldwide HIV infection is by far the most significant cause of immunosuppression, with an estimated 40 million infected individuals in 2001 of whom 70% are from Sub-Saharan Africa [15]. Overall, the greatest burden of respiratory disease in HIV infected individuals is that of tuberculosis with recent estimates from some Sub-Saharan countries that 70% of patients with active tuberculosis are also co-infected with HIV [16]. Tuberculosis is the leading cause of death among people with HIV infection, accounting for a third of deaths world-wide [16]. In resource-poor settings, the burden of tuberculosis is a mixture of reactivation and re-infection, with the latter thought to be increasingly more important in TB endemic areas.
HIV co-infection dramatically increases the risk of reactivation in those who are infected with TB but do not have clinical disease. It has been estimated that the rate of reactivation of primary TB is only 5-10% for the lifetime of a non-HIV infected individual [18]. In those co-infected with HIV the annual risk of developing active disease ranges from 5% to 15% [19-21]. These features have been highly significant in fuelling the TB pandemic in resource-poor countries, particularly in Sub-Saharan Africa. The high prevalence of TB in HIV-infected patients is not confined to the developing world. Increasing migration and immigration of persons from such countries has contributed to rising rates of TB in the West [22-24]. A particular concern is that of multi-drug resistant TB in HIV infection that has extremely high mortality rates [25].

In addition to increased susceptibility to mycobacterial infections, HIV+ patients are also at increased risk of *Pneumocystis carinii* pneumonia (PCP) which historically occurred in 60-80% in the resource-rich world prior to the advent of anti-retroviral and anti-pneumocystis therapy [26]. However, infection with this opportunistic pathogen is rare in adults from resource-poor settings, a finding that is largely explained by death from other diseases before a sufficient drop in CD4 count is reached to increase the risk of PCP [27]. In addition, HIV-infected adults and children are at increased risk of developing bacterial pneumonia [28, 29] and bacteraemia complicating this [30, 31].

By contrast with the HIV-infected population, where *cytomegalovirus* (CMV) infection is a rare respiratory pathogen [32, 33], patients who have undergone bone marrow transplantation (BMT) are known to be particularly at risk of *cytomegalovirus* pneumonitis (CMV-P). Infection with this pathogen had historical mortality rates of 30-80% until the recent introduction of effective prophylactic therapy [34]. Fungal infections, particularly with *aspergillus* species are also frequent infectious hazards in the early post transplant period characterized by neutropenia [34]. In lung transplant patients, CMV-P is also a well-recognised infectious complication [35].

Therefore, one of the characteristic features of respiratory infections in these groups reveals that different patterns emerge in the types of respiratory pathogens between patients who are immunocompromised due to HIV infection from those that have had BMT or solid organ transplantation. Fungal infections and cytomegalovirus frequently cause respiratory infections in BMT patients, but only rarely in those with HIV, whilst PCP and tuberculosis are more common in HIV infection than following BMT [34].

Underlying these clinical presentations are various defects in the host immune response that, in combination with the direct pathogenic effects of the organism, lead to
different clinical outcomes. Unfortunately, relatively few studies have examined the processes of the immune response in the lung in humans, but instead, investigations frequently extrapolate from the findings seen with cells taken from peripheral blood samples with the assumption that these are equally applicable to the responses in tissues.

Interest in the lung as a distinct immunological site has been stimulated by the investigation of diseases such as sarcoidosis in which lung involvement is a dominant clinical presentation. Considerable effort has been invested over the last two decades in determining the leukocyte differentials and CD4/CD8 lymphocyte ratios in the lung in patients with lung disease. The impetus behind this drive was threefold. First, it was discovered that the lymphocyte proportions obtained in BAL were similar to those obtained from lung biopsy specimens in sarcoidosis patients, thus lending credence to the use of BAL as an investigative sample [36, 37]. Second, sarcoidosis was shown to be characterized by a BAL lymphocytosis and a raised CD4/CD8 ratio when compared to healthy controls. [38, 39]. Lastly, it was documented that the changes noted in BAL were largely absent in the blood [38]. Therefore, the investigation of cell populations in the lung was thought to be of diagnostic relevance for diseases such as sarcoidosis and provided a further impetus to study BAL lymphocyte differentials in other respiratory diseases such as TB [40-42], cryptogenic organising pneumonia [43] and pulmonary fibrosis [44-46].

One of the features that has handicapped the investigation of lung immunology has been a conservatism in adopting new investigative tools and the consequent reluctance for introducing new concepts into the evaluation of disease processes. For example, flow cytometry, which has been the gold standard for enumerating CD4 counts and CD4/CD8 ratios in blood for twenty years [47], has yet to be adopted as a standard technique for BAL lymphocyte analysis. Most of the studies investigating BAL CD4/CD8 lymphocyte ratios have involved the use of immunofluorescence or peroxidase-anti-peroxidase staining. These techniques are time consuming and suffer from inaccuracies due to the low number of cells routinely counted. Furthermore, such a cumbersome technology does not allow the convenient application of new ideas that aim to solve complex problems of immunoregulation at the relevant tissue sites, in this case the lung.
1.2 Flow cytometry and CD45 panleukogating

Flow cytometry (FCM) is an alternative investigative tool to cytocentrifuge preparations (cytospins) that has several advantages. First, it analyses data for thousands of events and therefore reduces errors due to manual counting of small cell numbers. Second, it is fast as FCM can be performed immediately after filtering, centrifugation and staining of BAL samples. Third, and most importantly, the extensive experience of analyzing leukocytes in blood with FCM has resulted in the development of convenient and precise techniques for determining leukocyte differential counts. The key strategy that has recently emerged has been morphospectral analysis using the leukocyte marker, CD45 [48-50]. Importantly, these methods are readily exploitable for the analysis of other tissue samples. In the past, it has been well documented that CD45 staining was optimal to differentiate lymphomas from anaplastic carcinomas in tissue sections by immunohistology [51, 52]. In BAL, the use of CD45 enables leukocytes to be differentiated from non-leukocyte components such as mucoid particles and epithelial cells. Here, the adoption of CD45 pan-leukogating is particularly important because in BAL the intrinsic cell parameters measured by FCM, size and granularity are not sufficient to distinguish between the different leukocyte components and contaminating debris (figure 1.1)

**Figure 1.1**

FCM dotplots of fresh lysed whole blood and BAL. The intrinsic parameters of the acquired events measured are forward scatter (FSC, size, y axis) and side scatter (SSC, granularity, x axis). In the lysed blood, distinct leukocyte populations of lymphocytes, monocytes and granulocytes are demonstrated. In BAL, no clear populations are determined by these characteristics.
The concept of using CD45 during BAL analysis is not new. Several investigators have adopted a gating approach that included CD45 for distinguishing lymphocytes from the rest of the leukocyte pool in BAL [53-55]. However, these earlier methods have included unnecessary complications that masked the advantages of using CD45 [54]. An even more serious problem has been that in these previous studies the discrimination between the relevant leukocyte components of the BAL fluid such as neutrophils and eosinophils was neglected by FCM. This omission has been a significant factor in ensuring that cytospins have generally remained the dominant method for BAL leukocyte differential analysis.

Alveolar macrophages have also posed particular problems for flow cytometric evaluation due to their autofluorescence [56] and heterogenous light scatter characteristics. Some investigators have attempted to overcome the autofluorescence by quenching with gentian violet [57]. However, it has remained unclear whether such techniques have rendered these treated cells more amenable to phenotypic analysis by FCM. A further problem has been the lack of a 'bona fide' surface marker that would identify macrophages in their various stages of differentiation. The only likely candidate for the role of a pan-macrophage marker is the transmembrane glycoprotein CD68 [58]. Unfortunately, this marker is only suitable for histological or intracellular staining as the molecule is not well expressed on the membrane of intact macrophages when studied in suspension. This fact has led to an extra complication for the use of FCM to characterize alveolar macrophages since an additional permeabilisation step is required for adequate CD68 staining. As a result of these problems most, but not all analyses of alveolar macrophages have still been performed by cytospin preparations.

In summary, despite the fact that FCM has been refined, simplified and accepted as the gold standard method for the determination of leukocyte differentials in blood, this technology has not yet been adequately applied to BAL. Therefore, the first aim of this thesis was to develop a flow cytometric system that could distinguish all the relevant leukocyte components in BAL. In particular, it was felt necessary that such a system should be simpler, faster and more precise than the existing cytospin methods and thus provide an impetus for adopting FCM as the routine diagnostic tool for BAL analysis.

This initial aim provided the platform for the further investigation of BAL by FCM and the logical development of the other aims of this thesis. The second aim was to investigate the differences between the lung and the blood T cell responses in terms of both the major subsets of these lymphocytes and their particular phenotypic
characteristics found in each site. This second aim sought to determine to what extent the lung was a distinct immunological compartment when compared to the blood. The third aim of the thesis was to apply this comparative technique for the investigation of antigen-specific responses in both the lung and blood compartments by making use of recent advances in immunological techniques to detect such T lymphocyte responses.

1.3 Techniques for detecting antigen-specific T lymphocytes.

Over the last six years there have been major advances in cellular immunology, the most important of which has been the development of several techniques for the accurate determination of antigen-specific lymphocytes [59-61]. This has been a revolutionary step as it has enabled the study of the functional performance of antigen-specific CD4 and CD8 T lymphocytes in vivo and also provided valuable insights into the nature of immune responses to pathogens. Experiments using class I MHC tetramers bound to Epstein-Barr virus (EBV) epitopes have demonstrated huge EBV-specific CD8 responses during acute infection that previous limiting dilution techniques had markedly underestimated [62]. In the field of HIV, the detection of HIV-specific responses by tetramers and cytokine production methods such as the ELISPOT have been instrumental in understanding how the immune system responds to the virus [61, 63]. Each of these techniques has both advantages and disadvantages that are relevant to their application as research tools (table 1.1).

Tetramers are major histocompatibility (MHC) class 1 molecules folded into a tetrameric complex bound together with streptavidin to which relevant peptides can be attached. This structure forms a stable unit that binds CD8+ T lymphocytes that recognize the MHC-restricted peptide. This tetrameric complex has the advantage that it binds specific CD8+ T lymphocytes with greater avidity than the natural monomeric complex [64, 65]. The addition of a fluorochrome allows the CD8-tetramer complex to be analysed by FCM [66].

To date the majority of tetrameric complexes have been made with class 1 MHC molecules, although most recently class 2 MHC tetramers have also appeared as research tools [67]. The advantages of using tetramers are that the peptide-specific CD8 lymphocytes and with class 2 tetramers, CD4 lymphocytes can be directly visualized by FCM and the phenotype of these cells analysed using further discriminating monoclonal antibodies. Nevertheless, some investigators have questioned the functional ability of the tetramer-binding cells [68-70].
Table 1.1 Advantages and disadvantages of different methods for determining antigen-specific lymphocyte responses

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Tetramer</td>
<td>1. Rapid detection (1 hour) of Ag-specific response by FCM</td>
<td>1. Only measures response to peptide present on tetramer which may not be immunodominant</td>
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<td></td>
<td>2. Phenotypic analysis possible</td>
<td>2. Predominantly only CD8 responses since very few class-2 tetramers exist</td>
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<td>3. Functionality of the cells not determined</td>
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<td>4. HLA restriction</td>
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<td>5. Tetramer binding is temperature dependent</td>
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<td>6. Requires a flow cytometer</td>
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<tr>
<td>Elispot</td>
<td>1. Functional responses measured.</td>
<td>1. Cannot distinguish which lymphocyte (CD4 or CD8) is responding</td>
</tr>
<tr>
<td></td>
<td>2. Low tech- responses can be assessed with a microscope</td>
<td>2. Only measures the secretion of a single cytokine thereby may underestimate the Ag-specific response</td>
</tr>
<tr>
<td></td>
<td>3. Can use a variety of stimulatory antigens so HLA restriction not an issue</td>
<td>3. Phenotypic analysis not possible</td>
</tr>
<tr>
<td>Flow cytometric</td>
<td>1. Functional responses measured</td>
<td>1. Requires a flow cytometer.</td>
</tr>
<tr>
<td></td>
<td>2. Can determine the responding lymphocyte subset</td>
<td>Phenotypic analysis and multiple cytokine detection is dependent on the type of machine (number of lasers) and the number of fluorochromes used</td>
</tr>
<tr>
<td></td>
<td>3. Phenotypic analysis possible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Can distinguish a variety of different cytokines synthesized</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. Can use a variety of stimulatory antigens so HLA restriction not an issue</td>
<td></td>
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</table>
The addition of a peptide stimulation step following tetramer staining has overcome this problem by enabling the analysis of cytokine responses in the tetramer binding cells [71].

The major disadvantage of using tetramers to study CD8 lymphocytes is the HLA-restriction of the response. Subjects must share the HLA haplotype of the tetramer and there is no guarantee that the response generated is an immunodominant one. Pathogens contain multiple epitopes that stimulate different responses between individuals. Thus, in order to approximate the natural response to many pathogens a battery of different HLA-tetrameric complexes would need to be constructed. Fortunately, some well-studied viruses such as cytomegalovirus (CMV) appear to generate dominant responses to conserved epitopes of the CMV matrix protein pp65 [72]. This restriction of responses has facilitated the use of tetramers to study the immune response to this pathogen in various clinical settings [73-75]. The responses to viruses such as HIV are more complex and tetramer studies using separate HIV epitopes will only measure part of the total immune response against the virus. These limitations, in addition to the fact that tetramers are expensive and difficult to construct make them likely to have a limited role beyond that of purely applied scientific research.

The two main additional well-standardized techniques for analysing antigen-specific responses both detect cytokine responses following incubation with antigen. Both techniques more closely mimic the natural immune response in the sense that antigen, added to the culture medium is presented to cognate T lymphocytes by antigen-presenting cells. The concept underlying these systems is that T cells, either CD4 or CD8, that recognize antigen in the context of relevant MHC molecules, rapidly start synthesizing cytokines. These cytokines can then be measured intracellularly by FCM [60], visualized as spot forming colonies following secretion into a gel matrix containing anti-cytokine antibodies [76], or detected by ELISA [77]. These methods both have the advantage over the standard tetramer-binding assay that they directly measure the functional responses of antigen-specific cells.

A potential problem with the cytokine production methods for quantifying the antigen-specific lymphocyte populations are that a variety of different cytokines may be produced by these cells on encounter with antigen. Conventionally, interferon-γ (IFN-γ) has been measured by ELISPOT [61, 78] although this system can be used to detect other cytokines such as interleukin-12 (IL-12) released from monocytes [79]. The flow cytometric technique has also predominantly measured type-1 cytokine responses,
including tumour necrosis factor-α (TNF-α) [80]. However, focusing on such responses ignores other cytokines that may be produced and therefore may underestimate the total number of antigen-specific cells. This is a particularly important shortfall of the ELISPOT method where only one cytokine response is measured. The FCM method has the advantage of being able to discriminate a variety of different cytokine responses in addition to providing a phenotypic analysis of responding cells. Whilst current flow cytometers widely available on the market are set up for three of four colour analysis, industry is rapidly responding with interest to this powerful tool. As a result machines are in use that can perform 11 colour analysis, enabling the measurement of a large number of different cytokine responses in addition to phenotyping the responding cells [81]. It is likely that in the future cheaper multi-parameter flow cytometers will become widely available.

The major advantage of the cytokine-production methods over the tetramer assay for the detection of antigen-specific lymphocytes is that both CD4 and CD8 lymphocyte responses can be measured. In these assays the size of the stimulating antigen determines which T lymphocytes are preferentially stimulated. Complex antigens are phagocytosed and then presented in the context of class-2 MHC molecules [82]. Studies using peptides, rather than complex soluble antigens have demonstrated that larger peptides of 15 amino acids or more stimulate CD4 lymphocyte responses whilst CD8 responses are optimally stimulated by short peptides of between 8-12 amino acids [83]. By constructing overlapping libraries of peptides of varying lengths to use as the stimulating antigens the sum total of the CD4 and CD8 responses can be estimated [83, 84]. This technique has the great advantage that it overcomes the problem of HLA-restriction of responses.

Nevertheless, despite the proliferation of recent studies examining antigen-specific responses, these have been, with few exceptions [85] confined to looking at whole blood, or peripheral blood mononuclear cells (PBMC). In particular, there have been few attempts to examine antigen-specific responses in the lung, despite the high burden of pulmonary pathology. The reason behind this undoubtedly relates to the difficulty in obtaining lung specimens for examination when compared to the ease of evaluating peripheral blood. The investigation of lung responses in humans requires a BAL specimen in order to obtain sufficient leukocytes for immunological analysis. Fortunately, BAL is often routinely performed in cases with suspected respiratory infections where a diagnosis is not rapidly obtained from sputum samples or where an unusual organism,
such as *mycobacterium tuberculosis* is suspected. The threshold for performing BAL is lower in immunocompromised patients because the range of potential pathogens is greater and the treatment options are more complex. Therefore, BAL performed on these patients should provide an adequate specimen for both routine laboratory testing in microbiology, virology and cytology in addition to an aliquot for cellular analysis.

1.4 Overview of recent developments in understanding lung immune responses

An integrative understanding of lung immune responses has been elusive, in part due to the paucity of knowledge of the role played by antigen-presenting dendritic cells (DC) in orchestrating the immune response. Animal studies have demonstrated that DC reside throughout the respiratory tract in epithelial tissue [86] and more recently the function of DC’s has been more clearly defined.

It is now well documented that DC’s determine the type of immunological response by secreting cytokines that influence the subsequent development of CD4 and CD8 effector phenotypes. Interleukin 12 (IL-12) is the key cytokine determining differentiation towards Th1 responses [87-89] and interleukin-10 (IL-10) drives Th2 responses. The signals that encourage these critical cytokines to be produced by DCs are unclear, however, there is evidence that lung DCs in the rat preferentially stimulate Th2 responses and require additional signals such as TNF-α to switch to IL-12 production [90]. Furthermore, there is a growing body of evidence demonstrating that IL-12 production can be suppressed by a variety of microenvironmental tissue factors such as prostaglandin E2 (PGE2) [91], nitrous oxide [92] and histamines [93], as well as by drugs such as β2 agonists [94]. Although these studies were performed on blood monocytes and macrophages rather than alveolar cells, the findings are suggestive that a number of different mechanisms exist *in vivo* to control Th1 responses.

It has been argued that type-1 immune responses in such a delicate tissue as the lung must be carefully controlled as the foreign antigen load is high and there is a potential for damaging the fragile alveolar compartment vital for gas exchange [95]. Indeed, sarcoidosis, a disease characterized by strong type-1 responses and granuloma formation in the lung is associated with lung fibrosis, a restrictive lung defect and eventual respiratory failure in severe cases.
Most of the studies of DC and macrophage function have been performed on cells isolated from lung epithelial tissue sections in animal models. However, most antigens that escape the mucociliary escalator in the large airways will be likely to first encounter alveolar macrophages that comprise approximately 90-95% of the alveolar cells [39]. These cells phagocytose the antigens, but their role as antigen presenters is uncertain. Several studies have suggested that these cells are poor antigen presenters and argue, like Holt, that this could be an adaptive response to minimise lung injury [96, 97]. However, other investigators have demonstrated that alveolar macrophages are good antigen presenting cells [98]. Recently, this issue has been resolved by the demonstration of low percentages of cells with phenotypic and functional characteristics of DCs that were distinct from the main macrophage population from BAL in humans [99, 100]. It is likely that following lung infection, DC recruitment into BAL from epithelial tissue is enhanced. Evidence from animal models suggests that this population of DCs that is initially rare can be increased dramatically by intratracheal BCG inoculation [101]. Intriguingly, infection with BCG or with live *mycobacterium tuberculosis* (MTB) also resulted in maturation and activation of the DCs [101-103].

Taken together, these findings suggest that antigen presentation occurs predominantly in the alveolar space. It is likely that signals, such as TNF-α and GM-CSF released by macrophages that have phagocytosed antigen encourage both the migration into and the maturation of DCs in the alveoli. The antigen-loaded DCs must then migrate back into lung epithelial tissues and thence to the regional lymph nodes. The key events taking place in the immune response to mycobacteria are summarised in figure 1.2

Within the draining lymph nodes the key aspects of lymphocyte recruitment, proliferation and maturation into effector cells is determined. The role of chemokines in the recruitment of lymphocytes both to the lymph nodes and then to the lung is becoming better understood [104, 105] and clearly plays a crucial role in the inflammatory response. Of considerable interest has been the recent discovery that the chemokine receptor CCR7, which is expressed on naïve and a subset of memory T cells is also upregulated during DC maturation [106]. The localization of naïve T cells and DCs in the lymph node is then mediated by the expression of two T cell zone expressed chemokines, secondary lymphoid tissue chemokine (SLC) and EBL1 ligand chemokine (ELC) which bind to CCR7 [107, 108]. Thus brought together in the T cell zones of the
Figure 1.2

Cartoon of immune response to *Mycobacterium tuberculosis* (TB). Macrophages release TNF-α on encounter with TB, resulting in migration into and maturation of dendritic cells (DC) in the alveolar space. Antigen-loaded DC orchestrate antigen-specific naïve and memory T cells maturation to effector cells in bronchial associated lymphoid tissue (BALT) under the influence of IL-12. Secretion of IFN-γ and TNF-α by T cells are crucial for the formation of granulomas to control TB.
lung lymph nodes, naïve CD4 and CD8 T lymphocytes that recognize antigen presented in the context of relevant class-1 and class-2 MHC on mature DCs will undergo proliferation. These proliferating, antigen-specific lymphocytes then develop their effector phenotype under the influence of the DC derived cytokines, IL-12 or IL-10. Consequent upon encounter with antigen, CD4 and CD8 lymphocytes undergo changes in their surface markers as well as in their expression of cytokines. The CD45 isoform changes from RA+ RO- to RA- RO+ [109, 110] and in CD8 lymphocytes there is a progressive loss of CD27 [111, 112]. Both CD4 and CD8 lymphocytes emigrating from the lymph nodes lose CCR7 expression [113].

One of the final important pieces in the immunological jigsaw puzzle has been the discovery of chemokine receptors on different lymphocyte subsets that mediate the recruitment of these cells to the sites of infection or inflammation. CD8 lymphocytes with predominant Th1 characteristics have been demonstrated to express CXCR3 receptors and to accumulate in the lungs of HIV infected subjects [114]. By contrast Th2 type lymphocytes may preferentially express receptors for different chemokines such as CCR3 and CCR4 [104].

These studies, taken as a whole have contributed greatly to our understanding of the immune response to pathogens in the lung. A picture has emerged of the role of antigen presenting cells, interactions in regional lymph nodes and the recruitment and differentiation of lymphocytes to the lung. However, there have been only a few attempts to characterize the antigen-specific lymphocyte populations in the lung.

The immune responses to tuberculosis in the lung are undoubtedly the best studied of all lung infections in humans. Investigators have examined the different leukocyte populations found in radiologically normal and abnormal lung [41, 115], thus giving insights into the pathogenesis of the disease. More interestingly, two studies have estimated the antigen-specific component of the lung responses by measuring lymphocyte proliferation to TB antigens. Both studies separated T lymphocytes from BAL and incubated them with either irradiated PBMC or isolated autologous monocytes in the presence of various MTB antigens. Increased proliferative responses to tuberculosis antigens in BAL, but not PBMC measured by [3H]-methyl thymidine incorporation were demonstrated when T cells from TB patients were incubated with TB antigens [116, 117].
These complex experimental designs presumably reflected fears that the macrophages from BAL would suppress the antigen-specific responses. Indeed, in one of the studies the authors demonstrated that addition of alveolar macrophages suppressed the BAL T cell proliferative responses to phytohaemaglutinin (PHA) from TB patients [117]. The later study also used an ELISPOT system to measure IFN-γ, IL-4 and IL-10 responses to PPD a in a subgroup of six patients. This is the first study to utilize one of the new antigen-specific techniques to examine lung immune responses. The authors demonstrated increased IFN-γ spot-forming colonies in the BAL from TB patients, but not from BAL from healthy subjects [117]. In three patients with TB, BAL was taken from radiologically unaffected areas of the lung and in these samples the number of spot-forming colonies were similar to those in the healthy controls. In this paper the dominance of anti-TB responses in the lung, but not in the blood has been clearly demonstrated by both the proliferative assays and the ELISPOT tests.

However, the ELISPOT system may not be the optimum technique for the delineation of BAL antigen-specific lung responses. The proportions of lymphocytes and the CD4/CD8 ratios may be highly variable in BAL from patients with active TB [40, 115]. Since complex antigens such as PPD will predominantly stimulate CD4 lymphocytes in the short incubation period of the ELISPOT assay, then the number of spots detected by this method will be depend on the proportion of CD4 lymphocytes in the BAL sample. For example, a low number of spot-forming colonies could be obtained from BAL from a tuberculous cavity in which the predominant leukocyte subset are neutrophils and only a small proportion CD4 lymphocytes. In fact, the proportion of antigen-specific CD4 lymphocytes from such a sample could be very high but this would be more accurately determined by a flow cytometric system.

A flow cytometric experimental system has been used to examine lymphocyte responses in the lung from mice infected with MTB [118]. Following intravenous inoculation with *M. tuberculosis*, lungs and spleens were removed at different time points following infection and mononuclear cells separated by density centrifugation. The tissue cells were then incubated with brefeldin A to prevent the secretion of cytokines and the cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Following incubation, the cells were permeabilised and intracellular cytokine staining in combination with surface staining for CD4 and CD8 was performed. The proportion of IFN-γ producing T lymphocytes from the lung and spleen were then measured by FCM.
In resistant C57BL/6 mice infected with virulent *M. tuberculosis*, there was an early and persistent production of IFN-γ by CD4 and CD8 lymphocytes in the lung that controlled the mycobacteria. By contrast, in susceptible mice, these IFN-γ producing responses in the lung were both delayed and attenuated, and there was failure to control the mycobacterial load. This study elegantly demonstrates the importance of IFN-γ producing lymphocytes in the control of TB in a murine model and supports the previous studies using IFN-γ knockout mice [119]. However, in this study lymphocyte activation was achieved in cells taken from the Tb-infected mice by using phorbol myristyl acetate (PMA) and ionomycin instead of specific antigens in order to boost cytokine synthesis. The use of such powerful immune activators may by-pass certain physiological steps that occur in antigen-specific systems and therefore these observations may generate a misleading picture of the true antigen-specific cytokine response.

The conclusion of this review is therefore that the technology exists for the detection of antigen specific responses in a tissue fluid such as BAL. A consideration of the merits and disadvantages of each method has led to the conclusion that the optimum technique is to detect intracellular cytokine synthesis following incubation with antigen by flow cytometry. This is because the ELISPOT assay suffers from the lack of information about the type of cells that respond by cytokine synthesis and the detection of only one cytokine in the secreted product. This is likely to make this method far less sensitive than FCM when using a tissue fluid such as BAL as the proportion of lymphocytes may be highly variable during episodes of respiratory disease. The limitations imposed by HLA-restriction and the current inability to measure CD4 lymphocyte responses that would require class-2 tetramers excludes the direct antigen binding assays from use in this investigation.

### 1.5 Summary of rationale and aims

Studies from the 1980’s, that focused on the lung as a target of immunological investigation have confirmed that the lymphocyte responses in BAL differ considerably from those in the blood. This holds true both for the simple lymphocyte percentages and CD4/CD8 ratios demonstrated in sarcoidosis and also for the recently published fledgling antigen-specific studies in patients with tuberculosis. These early comparative studies in patients with sarcoidosis have demonstrated close correlations between the lymphocyte percentages in BAL and those seen in biopsy specimens from the lung interstitium.
Consequently, samples of BAL can be regarded as a 'window' for lung immunity. From these findings a strong case can be made that lymphocyte responses in BAL are likely to be especially informative both in terms of understanding immune responses to pathogens and also as diagnostic tools.

These investigations into lung immune responses are particularly topical due to the recent development of exciting new techniques in the field of immunology. The most important of these has been the ability to precisely and rapidly detect antigen-specific responses and flow cytometry has emerged as optimum tool for this purpose.

The main impetus for this thesis was the exploration of the immune responses to pathogens in BAL by flow cytometry. Encompassing this broad aim were three objectives. First, to establish a simple and reliable panel for the detection of the most clinically relevant parameters in BAL by flow cytometry. This was an important primary objective since a narrow focus on lymphocytes alone would have ignored other leukocyte responses that are relevant both for immunopathological and diagnostic reasons.

The second objective was to examine more closely T lymphocyte differentiation by investigating the various phenotypic alternatives of these populations during bacterial and viral infections in the lung and the blood. Although it has been previously demonstrated that the lung serves as a repository for T cells of 'memory' type, it has not, so far, been investigated whether these cells can undergo local stimulation and show special alterations in phenotypic and activation markers. Such investigations are timely in the light of recent advances that have generated a more complete picture of lymphocyte differentiation patterns.

The third objective was to introduce the tests of antigen-driven stimulation of cytokine synthesis into clinical diagnosis using BAL samples. In this area the most important task was to assess whether such a system would be relevant for the diagnosis of tuberculosis.

Altogether, these objectives represent the first comprehensive array of technical innovations that aim to place clinical flow cytometry of the lung using BAL samples into the realms of practical thoracic medicine.

1.6 References


89. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998;8:275-83.

90. Stumbles PA, Thomas JA, Pimm CL, et al. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require


2. Chapter 2

Methods
2.1 Introduction
The methods described in this chapter cover the techniques used in the thesis as a whole. Bronchoscopy and cytospin preparation are commonly used and the details of these methods are well established and therefore given only a brief description here. By contrast, considerable attention has been devoted in this thesis to describing flow cytometry in general and CD45-directed gating in particular. The method for determining antigen-specific analysis in BAL has also been described in detail including modifications of this technique to enable it to be applied to tissue fluids such as BAL.

2.2 Fibreoptic bronchoscopy and bronchoalveolar lavage
Bronchoscopy was performed in a fully equipped endoscopy suite at the Royal Free Hospital. British Thoracic Society (BTS) guidelines [1] regarding the safe practice of bronchoscopy were adhered to. Bronchoscopies were generally performed through the oral, rather than nasal approach which was more comfortable for the patient. Bronchoalveolar lavage (BAL) was performed with a maximum of 200ml of warmed, sterile 0.9% normal saline introduced in aliquots of 20 or 30ml. The bronchoscope was wedged into a subsegmental bronchus directed to an area of radiological abnormality. In patients or control subjects with radiologically normal lung parenchyma, standard BAL was performed from the right middle lobe.

Bottles for acquiring BAL were siliconized glass containers that had been autoclaved to ensure sterility prior to the procedure. Immediately following acquisition of the sample, the bottles were placed on ice and sent to the laboratory.

2.3 Preparation of BAL
The BAL specimen was kept on ice and all samples were analysed within two hours of their acquisition. BAL was performed for the investigation of respiratory disease and the appropriate samples were therefore sent to the relevant diagnostic laboratories. BAL specimens were divided, placed into universal containers and sent to microbiology, cytology and virology laboratories in most cases. The remaining BAL was used for immunological analysis. BAL was centrifuged at 430g for 8 minutes and decanted. The pellet was resuspended in phosphate buffered saline (PBS) and filtered through a 100μm filter (CellTricks, Partec GmbH, Munster, Germany) and centrifuged again. The pellet was resuspended up to 1ml in either culture medium (RPMI 1640 with 10% FCS)
or PBS depending on whether simple leukocyte differentials were to be determined or whether further culture and functional assays were planned.

2.4 Cytospins

Cytospin preparations were made in order to obtain BAL leukocyte differentials which could then be compared with those obtained by a flow cytometric method. Following the washing and filtering of BAL described in 2.3 above, an absolute cell count of the number of leukocytes/ml was obtained using the CytorroneAbsolute flow cytometer (see below). A 50μl aliquot of the BAL suspension containing 3-5 X 10^6 cells/ml was then used to prepare a cytospin slide by standard methods. 50μl of BAL was introduced onto glass microscope slides placed within a cytospin machine (Shandon cytospin 11, Shandon scientific Ltd, Runcorn, UK) and spun for two minutes at 800 rpm. The slides were then air-dried for one hour and fixed for ten minutes in a 50:50 mixture of chloroform and acetone before air drying again.

The slides were then stained using a modified May-Grunwald Giemsa stain (DiffQuik), left to dry and then mounted in DPX (BDH Chemicals Ltd, Poole, Dorset). Formal cell differentiation into lymphocytes, alveolar macrophages, neutrophils, eosinophils and any other cell types was then performed by light microscopy (figure 2.1) by an expert cytologist. 500 leukocytes were counted per sample and the leukocyte differentials recorded.

2.5 Immunofluorescence staining

In several samples, immunofluorescence staining was performed on cytospin preparations in order to validate the use of antibodies to discriminate eosinophils and neutrophils by FCM. Cytospins were made as described above but were not fixed and dried. Immunofluorescence staining was performed in a moist staining chamber to ensure that the cytospins did not dry out. 50μl of the following antibodies: CD15 FITC (Dako, Ely, UK) and CD23 FITC (Caltag Medsystems, Towcester, UK) both at a dilution of 1:10 with PBS were added to the cytospin preparations. Staining was performed in the dark for 45 minutes and then the cytospins were washed twice with PBS before fixing in 4% paraformaldehyde for five minutes. After fixation, the slides were washed again with PBS and then examined using a fluorescence microscope with barrier filters appropriate for FITC conjugated antibody staining.
Figure 2.1

Digital photographs of stained cytospin preparations from BAL at 400x magnification. The morphological and staining characteristics of the major BAL cell populations—alveolar macrophages, lymphocyte and neutrophils (a) and eosinophils (b) are shown.
2.6 Flow cytometry: general introduction and gating strategies

2.6.1 General characteristics of the flow cytometers used

FCM was performed by both three colour (CytoronAbsolute, Ortho diagnostics, High Wycombe, UK) and four colour machines (FACSCalibur, Beckton Dickinson, San Diego, California, USA). The CytoronAbsolute acquired a known volume of sample and therefore an absolute count of the number of cells acquired was determined by volumetry. From this and the total volume of the wash-outs, the absolute number of cells in the original sample could be calculated. This flow cytometer was employed when absolute cell counts were required for antigen-specific analysis (see below). The FACSCalibur machine was utilized to define the percentages of the cells expressing a given phenotype within a population with the increased discrimination of 4-colour immunofluorescence. Instead of using microspheres on the FACSCalibur, the absolute counts on the Cytoron in parallel samples were obtained for major populations of CD4 and CD8 T lymphocytes when absolute numbers were required.

2.6.2 Mechanisms of analyte discrimination by FCM

Flow cytometry discriminates cells or other analytes by the virtue of both their size and granularity and also by detecting antibodies bound to their surfaces or intracellular components. Cells in suspension pass through a laser beam and this beam is scattered dependant on the physical qualities of the cells. Size is measured by the forward deflection of the laser beam when it hits the particles and is referred to as the forward scatter (FSC). Some of the laser beam is deflected at right angles and the amount of this reflected light corresponds to the intracellular features or granularity of the cell. This light, detected by a separate photomultiplier is referred to as side scatter (SSC). Taken together, size and granularity refer to the intrinsic qualities of a cell (or other analyte). These intrinsic features can be used to differentiate the major leukocyte populations in lysed whole blood (figure 2.2).

The second mechanism by which FCM can discriminate cells is through the detection of light released by fluorochrome labelled monoclonal antibodies. Different fluorochromes exist that absorb light from the laser beam and emit fluorescent light within a particular wavelength band that is detected by different photomultiplier detectors. Thus cells may be distinguished by their fluorescence characteristics.
dependant on which monoclonal antibodies are bound to their surface. This fluorochrome labelled antibody discrimination of a cell relates to its extrinsic features. The investigation of both the intrinsic and extrinsic characteristics of an analyte may be termed morphospectral analysis.

**Figure 2.2**

FCM dotplot of lysed whole blood demonstrating that the intrinsic cell parameters size (forward scatter, FSC) and granularity (side scatter, SSC) can be used to distinguish lymphocytes (a), monocytes (b) and granulocytes (c)

The number and type of different fluorochromes that can be used is dependant on their various absorption and emission spectra as well as the number of lasers present in the machine. A single laser machine such as the CytoronAbsolute can perform analysis with three fluorochromes, whereas with the FACSCalibur, the two lasers allow the routine use of four fluorochromes (table 2.1). The use of four fluorochromes is not only more informative than three colour analysis but is also more economical because fewer tubes are required to investigate complex patterns of differentiation antigen expression. The four colour analysis is also important in BAL where lymphocyte populations may be scanty.
Table 2.1 Fluorochromes available for use between the CytoronAbsolute and FACSCalibur flow cytometers

<table>
<thead>
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<th>Detector</th>
<th>Cytoron</th>
<th>FACSCalibur</th>
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</thead>
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<tr>
<td>2</td>
<td>PE²</td>
<td>PE</td>
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<td>3</td>
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<td>PerCP, PEcy5.5, PEcy7</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>APC⁴</td>
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</table>

Footnotes
1. FITC=Fluorescein isothiocyanate
2. PE=Phycoerythrin
3. PerCP=Perididine chlorophyll protein
4. APC=Allophycocyanin

2.6.3 CD45 directed panleukogating in blood and BAL
A number of different strategies have been developed for the discrimination of leukocyte subsets by FCM. Lymphocytes, monocytes, neutrophils and eosinophils all carry unique antigens by which they may be distinguished flow cytometrically using monoclonal antibodies. However, the use of large numbers of different fluorochrome labelled antibodies often introduces unnecessary complications and cost to the analysis. More recently, the features of CD45 staining, well established since 1979 [2] when the first CD45 reagent was made are coming back to prominence for two reasons: their simplicity and their reliability on stored samples. These simple protocols investigate both the intrinsic and extrinsic parameters of analytes. CD45-directed gating allows leukocytes to be distinguished from non-leukocyte parenchymal cells and debris, a factor that is particularly important for the analysis of tissue fluids such as BAL where many of the acquired events are not leukocytes.

One of the basic features of CD45 staining is that on leukocyte populations in blood, CD45 is expressed at high density on lymphocytes with medium density on monocytes and more weakly on granulocytes. This feature, combined with the differences in the
intrinsic parameters of these populations allows CD45 and side scatter to precisely differentiate these major populations in lysed whole blood (figure 2.3).

It is not known however, whether these features of discriminating intensity of staining are maintained in BAL samples where in this thesis the various aspects of CD45 labelling will be investigated (see below). It appears that when using BAL lymphocytes form an easily gateable population but the alveolar macrophages cannot be easily distinguished from the granulocytes since macrophages have very heterogenous side scatter characteristics (figure 2.4). Clearly the preliminary observations indicate that additional granulocyte markers such as CD15 may be needed to achieve macrophage-granulocyte discrimination.

**Figure 2.3**

FCM dotplot of CD45 panleukogating against side scatter (SSC) to differentiate the major leukocyte populations in lysed whole blood. Lymphocytes (a) have low side scatter and express CD45 brightly whilst granulocytes (c) have high side scatter but are CD45 dim. Monocytes (b) are intermediate for both characteristics.
2.6.4 Gating strategy to identify bronchial epithelial and squamous cells in BAL by FCM

Effective BAL adequately samples the alveolar cellular component of the washed lobe. However, BAL may be of variable quality and often specimens may include high proportions of bronchial epithelial and squamous cells, indicating sampling from the airways rather than the alveoli. These cells are readily identifiable by light microscopy, thereby enabling the cytologist to comment on the adequacy of the BAL sample. Therefore, it was felt that that a flow cytometric system should be devised that could also identify these cells from the non-leukocyte component.

An epithelial marker conjugated to FITC (Ber-EP4, Dako) was used in tandem with CD45. This epithelial antigen consists of two glycoproteins of 34 and 39 Kda and is expressed on a broad range of epithelial tissues, but not on mesothelial cells [3]. In poor quality BAL the epithelial+ cells could be gated and were demonstrated to be CD45- (figure 2.5).
Figure 2.5

FCM dotplots and images of cytospins of BAL indicating upper airways cellular contamination (a,c) and a good alveolar specimen (b,d). The gated populations in the dotplots represent the epithelial+, CD45- bronchial epithelial and squamous cell component. The staining aligned around the 45° is a feature of non-specific labeling, including dead cells and debris.

2.6.5 General gating strategy for lymphocyte phenotypic analysis: primary immunological gating

For all lymphocyte phenotypic analysis by FCM, primary immunological gating using the relevant discriminatory monoclonal antibody (CD4, CD8 or CD3) against side scatter was performed. These gated events were subsequently sent to a lymphoid scatter gate. Events that lay outside this gate were excluded from the analysis. Care was taken to include larger lymphoid blast cells (figure 2.6). The events with lower forward scatter
lying outside this gate were assumed to be apoptotic lymphocytes. This strategy of primary immunological gating followed by back gating to assess scatter characteristics has been demonstrated to be the optimum method for flow cytometric discrimination of lymphocyte and leukocyte subpopulations [2, 4]. Further strategies for lymphocyte gating are discussed in the relevant results chapters.

Figure 2.6
Flow cytometric gating strategy for lymphocyte subsets for phenotypic analysis. Primary immunological gating of CD4+ events with low side scatter (R1, plot a) are then sent to a forward scatter, side scatter plot (b) to ensure that they lie within a characteristic lymphoid gate (R2). Events with high FSC within R2 are blast cells.

2.7 Flow cytometry: Reagents, panels and protocols

2.7.1 Reagents and panels for three and four colour FCM
The monoclonal antibodies, their manufacturers and the fluorochromes to which they were conjugated to are shown in table 2.2. All antibodies were used in optimised pre-titrated saturating concentrations.
Table 2.2 Monoclonal antibodies used in this thesis

<table>
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The monoclonal antibodies were used in panels of three reagents for the Cytoron, or four reagents for the FACSCalibur. On the Cytoron, the following panels were used:

1. CD45 FITC / CD15 PE / 7-AAD
2. CD4 FITC / CD8 PE / CD3 PEcy5

For the FACSCalibur the following panels were used:

3. CD23 FITC / CD15 PE / CD45 APC
4. Epith FITC / CD45 APC
5. CD15 FITC / CD4 PE / CD8 PEcy7 / CD45 APC
6. CD4 FITC / CD8 PE / CD3 PEcy7 / CD45 APC
7. CD56 FITC / CD22 PE / CD3 PerCP / CD45 APC
8. CD27 FITC / Perforin PE / CD8 PerCP / CD45RA APC
9. CD27 FITC / CD8 PE / CD3 PerCP / CD45RA APC
10. KI67 FITC / CD38 PE / CD8 PerCP / CD45RA APC
11. CD4 FITC / IFN-γ PE / CD3 PerCP / TNF-α APC

2.7.2 Protocols for staining of fresh whole blood and BAL

Parallel blood and BAL samples were run using panels 1 and 2 above to determine the leukocyte subsets and CD4/CD8 ratios in these fluids. 50μl of whole blood or 50μl of prepared BAL (see 2.3) was added to the antibody panels in separate flow cytometry tubes. The samples were stained at room temperature in the dark for 15 minutes. Following this 950μl of lysis buffer (ammonium chloride 8.26%, potassium bicarbonate 1% and EDTA tetra sodium salt 0.036%, pH 7.5) was added to the blood and 950μl of PBS added to the BAL. The samples were left for a further 15 minutes at room temperature to allow lysis of the red cells and then run immediately on the Cytoron using an absolute counting protocol.

Absolute cell counts of the CD4 and CD8 lymphocyte subsets as well as the CD4/CD8 ratio, and the percentages of lymphocytes granulocytes and monocytes/macrophages were calculated for both blood and BAL (see chapter 3 for further discussion)

2.7.3 Intracellular staining by FCM: fixation and permeabilisation of cells

In addition to characterising the phenotype of cells by their surface antibody staining, intracellular components could also be identified following permeabilisation of the cells. This technique was necessary for demonstrating the presence of cytokines following
incubation with antigen or for the presence of cytotoxic markers such as perforin or nuclear markers of cell proliferation such as Ki67. Several different techniques exist for the permeabilisation of cells [5].

In this study fixation and permeabilisation was performed using fix and perm (Caltag). 200µl of reagent A (fix) was added to 500µl of whole blood or BAL in a universal container and left for 15 minutes in the dark at room temperature. The samples were then washed in PBS at 430g for 8 minutes. Following the wash step, the containers were decanted allowing red cells to separate away from the cell pellet. 200µl of reagent B (perm) was then added, the samples vortexed and then incubated in the dark for a further 15 minutes. Following a second wash step, the pellet was resuspended up to 200µl with PBS and then added to the relevant antibody panel requiring intracellular staining (panels 5 and 6). Staining was performed at 4°C for 30 minutes and followed by a final wash step. By this stage almost all red cells were removed during the decanting phases. Samples were run on the FACSCalibur machine.

2.8 Measurement of antigen-specific responses: cytokine synthesis assay

2.8.1 General introduction to the method

Several investigators have described methods for the detection of antigen specific lymphocyte responses by measuring the production of cytokines in response to antigen added in assays of whole blood or PBMC [6-9]. These assays used short (6-48) hour incubations and the cytokines synthesised were measured by ELISA [6], visually by detecting spot forming colonies in a gel matrix [8, 10], or by flow cytometry [7].

The underlying assumption in all of these systems is that cognate T lymphocytes present in the blood samples respond to antigen by producing cytokines. Therefore the measurement of the proportion of lymphocytes synthesising cytokines is a measure of the antigen-specific response. The caveat to this assumption is that a variety of different cytokines may be produced following the encounter of cognate lymphocytes with antigen and usually only one, or sometimes two cytokines are actually measured. This method may therefore underestimate the true population of antigen-specific responses.

Nevertheless, these techniques have distinct advantages over the use of class 1 HLA tetramers to detect antigen-specific responses since they do not require HLA matching.
of the subject with the tetramer. Moreover, the detection of cytokine synthesis gives an indication of the functionality of the responding lymphocytes.

FCM is a powerful technique that enables the rapid and accurate elucidation of both the subtypes of responding lymphocyte (CD4 or CD8) as well as being able to discriminate a number of different cytokines synthesised by the addition of further fluorochrome labelled anti-cytokine antibodies.

The flow cytometric method, first described by Suni, Picker and Maino [7] used a whole blood assay with a short incubation period of six hours in total before the samples were lysed, fixed and permeabilised and stained prior to acquisition on the flow cytometer. Integral to this method was the addition of the co-stimulatory antibody CD28 to augment the cytokine responses and the secretion blocking agent Brefeldin A to keep the synthesised cytokines within lymphocytes [11, 12], and therefore optimise their detection by FCM following cell permeabilisation.

The aim was to modify and simplify the existing described method so that it would be appropriate for use in bronchoalveolar lavage. One important consideration was that the timings of the assay should be appropriate for the analysis of samples of BAL that were collected following the routine clinical bronchoscopy lists.

The following issues were addressed when adapting this method for the detection of antigen specific responses in BAL:

1. What was the optimum incubation time with antigen in order to maximise cytokine synthesis?
2. Should a marker of cell activation such as CD69 be used in BAL?
3. What was the dose response curve for PPD?
4. What was the best sequence of staining of surface antigens in order to achieve optimum flow cytometric discrimination of the T lymphocyte subpopulations?

2.8.2 Time course experiment for cytokine synthesis following incubation with PPD

In this experiment a single patient with tuberculosis was investigated and the BAL CD4 IFN-γ and TNF-α synthetic responses were measured at different time points following incubation with PPD, ESAT-6 or no antigen. A standard initial 2 hour incubation was performed to allow antigen presentation and then 5μg of brefeldin A was added. Samples were then incubated for a further 4, 8, 16, 24 or 36 hours and the cytokines synthesized were measured by FCM for each of the different time points.
The time course experiment demonstrated several important features of this assay that were relevant to a precise determination of the antigen-specific response by FCM. The first feature was that the CD4 molecules were progressively downregulated with increasing incubation time so that at later time points it was difficult to distinguish CD4+ from CD4- events (figure 2.7). By contrast, CD3 molecules were well preserved on the lymphocyte surface (MFI at four hours 280.9 and at 36 hours 174.0). The second feature was that the scatter characteristics of the lymphocytes changed with time.

Figure 2.7

Decline in mean fluorescence intensity (MFI) of CD4 expression on BAL lymphocytes demonstrated graphically and by FCM dotplots with incubation time following brefeldin administration. At 36 hours CD4+ lymphocytes were difficult to distinguish from CD4- events.
At up to 8 hours incubation well preserved lymphoid scatter was noted, but by 24 hours many of these cells had increased side scatter. A viability dye was not used in these experiments, but many of these lymphocytes may have been undergoing apoptosis.

The percentage of CD4+ T lymphocytes synthesizing either IFN-γ or TNF-α was determined in CD3+ CD4+ lymphocytes (figure 2.8). The IFN-γ and TNF-α responses were maximal at 8 hours, but did not start to decline until after 24 hours incubation. It is difficult to interpret why there was a drop in the 16 hour response.

**Figure 2.8**

IFN-γ and TNF-α responses to PPD in BAL at different incubation times following the addition of brefeldin to the culture medium. The control samples had no antigen added and show low responses.

The conclusion of this time course experiment is that the optimum incubation time for maximizing the CD4 cytokine responses, lies between 8 and 24 hours following the addition of brefeldin. However, CD4 down-regulation at longer incubation periods is a significant problem for the accurate gating of CD4 lymphocyte responses. In addition, it is not clear to what extent apoptosis may become a problem with longer incubation. The final important factor was that the assay should be appropriate for routine analysis. Therefore, BAL specimens were incubated with brefeldin for two hours and then for an additional 14 hours overnight to allow the practical evaluation of the responses the following day.
2.8.4 Use of CD69 in BAL

The proportions of T lymphocytes responding to antigens has traditionally been measured by determining the proportion of lymphocytes (CD4 or CD8) that have both synthesised the cytokine of interest in addition to expressing the activation marker CD69 [7, 13, 14]. Despite the convention of using CD69 as an activation marker, it is not clear that this provides any additional useful information than the cytokine synthetic response alone for determining the antigen-specific response. In fact, some cognate cells may take up to 3 days to maximally express this marker after encounter with antigen [15], so exclusively counting the cells both expressing CD69 and cytokine may underestimate the true antigen-specific response. A more serious problem with the use of CD69 in

**Figure 2.9**

Histograms of expression of the activation marker, CD69 on CD4 lymphocytes (a,c) and CD8 lymphocytes (b,d) from fresh, unactivated BAL from a healthy control subject and a patient with pulmonary sarcoidosis. Very high percentages of CD69 expression are demonstrated on both CD4 and CD8 lymphocytes.
tissue specimens such as BAL is that a high proportion of unactivated T lymphocytes express this marker. Almost three-quarters of CD4 and CD8 BAL lymphocytes in a healthy individual and even more in a patient with sarcoidosis expressed CD69 when the BAL preparations were examined fresh without the addition of antigen (figure 2.9). These findings render CD69 unsuitable for use as an activation marker in BAL.

2.8.5 Dose response curve for purified protein derivative

Different doses of PPD were added to BAL as the stimulatory antigen in order to establish the optimum dose. The parameters measured by the standardised flow cytometric cytokine synthesis assay were both the percentage of lymphocytes producing cytokines and the mean fluorescence intensity (MFI) of the synthesised cytokines. Using the standardised gating strategy described above, the percentage of CD4+ T lymphocytes producing either IFN-γ or TNF-α following incubation with PPD was measured in comparison with the control samples to which no antigen was added. The following doses of PPD were used 1μg, 2 μg, 5μg, 10μg and 20 μg. The standard 16 hour incubation was used for all samples and the percentages of CD4+ T lymphocytes staining for intracellular IFN-γ and TNF-α were measured for each dose of antigen by FCM. The dose titration was performed on two patients with TB and the response reached a plateau at a dose of 10μg of PPD in one patient and 5μg in the other (figure 2.10). For each dose of stimulatory antigen, the percentage CD4+ T lymphocytes synthesising TNF-α was greater than those synthesising IFN-γ (figure 2.10).

The MFI for the relevant cytokine was determined by flow cytometry using winMDI software (M Trotter, free software). Single parameter histograms of cytokine synthesis by CD4+ T lymphocytes were used to allow accurate gating of the positive events and the mean values of these events calculated using the software. The MFI increased with increasing doses of PPD (figure 2.9). What was most noticeable was the much greater MFI for TNF-α expression than for IFN-γ, particularly in one subject. However the increased fluorescence noted with the APC-conjugated TNF-α antibody cannot be directly correlated with increased production of that cytokine when compared to IFN-γ since APC fluorescence is greater than PE.

Based on these observations a dose of 10μg of PPD was chosen although 5μg could have been adequate.
Figure 2.10

The percentage of CD4 lymphocytes synthesizing IFN-γ and TNF-α in response to different doses of PPD (dose in μg) in BAL from two patients with TB (top two graphs). The bottom two graphs demonstrate the mean fluorescence intensity (MFI) of the synthesized cytokines.
2.8.6 Optimisation of antibody surface staining sequence

Following incubation, the surface antigens used to delineate the lymphocyte subsets may become downregulated, thus affecting the ability of monoclonal antibodies to reliably distinguish such populations. This process of downregulation may be exaggerated by permeabilisation of the cells. Therefore, the MFI of CD4 and CD8 expression was compared when antibodies against these antigens were added before fixation and permeabilisation (pre-staining), or when added at the same time as the cytokine antibodies.

There was a clear advantage for CD4 discrimination with pre-staining as compared to staining with the cytokines (figure 2.11). No difference was noted for CD8 staining between these two sequences.

![Figure 2.11](image)

**Figure 2.11**

FCM dotplots of BAL following 16 hour incubation demonstrating that optimum CD4 discrimination is achieved when surface staining is performed prior to fixation and permeabilisation (a) rather than after this process (b).

2.8.7 Method for the detection of intracellular cytokine synthesis in whole blood and BAL

Aliquots of the BAL suspension containing $1 \times 10^5$ CD4+ lymphocytes in 1ml of culture medium were placed into sterile 5ml polypropylene tubes (Thermo Life Sciences, UK). In addition, 1ml of peripheral blood from the same patient collected into lithium heparin
tubes was also placed into polypropylene tubes. To one of the BAL and blood samples, 10μg of PPD (Statens Serum Institute, Copenhagen, Denmark) was added. The other tubes were unstimulated control samples. The samples were incubated for two hours at 37°C and 5% CO2, after which time 5μg of Brefeldin A (Epicentre Technologies, Cambridge, UK) was added and the samples incubated for a further 14 hours.

Following incubation, the samples were vortexed vigorously to detach cells from the walls of the tube. First, lymphocyte surface markers were stained using CD4-FITC (Royal Free Hospital) and CD3-PerCP (Becton Dickinson) for 15 minutes in the dark at room temperature and the samples were washed. Fixation and permeabilisation of the cells was performed as described in 2.8.3 above using Fix-and-Perm (Caltag). Following this, IFN-γ-PE (Caltag) and TNF-α-APC (Becton Dickinson) were added and the samples stained at 4°C for 30 minutes, followed by a final wash step. The acquisition and analysis of the stained preparations is described in chapter 7.

2.8.8 Antigens used for the cytokine synthesis assay
The cytokine synthetic responses to a variety of antigens other than PPD were assessed in both BAL and blood (table 2.3).

Table 2.3 Antigens and substances used to stimulate cytokine synthesis

<table>
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<tr>
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<th>Indication</th>
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<td>Sigma Aldrich</td>
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<td></td>
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<tr>
<td>Purified protein derivative</td>
<td>TB-specific responses</td>
<td>Statens serum institute</td>
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<tr>
<td>(PPD)</td>
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<td></td>
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<tr>
<td>Tetanus toxoid</td>
<td>Control antigen</td>
<td>Pasteur Merieux</td>
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2.9 Statistics
Most of the data generated such as leukocyte differentials in BAL, and cytokine synthetic responses were not normally distributed. Therefore median values and inter-quartile ranges or full ranges were recorded in the text. Comparisons between data sets were made using Mann-Whitney analysis.
Results generated using different techniques, for example the BAL leukocyte differentials by cytospin and FCM were compared using both the Spearmans correlation coefficient, and by Bland-Altman analysis.

2.10 References


Chapter 3

Comparison of Flow Cytometry with Cytospin for the Determination of Bronchoalveolar Lavage Leukocyte Populations in Patients Investigated with Respiratory Disease
3.1 Introduction

Bronchoalveolar lavage is a recognized procedure for the diagnosis of respiratory disease of both infective and inflammatory etiologies. Not only does the directed washing of an affected area of lung provide specimens that may enable a microbiological or cytological diagnosis to be made, but effective BAL also samples the cellular component of the lower bronchial tree. Increases in BAL cell lymphocyte populations have been found to be helpful in the diagnosis of inflammatory conditions such as sarcoidosis [1, 2], interstitial lung diseases [3] and in cryptogenic organizing pneumonias [4], while increasing neutrophil concentrations in BAL may be evidence of a bacterial infection [5, 6], in types of pulmonary fibrosis [7], or as a response to lung transplantation [8, 9].

The existing standard method for the evaluation of BAL leukocyte populations is predominantly through the investigation of cytospin preparations. Simple differential counts of lymphocytes, macrophages and granulocytes can be achieved by counting stained cells by microscopy. Further identification of subpopulations of lymphocytes or macrophages can also be performed by the use of immunoperoxidase [10] or immunofluorescence staining.

Similarly, flow cytometric (FCM) techniques have been developed to distinguish the BAL leukocyte component from the non-cellular and non-leukocyte events. Several investigators have used the pan-leukocyte marker, CD45 to determine the lymphocyte events acquired by FCM [11, 12]. Lymphocytes form homogenous populations of small cells that express high levels of CD45 and are easily gateable by FCM. Whilst lymphocytes can be accurately determined by CD45 expression and light scatter characteristics, this is not true of neutrophils, eosinophils and macrophages. Macrophages in particular are notoriously difficult to distinguish by FCM, although fortunately the enumeration of the total macrophage pool in BAL is rarely of clinical significance. By contrast, BAL neutrophilia and eosinophilia are diagnostically important. A granulocyte marker, CD15 was therefore used to distinguish these cells from the macrophages by FCM. The macrophage population was then derived as those CD45+ events remaining after subtraction of the gated lymphocytes and granulocytes.

The main aim of this study was to develop a simple flow cytometric method for determining the proportions of the clinically relevant leukocyte populations in BAL. The results obtained by FCM were then compared with those from cytospin preparations under optimum conditions using a highly experienced cytologist counting 500 cells, A
significant improvement on the standard visual counts in which 200 cells are routinely counted. A further aim of this study was to assess whether both techniques could reliably determine BAL leukocyte differentials on samples frozen in liquid nitrogen.

3.2 Material and methods

3.2.1 Subjects
Patients undergoing bronchoscopy for the diagnosis of suspected non-malignant respiratory disease were included. 100 BAL were performed on 92 patients. 53 BAL were performed on HIV-infected patients. The remaining 47 episodes included four BAL undertaken on patients following bone marrow transplantation, three on patients immunocompromised with haematological malignancies and two on subjects in intensive care.

3.2.2 Bronchoalveolar lavage
Bronchoscopies were performed under sedation using a flexible bronchoscope wedged into a subsegmental bronchus. BAL was site-directed in cases with radiologically defined areas of abnormality, but otherwise the right middle lobe was washed. Sterile normal saline was introduced through the bronchoscope to a maximum volume of 200ml and the fluid aspirated into a siliconized glass container on ice. BAL specimens were analysed within two hours of their acquisition. Aliquots of BAL were sent to the relevant laboratories and the remaining fluid (normally >25ml) was centrifuged at 430g for 8 minutes, decanted and the pellet resuspended. The sample was then filtered through a 100μm filter (Cell Trics, Partec GmBH, Münster, Germany), centrifuged again and the pellet resuspended in 1 ml of Phosphate buffered saline (PBS).

3.2.3 Flow cytometry
50μl of the BAL suspension was added to a flow cytometry tube containing the following monoclonal antibodies; CD45-FITC (Beckton Dickinson, Oxford, UK), CD15-PE (Caltag Medsystems, Towcester, UK) and 7-AAD (7 amino-actinomycin D, Pharmingen, San Diego, California, USA). These antibodies were pretitrated and added in saturating concentrations. The samples were stained at room temperature in the dark for 15 minutes and PBS added to a volume of 1ml. In samples that were visibly bloodstained, lysis buffer (0.17M NH₄Cl) was added instead of PBS and left for 15 minutes to ensure
red cell lysis. Samples were run on a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, New Jersey, USA) using an absolute counting protocol.

List mode data were analysed using ImmunoCount-2 software. Primary immunological gating of CD45+ events against side scatter was performed and a tight gate placed around the low side scatter lymphocytes (Figure 3.1, plot a). Second, a dot plot of CD45 against CD15 was produced and a gate placed around the CD45+, CD15+ granulocytes (Figure 3.1, plot b). The percentages of the lymphocyte and granulocyte populations were calculated as the number of gated events divided by the total number of CD45+ events.

Figure 3.1

FCM dotplots of BAL demonstrating CD45 pan-leukogating (R1, plot a) to differentiate leukocytes from debris. Lymphocytes (R2, plot a) express CD45 brightly and have low side scatter. Granulocytes can be distinguished from macrophages within the panleukogate by their expression of CD15 (R3, plot b).

The macrophage pool was derived from the total number of CD45+ cells after subtraction of lymphocytes and granulocytes.

In selected patients in whom eosinophils were identified by cytospin a further aliquot of BAL was stained with the following antibodies: CD23-FITC (Caltag), CD15-PE (Caltag) and CD45-APC (Pharmingen). The samples were run on a FACScalibur flow cytometer (Becton Dickinson) after a wash step following antibody staining. CD15+ granulocytes within the CD45 pan-leukogate were gated and sent to a CD45 CD23 dotplot (Figure
3.2). Eosinophils were characterized by the dual expression of CD15 and the IgE receptor antigen, CD23, whereas neutrophils were CD23 negative.

Figure 3.2

FCM dotplots and a photomicrograph of BAL from a patient with an eosinophilia. CD45+ CD15+ granulocytes (R2) are demonstrated to be predominantly CD23+ eosinophils (R3) with few CD23- neutrophils (R4, plot b). The photograph (c) confirms eosinophilia in the cytospin preparation.

The accuracy of the BAL lymphocyte gating strategy of CD45 expression and scatter characteristics was compared with lymphocyte enumeration by counting the sum of the various lymphocyte subsets in BAL by FCM (lymphosum).
In 15 samples, BAL was stained with T cell (CD3), B cell (CD19) and NK cell (CD56) markers in addition to CD45. A cocktail of the following antibodies were used: CD56-FITC (Beckton Dickinson), CD19-PE (Beckton Dickinson), CD3-perCP (Beckton Dickinson) and CD45-APC (Beckton Dickinson). The samples were stained as described above and washed before running on a FACScalibur. First, Lymphocyte percentages were derived by CD45 expression and side scatter characteristics as described above. These values were then compared with the sum of the percentages of T, B and NK cells. Individual lymphocyte subsets were calculated by gating the number of CD3, CD56 or CD19 bright events with lymphoid side scatter characteristics (figure 3.3).

**Figure 3.3**

FCM dotplots of BAL lymphocyte determination by CD45 expression and light scatter (a) compared with gating strategies to determine the numbers of NK (b), T (c) and B (d) lymphocytes. The numbers of each of the lymphocyte subsets were expressed as a percentage of the total CD45 panleukogate (R1). These percentages were added together to give a sum value of the lymphocyte percentage and this was compared with the lymphocyte percentage derived from plot a. NK cells (b) were CD56+ but CD3−.
NK cells were only counted as such if they were CD56+ but CD3-. The numbers of each lymphocyte subset were then expressed as a percentage of the total number of CD45+ events and these were added together to give the total lymphocyte percentage.

3.2.4 Cytospin
BAL was adjusted to a concentration of 2-5 x 10^5 CD45+ cells/ml. 100μl of BAL was centrifuged for 2 minutes at 800rpm in a cytospin machine (Shandon, Runcorn, UK) and the resultant slide air dried, fixed in chloroform and acetone for 10 minutes and stained with Haematoxylin and eosin. 500 cells were counted by light microscopy and the BAL cells differentiated by morphological and staining characteristics. The cytologist was blinded to the BAL differentials achieved by FCM.

3.2.5 Freezing and thawing of BAL
15 BAL’s were frozen in liquid nitrogen after the samples were run on the flow cytometer as described above. 500μl of the BAL suspension was added to 500μl of freeze mixture (10% DMSO in 20% fetal calf serum in RPMI 1640) in a cryovial. The sample was vortexed rapidly and then placed in the vapour phase of liquid nitrogen to ensure freezing at the rate of 1°C/minute. After 12 hours the samples were stored in liquid nitrogen. For reanalysis after freezing, the samples were rapidly defrosted by pipetting with warm RPMI and 10% FCS into a universal container. The thawed sample was then centrifuged, decanted and the pellet resuspended in RPMI and FCS before a further centrifugation step to ensure complete removal of the freeze mixture.

3.2.6 Immunofluorescence staining of BAL
Cytospins were prepared from a sample with a BAL neutrophilia and a further sample with an eosinophilia. After fixation, the cytospins were stained with either 5μl CD15-FITC (Dako) or CD23-FITC (Caltag) in 45μl of PBS. The stained preparations were examined using a fluorescence microscope.

3.2.7 Statistical analysis
Comparisons between FCM and cytospin cell differentials were made using Pearsons correlation. Bland-Altman plots were used to analyse the degree of variation between the two techniques. Coefficients of variation (CV) were assessed by the parallel analysis of 10 cytospins and 10 FCM tubes from the same BAL specimen. This process was
undertaken with 5 different BAL samples. The microscopist was unaware that the cytospins were from the same BAL specimen. The CV's for cytospin and FCM were estimated using the analysis of variance (ANOVA), after controlling for the difference between the mean percentage of each leukocyte subset for the different samples. Otherwise, data was expressed as mean values with 95% confidence limit adjustments included.

### 3.3 Results

#### 3.3.1 BAL diagnoses

46 BAL specimens yielded a diagnosis of which tuberculosis was the most common, occurring in 21 cases. A bacterial organism was only cultured in 8 specimens, partly reflecting prior antibiotic usage. For the HIV-infected population, the most common BAL diagnosis was tuberculosis, occurring in 9 cases. *Pneumocystis carinii* was found in 4 patients and rare diagnoses included strongyloidiasis, cryptococcosis and cytomegalovirus infection. One patient was co-infected with *pneumocystis carinii*, *mycobacterium tuberculosis* and *cytomegalovirus* and went on to have 3 further BAL. The median blood CD4 count in the HIV-infected subjects was 78 cells/μl.

#### 3.3.2 BAL leukocyte differential counts by FCM

The median recovery of saline introduced during BAL was 90ml (50%). Using CD45 to differentiate the leukocyte from the non-leukocyte populations, a mean of 61.6% (95% Cl: 56.6% to 66.6%) of the events acquired by FCM were leukocytes when using fresh BAL. The majority of the CD45 negative events were non-cellular debris, although a variable proportion consisted of bronchial epithelial cells and squamous cells. The mean absolute number of leukocytes counted by FCM was 9305 cells (95% Cl: 7740 to 10870).

There was a marked variability in the leukocyte proportions between different patients with lymphocyte percentages varying from 0.3% to 86% of CD45+ events and the granulocyte percentages varying from 0.2% to 94%.
3.3.3 7-AAD expression in BAL

The mean number of CD45+ events that co-expressed the dead cell marker 7-AAD was 37.2% (95% CI: 32.7% to 41.7%). When these 7-AAD+ events were further analysed, the majority were found to be non-lymphoid. 7-AAD+ lymphocytes maintained their light scatter characteristics and formed a similar gateable population to 7-AAD- lymphocytes (figure 3.4).

![Figure 3.4](image)

**Figure 3.4**

FCM dotplots of fresh BAL demonstrating CD45 expression and light side scatter before (a) and after (b) gating by the dead cell marker, 7-AAD (R3, histogram c). CD45+ events that co-express the dead cell marker 7-AAD (b) maintain light side scatter characteristics and lymphocytes (R2) can be as easily differentiated from non-lymphocytes as in the non-7-AAD gated leukocytes (a).

3.3.4 Correlation between leukocyte differentials by FCM and cytospin

The correlation between each of the leukocyte proportions in BAL enumerated by FCM and cytospin were close with R values of 0.92 for lymphocytes, 0.95 for granulocytes and 0.86 for macrophages (figure 3.5). Bland-Altman analysis (figure 3.6) demonstrated...
Figure 3.5

Correlation plots comparing the enumeration of BAL lymphocytes, granulocytes and macrophages by flow cytometry and cytospin.
Figure 3.6

Bland-Altman plots comparing the enumeration of BAL lymphocytes, granulocytes and macrophages by flow cytometry and cytospin.
a small but statistically significant tendency by cytospin to underestimate the percentage of lymphocytes by 3% (95% CI: -3.7% to -2.2%) when compared to FCM. There was a significant tendency for cytospin to overestimate the percentage of macrophages by 3.4% (95% CI: 1.3% to 5.5%). For granulocytes, the overestimation on cytospin was 2.4% (95% CI: 0.9% to 3.9%).

3.3.5 Coefficient of variation between FCM and cytospin

The coefficient of variation for lymphocyte determination was 2.67% by FCM and 13.3% by cytospin, whilst for macrophages the figures were 2.60% and 10.9% and for granulocytes, 2.78% and 23.0% respectively (table 3.1).

Table 3.1 Coefficients of variation for BAL lymphocyte, macrophage and granulocyte percentages derived by flow cytometry and cytospin

<table>
<thead>
<tr>
<th>BAL Leukocyte subset</th>
<th>FCM</th>
<th>Cytospin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>2.67%</td>
<td>13.3%</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.60%</td>
<td>10.9%</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>2.78%</td>
<td>23.0%</td>
</tr>
</tbody>
</table>

3.3.6 Comparison between fresh and frozen BAL for leukocyte subset determination by FCM

Frozen BAL specimens were analysed using the FCM method described above and the leukocyte differentials were compared with those obtained previously from the same fresh BAL sample. A correlation of 0.978 was achieved for lymphocytes (n=11), with Bland Altman analysis demonstrating limits of agreement between 11.9% and -7.4% for lymphocyte percentages derived from fresh and frozen samples. Similar close correlations were reached for granulocyte and macrophage proportions by FCM. In cytopins made from frozen BAL samples lymphocytes retained their morphology, but it was difficult to count macrophages and neutrophils due to the deterioration of their cellular architecture.
3.3.7 Comparison between BAL lymphocyte percentages obtained by CD45 and light scatter with the sum of the lymphocyte subsets by FCM

Excellent correlations between lymphocyte enumeration by CD45-light scatter and lymphosum were obtained (r=0.9986). Bland Altman analysis demonstrated very close limits of agreement between these two methods (figure 3.7) thus confirming the adequacy of the CD45-light scatter method of calculating BAL lymphocyte percentages.

Figure 3.7

Correlation plot (a) and Bland Altman plot (b) for the total percentage of lymphocytes in BAL derived by CD45 expression and low side scatter compared with the lymphocyte percentage derived by adding the percentages of T, B and NK cells (lymphosum).
3.3.8 Immunofluorescence staining of BAL

Positively stained cells with characteristic granulocyte morphology were demonstrated using both CD23 and CD15 reagents in samples with an eosinophilia (figure 3.8). As expected, CD23 staining was negative on CD15-positive granulocytes in samples with a neutrophilia. These findings confirm that CD23 allows eosinophils to be distinguished from neutrophils and validates the FCM gating strategy (Figure 3.2).

![Figure 3.8](image)

Immunofluorescence staining of BAL from a patient with a BAL eosinophilia. Green fluorescent cells with a characteristic eosinophil appearance are noted following staining with CD23 FITC (a) and also following staining with CD15 FITC (b).

3.4 Discussion

Cytospin remains the most frequently used method of analysing BAL leukocytes, despite the fact that FCM readily discriminates lymphocytes, macrophages and granulocytes in addition to providing details about lymphocyte subsets. Previous studies using FCM have provided a biased view of BAL analysis by concentrating on the characteristics of the easily distinguishable lymphocytes and their subsets [11-13]. Such an approach has failed to provide information on the relative proportions of the clinically
important granulocyte component. Here a more complete picture has been provided by recording the proportions of lymphocytes, granulocytes and macrophages in BAL in patients with respiratory disease.

The precision of cytospin versus FCM has not previously been compared under optimal conditions. In this study cytospins were evaluated by a cytologist of 15 years experience who counted 500 leukocytes in each preparation. FCM was undertaken using CD45 directed gating to distinguish leukocytes from debris and epithelial cells. The use of CD45 panleukogating and light scatter characteristics has been established as the optimum method for lymphocyte enumeration in blood [14]. The addition of a granulocyte marker is required in BAL, but not blood, because light scatter and CD45 expression alone cannot reliably distinguish granulocytes from macrophages.

Some investigators have argued that the best strategy for BAL lymphocyte analysis is to eliminate damaged cells from the analysis by using a DNA dye, LDS-751 after first gating the CD45+ low side scatter lymphocytes [12]. Whilst the exclusion of damaged cells may be necessary for functional and phenotypic analysis of lymphocytes, our study demonstrates that CD45 expression and light scatter characteristics are remarkably robust for BAL leukocytes and are not significantly affected by freezing. Moreover, the exclusion of damaged cells also skews the BAL leukocyte differentials. We used a nuclear dye, 7-AAD to recognize early apoptotic and dying cells [15]. In fresh BAL, the majority of 7AAD+ leukocytes were non-lymphoid, but the 7-AAD+ lymphoid and non-lymphoid components could still differentiated from each other by light scatter characteristics (figure 3.4). Thus, the exclusion of 7-AAD+ events will overestimate the proportion of lymphocytes and underestimate macrophages and granulocytes that are more prone to cell death.

Comparisons between FCM and cytospin were made using three statistical techniques. First, the coefficient of variation for each method in determining leukocyte subsets was assessed by the analysis of 10 parallel preparations of cytospins and FCM tubes from the same BAL sample repeated with 5 different samples. The coefficients of variation by FCM were considerably lower than by cytospin for each leukocyte subset (Table 3.1), demonstrating the superior precision of FCM. Such a finding is unsurprising as a mean of 9305 CD45+ events were counted by FCM in this study compared with 500 cells by cytospin. Second, correlation plots between the two methods for the enumeration of lymphocytes, granulocytes and macrophages were performed to demonstrate the excellent overall agreement of the mean values. Third, the two methods
were scrutinized using the Bland-Altman test (figure 3.6) to detect any consistent variation between the two techniques. Bland-Altman analysis demonstrated a slight selective accumulation of the larger macrophages and the reciprocal depletion of the smaller lymphocytes by cytospin when compared to FCM. Such a phenomena has previously been described when stimulated large blast cells preferentially accumulated on cytopsins at the expense of small lymphocytes [16].

The FCM method described here distinguishes granulocytes from macrophages by virtue of CD15 expression. CD15+ granulocytes consist of neutrophils, eosinophils and basophils. Basophils are rare populations in BAL, but the presence of eosinophils can be diagnostically helpful. Eosinophils were found to co-express both CD15 and the IgE receptor antigen, CD23 (Figure 3.2), whereas neutrophils were CD15+ but did not express CD23.

In summary, a simple flow cytometric technique has been described for distinguishing the BAL leukocyte populations that avoids the potential complications recorded by previous investigators [12]. This system, using primary immunological gating of CD45+ events in conjunction with the granulocyte marker CD15 and the eosinophil marker CD23 represents an effective antibody panel for the delineation of the clinically relevant leukocyte subsets in BAL with apoptotic markers such as 7AAD demonstrated to be unhelpful. Statistical analysis has confirmed close correlations between the leukocyte populations demonstrated by FCM and by cytospin preparations while also documenting that FCM is superior in terms of precision, reliability and robustness. These features, combined with its speed and the ability to perform simple additional lymphocyte phenotyping panels argue strongly in favor of FCM being adopted as a standard method for BAL analysis.

3.5 References


Chapter 4

Optimal Gating Strategies for Determining Bronchoalveolar Lavage CD4/CD8 Lymphocyte Ratios by Flow Cytometry
4.1 Introduction

The aim of this chapter was to compare different gating strategies for the determination of CD4/CD8 ratios in BAL with a 'gold standard' flow cytometric method. The impetus for this investigation was to generate a single, 4-colour monoclonal antibody panel that could determine the lymphocyte heterogeneity based on the CD4/CD8 ratios in addition to the relevant BAL leukocyte components discussed in the previous chapter.

Raised BAL CD4/CD8 lymphocyte ratios have been known to be associated with diseases such as sarcoidosis and berylliosis and respiratory physicians have found these parameters helpful in diagnostic decision making [1-4]. Nevertheless, these early studies have used cumbersome immunofluorescence or immunoperoxidase methods for lymphocyte subset analysis that are both time consuming and labour intensive. Flow cytometry has been demonstrated to be an alternative method for BAL lymphocyte subsetting [5-7]. However, it remains unclear what are the optimum combinations of antibodies and gating strategies in order to perform BAL lymphocyte subset analysis. Since the publication of these early studies on the use of FCM in BAL, there have been few subsequent published reports dedicated to exploring this issue. By contrast, there has been a considerable degree of research interest in the generation of simple new protocols for the determination of CD4 and CD8 lymphocyte subsets in blood [8, 9]. In view of these developments, a simplified, CD45 panleukogating system where CD45+ low side scatter lymphocytes were gated and the CD4 and CD8 subsets directly investigated without the use of the T cell marker, CD3. The CD4 and CD8 subsets derived by this method were compared with several different techniques. As a 'gold standard' comparator, a more complex panel including an anti-CD3 antibody and a precise gating strategy designed for optimum CD4 counting in blood was used [10]. In addition, the CD4/CD8 ratios were also analysed using a CD3 gating method, but without CD45 discrimination on a different flow cytometer.

4.2 Methods

4.2.1 Patients

Immunocompetent patients undergoing BAL for suspected respiratory disease were included. In addition, pleural fluid was also analysed from two patients. The majority of patients (table 4.1) had either sarcoidosis or tuberculosis. HIV+ patients were not included since the BAL CD4/CD8 ratios were reduced and
therefore any differences between the two techniques would have been less easy to detect.

4.2.3 Bronchoalveolar lavage and pleural fluid
BAL and pleural aspirations were performed for clinical indications. Standard techniques for BAL and pleural aspirations were undertaken. Aliquots of BAL and pleural fluid were sent to the relevant laboratories and the remainder was used to perform differential cell counts.

4.2.4 Handling of samples
BAL and pleural fluid samples were washed and filtered as described in chapter 3.

4.2.5 Flow cytometry
The following monoclonal antibodies were used in optimised, pretitrated saturating concentrations. For the first panel (method 1): CD4 FITC (Royal Free Hospital), CD8 PE (Cymbus), CD3 PECy7 (Caltag) and CD45 APC (Pharmingen). For the second panel (method 2): CD15 FITC (Cytognos, Salamanca, Spain), CD4 PE (Cymbus), CD8 PECy7 (Caltag) and CD45 APC (Pharmingen) were used. For the third panel (method 3): CD4 FITC (Royal Free Hospital), CD8-PE (Royal Free Hospital) and CD3-PECy5 (Dako). 50μl of BAL or pleural fluid was added to the different antibody panels and the samples stained at room temperature in the dark for 15 minutes. For the first two panels a wash step was then performed and the pellets resuspended up to a volume of 200μl with PBS-A before running on a FACSCalibur flow cytometer (Becton Dickinson). The wash step was omitted for the last panel and the samples were made up to 1ml with PBS-A before running directly on the CytomonAbsolute flow cytometer (Ortho diagnostics).

20,000 CD45+, low side scatter lymphocytes were acquired for each sample run on the FACSCalibur. The listmode data generated were analysed in the following fashion. For the first panel, the gating strategy adopted was the same as that described by Bergeron et al [10] . Briefly, CD45+ low side scatter lymphocytes were first gated and then sent to a second dotplot to discriminate CD3+ T cells with low side scatter from non-T cell lymphocytes. The CD3+ CD45+ events were then backgated to a CD45 side
scatter plot to ensure that apoptotic lymphocytes were excluded. Lastly, the lymphocytes were further scrutinised by their expression of either CD3 and CD4 or CD3 and CD8 (figure 4.1).

**Figure 4.1**

FCM dotplots demonstrating the optimum gating strategy for determining CD4/CD8 ratios (method 1). CD45+ low side scatter lymphocytes (R1) were analysed in terms of the CD3+ T cell component (R2). R2 events were confirmed to be lymphoid cells by backgating to a CD45 side scatter plot (R3). Finally, R3 events were scrutinized by their expression of CD3 and CD4, or CD3 and CD8. The number of events in the upper right hand quadrant of each of these latter dotplots was used to calculate the CD4/CD8 ratios.
For the simplified second panel (method 2), CD45+ low side scatter lymphocytes were analysed directly in terms of CD4 and CD8 expression (figure 4.2). The final 3-colour panel run on the cytoron was analysed as follows (method 3).

Figure 4.2

Dotplots demonstrating the simplified gating strategy for determining the BAL CD4/CD8 ratios (method 2). CD45+ low side scatter lymphocytes were sent directly to a second dotplot to differentiate the CD4 and CD8 components.

Figure 4.3

Dotplots demonstrating the gating strategy to determine the BAL CD4/CD8 ratios on samples run on the CytoronAbsolute (method 3). Cells with lymphoid forward and side scatter (R1) were analysed in terms of their CD3 expression (R2) and these CD3+ T lymphocytes were finally differentiated into CD4 and CD8 subsets.
Lymphocytes in BAL were gated on their intrinsic properties (figure 4.3) and sent to a further dotplot in which CD3+ T cells were gated. This CD3+ lymphoid population was then directly differentiated into its CD4+ and CD8+ components.

4.2.6 Statistics
The different methods for determining the BAL CD4/CD8 ratios were compared by Spearmans correlation coefficient and by Bland Altman analysis.

4.3 Results
4.3.1 Diagnoses in the study population
31 subjects were included in the analysis (table 4.1). Of these 15 had *mycobacterium tuberculosis* diagnosed by culture confirmation and in one *mycobacterium avium intracellulari* was grown. In 6 patients, sarcoidosis was diagnosed by a combination of clinical suspicion, typical histological appearances on endobronchial or transbronchial biopsies and failure to culture *mycobacterium tuberculosis*. Two bone marrow transplant patients underwent BAL for respiratory symptoms and in one cytomegalovirus was detected by polymerase chain reaction (PCR). Cytomegalovirus was also detected in BAL from a patient with chronic renal failure (patient 20). In 7 patients, no diagnosis was determined from the BAL. Of the two pleural fluid specimens analysed, one was from a patient with tuberculosis and in the other a pathological cause was not identified.

4.3.2 Comparison of CD4/CD8 ratios determined by the ‘gold standard’ (method 1) with the simplified technique (method 2)
The BAL and pleural fluid CD4/CD8 ratios in the study population were compared using method 1 and method 2 as described above. Comparisons were made both by determining the correlation between the two methods (figure 4.4) and by Bland Altman analysis (figure 4.5). An excellent close correlation was achieved (r=0.992). More importantly, Bland Altman analysis demonstrated a very minimal difference between the two techniques for BAL and pleural fluid CD4/CD8 ratio determination. When compared to the method 1, the simplified gating strategy overestimated the CD4/CD8 ratio by only 0.08. Close levels of agreement were demonstrated between the two techniques.
Table 4.1 Demographic details, diagnoses and BAL and pleural fluid CD4/CD8 ratios by different methods in the study population

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
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<th>CD4/CD8 ratio Method 2</th>
<th>CD4/CD8 ratio Method 3</th>
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</table>

Footnotes: MAI = *Mycobacterium avium intracellulare*

NAD = nothing abnormal detected
Figure 4.4

Correlation plot for CD4/CD8 ratio determination between the complex technique (method 1) and a more simplified approach (method 2).

Figure 4.5

Bland Altman comparisons between method 1 and 2 for the determination of CD4/CD8 ratios.
with the differences ranging from +0.77 to −0.93. The widest limits of agreement for the CD4/CD8 ratios determined by these two methods were demonstrated in those with the highest values (mostly in patients with sarcoidosis). At lower values, the limits of agreement were tighter and there was very little variation when the CD4/CD8 ratios were <2.5.

4.3.3 Differences between the BAL and pleural fluid CD4/CD8 ratios measured by method 1 and method 3
This analysis was performed to assess whether a standard method for CD4/CD8 ratio determination in BAL and pleural fluid using CD3 to identify T lymphocytes performed as well as the 'gold standard', method 1. 24 BAL specimens and the two pleural fluid samples were analysed by both methods. The correlation between these two methods was close (R=0.988, figure 4.6).

Figure 4.6

Correlation plot between the CD4/CD8 ratios derived on the FACSCalibur by a complex gating system (method 1) and those derived by a different method on the Cytoron (method 3).
Bland Altman analysis demonstrated an insignificant underestimation of the CD4/CD8 ratio by the cytoron of 0.02. The limits of agreement were slightly wider (-1.1 to 1.1) than between method 1 and method 2 that were both performed on the FACSCalibur. However, at lower CD4/CD8 ratios the limits of agreement were much closer, and the wider limits of agreement were a feature of higher CD4/CD8 values.

Figure 4.7

Bland Altman plot comparing the CD4/CD8 ratios in BAL and pleural fluid between method 1 and method 3

4.4 Discussion

This study has been the first to assess the precision of flow cytometry for determining tissue fluid CD4/CD8 ratios by comparing different flow cytometric methods. As the 'gold standard', a recently published method using a combination of CD45-directed gating in conjunction with CD3 gating was used [10]. This gating strategy was designed as a universal template to produce accurate absolute CD4 and CD8 T cell counts, but was equally applicable for deriving the CD4/CD8 ratios. However, it was clear that optimum precision was not an overriding concern for CD4/CD8 ratio measurement in tissue fluids. Rather, the method should be able to distinguish between those with normal and high
ratios, with the upper limit of normal generally agreed to lie between 2.5 to 3 [11]. Therefore, the primary aim was to develop a simplified gating strategy with adequate precision to determine the CD4/CD8 ratios in BAL for clinical diagnostic purposes.

Interestingly, close agreement between these methods was demonstrated by Bland Altman analysis with the simplified method overestimating the CD4/CD8 ratios by only 0.08. More importantly, the limits of agreement between these two techniques varied between only 0.77 and -0.93, with the main variability occurring at the higher CD4/CD8 ratios. The conclusion, therefore, is that the simplified method offers no loss of precision for CD4/CD8 ratio analysis. Such a finding is reassuring, since it might be expected that the omission of a CD3 antibody could introduce an error into the CD4/CD8 ratios analysis. The reason for this is that NK cells, which weakly express CD8 but do not express CD3 may be erroneously included as CD8 cells in the simplified method. It is of interest, therefore that this analysis included a bone marrow transplant patient (patient 24, table 4.1), in whom BAL was performed only three months after the transplant. Since it is known that NK cells reconstitute early after transplantation followed by a slower recovery of T cells [12], the BAL cellular constituents were examined more closely in this patient in order to determine the NK cell component. NK cells, defined as CD56+ and CD3-, constituted 20.1% of the total CD45+ low side scatter BAL lymphocyte pool. Nevertheless, despite this high proportion of NK cells, the CD4/CD8 ratios determined by both the optimum and the simplified method were virtually identical. The explanation for this finding is that the gate to differentiate the CD8 component in the simplified method was placed to include only the CD8bright lymphocytes, thus excluding NK cells.

Both methods 1 and 2 described above were performed on the FACSCalibur flow cytometer. In this study an additional panel was run using a different flow cytometer, the CytoronAbsolute. This last method has been extensively employed for deriving CD4 and CD8 counts in both blood [13] and BAL [14] using a CD3 gating strategy without the use of panleukogating. Again, close correlations between the 'gold standard' method and the Cytoron method were observed for the generation of CD4/CD8 ratios. Bland Altman analysis demonstrated that the latter method underestimated the lymphocyte ratios by only 0.02 and the limits of agreement ranged between -1.1 to 1.1. As with the first comparison, the greatest variation occurred with the highest CD4/CD8 ratios.

Taken together, these observations demonstrate the remarkable precision of flow cytometry. Here three different gating methods were employed and two different flow cytometers used. Despite this, there was clinically insignificant variability between the
different techniques. It has been demonstrated in chapter 3 that CD45 directed gating, in conjunction with only one other antibody, CD15 to determine the granulocyte component in BAL was able to discriminate the major leukocyte subpopulations in BAL. Therefore, a single 4-colour antibody panel can be constructed enabling the differentiation of not only the lymphocyte, granulocyte and macrophage populations but also the CD4 and CD8 lymphocyte subset ratios. The following monoclonal antibodies were used: CD45, CD15, CD4 and CD8. This single panel could therefore provide the maximum clinically relevant information in a rapid and simple manner. Nevertheless, such a panel does not provide information on the relative proportions of neutrophils and eosinophils within the total granulocyte pool. The importance of eosinophils discrimination in BAL was discussed in the previous chapter where it was also demonstrated that monoclonal antibodies against the IgE receptor, CD23 could be used to discriminate eosinophils from neutrophils within the CD15+ granulocyte pool. Preliminary observations have suggested that CD23 may be included in the single panel by staining with CD15 and either CD4 or CD8 conjugated to the same fluorochrome. This is possible since the difference in the scatter characteristics between lymphocytes and granulocytes allow these two populations to be easily distinguished within the CD45 panleukogate. Whether such a panel is demonstrated to be equally reliable for lymphocyte and leukocyte subsetting remains to be determined.

In summary, it has been demonstrated here that CD45-directed morphospectral gating of lymphocytes is sufficient before CD4 and CD8 discrimination to assess the clinically relevant CD4/CD8 ratio in both BAL and pleural fluid. The ratios generated by this method varied by a clinically insignificant amount when compared to an optimal gating strategy. Therefore, these results have made feasible a simple, single panel protocol for the determination of both the major leukocyte components and the CD4/CD8 lymphocyte subsets in BAL.

4.5 References


Chapter 5

Bronchoalveolar Lavage and Other Tissue Fluid Leukocyte Differentials Assessed by Flow Cytometry in Patients with Distinct Clinical Syndromes
5.1 Introduction

In the previous chapters, flow cytometry was used to simplify and optimize the discrimination of both the BAL leukocyte differentials and CD4/CD8 ratios. In this chapter, these parameters were determined in BAL from a large number of patients investigated for respiratory disease. The results were then correlated with the clinical findings in order to assess their diagnostic relevance.

Such a study is not new as numerous previous investigators have examined BAL leukocyte differentials and shown certain characteristic features in a variety of clinical diseases such as sarcoidosis [1-6], tuberculosis [7-9] and interstitial lung diseases [10, 11]. Nevertheless, these previous studies have exclusively used cytospin techniques to determine the differentials. Although there have been some investigations using FCM on BAL from patients with various interstitial lung diseases, these have focused mainly on analysing either the CD4/CD8 T lymphocyte subset ratios [12, 13], or the lymphocyte proportions using CD45 panleukogating and light scatter characteristics [14, 15]. Whilst the BAL lymphocyte percentages and CD4/CD8 ratio are undoubtedly the most useful cellular characteristics for the diagnosis of sarcoidosis, the omission of details of the neutrophil component is serious as a BAL neutrophilia in this disease may be an adverse prognostic factor [16]. Similarly, raised neutrophils in interstitial lung diseases and tuberculosis may also be relevant to the disease process.

Therefore, a comprehensive FCM system has not previously been applied to the routine investigation of BAL in patients with respiratory disease and the leukocyte differentials thus derived assessed for their diagnostic significance. The same flow cytometric analysis was also performed on a small number of clinical specimens other than BAL in order to demonstrate that such a system has more widespread clinical applicability. These specimens included pleural, peritoneal, ascitic and cerebrospinal fluid.

5.2 Methods

5.2.1 Patients

Samples were analysed from patients undergoing routine bronchoscopy for the investigation of respiratory disease of presumed infectious or inflammatory aetiology. 5 healthy control subjects also underwent bronchoscopy (median age 38; range 25-56, 2 smokers, 3 non-smokers). A small number of specimens of pleural, peritoneal or
cerebrospinal fluid were also investigated after samples had been sent for routine diagnostic analysis. In total 167 subjects including the controls had BAL differentials measured by FCM. In addition, five had pleural fluid, one peritoneal fluid and one cerebrospinal fluid measured by the same procedure.

5.2.2 Bronchoalveolar lavage and bronchial biopsy

BAL was performed as previously detailed and according to British Thoracic Society guidelines. In cases with focal abnormalities detected on thoracic radiographs or computed tomograms, the BAL was site-directed to these areas. In those in whom either diffuse radiological abnormalities were noted, or the chest radiography was normal but respiratory pathology was still suspected, standard right middle lobe BAL’s were performed. In several cases with pulmonary tuberculosis, BAL was undertaken from both a radiologically abnormal and normal area. In these latter cases, 150ml of normal saline was instilled into the radiologically affected area and 50ml into the right middle lobe. 10ml of blood was collected into a lithium heparinised tube at the time of bronchoscopy.

In patients suspected of having sarcoidosis, endobronchial, and in most cases transbronchial biopsies were performed in addition to BAL.

5.2.3 Acquisition of pleural, peritoneal and cerebrospinal fluid samples

Samples obtained from pleural, peritoneal and cerebrospinal sites were obtained using standard sterile procedures by medical staff investigating patients with suspected clinical disease. In these cases, aliquots were sent to the relevant diagnostic laboratories and the remainder analysed by flow cytometry.

5.2.4 Routine analysis of Clinical Specimens

All samples other than those from the normal control subjects who underwent BAL were sent for routine analysis. Since the patients who underwent BAL and other diagnostic procedures were being investigated for presumed infectious or inflammatory conditions, an aliquot from all samples was sent to microbiology. Standard culture was performed with an additional Ziehl-Neelson smear for acid-alcohol fast bacilli, polymerase chain reaction (PCR) and culture on Lowenstein-Jensen medium if tuberculosis (TB) was suspected. Fungal culture was also performed in those at high risk such as bone marrow transplant patients. In almost all specimens a further sample was sent to cytology. Stained cytology specimens were examined for the presence of acid-
alcohol fast bacilli, pneumocystis carinii, fungal hyphae and viral inclusion bodies. In addition a comment was made on the relative proportions of the leukocyte populations although a formal leukocyte differential was not performed. Lastly, in BAL and other samples where a viral aetiology was considered, an aliquot was sent to virology where relevant immunofluorescence or enzyme linked immunoabsorbant (ELISA) assays were performed. In BAL samples, adenovirus, influenza and parainfluenza viruses as well as respiratory syncitial virus were routinely tested for. In selected samples from patients who had undergone bone marrow transplantation, or those severely immunocompromised due to HIV, a cytomegalovirus direct antigen fluorescent foci test (DEAFF) and cytomegalovirus PCR were performed. All HIV+ BAL specimens were sent to microbiology, cytology and virology.

Endobronchial, and transbronchial biopsies from patients with sarcoidosis were examined for the characteristic histological features of the disease. Some cases presenting with stage 1 pulmonary disease with hilar adenopathy underwent mediastinal lymph node biopsies following failed endobronchial and transbronchial biopsy procedures. Patients with suspected sarcoidosis also had their serum angiotensin converting enzyme (SACE) levels measured routinely in biochemistry.

The pleural, peritoneal and cerebrospinal samples were analysed in a similar manner. Formal leukocyte differential counts were determined in the microbiology laboratory for cerebrospinal and peritoneal fluid samples and expressed as the number of cells per mm$^3$. In addition, the protein and glucose concentrations were also determined in these samples. Further tests such as lactate dehydrogenase levels and pH were performed on pleural fluid samples.

5.2.5 Preparation of Specimens

BAL specimens were collected on ice and analysed within two hours. Aliquots were sent for routine analysis and the remainder was prepared for flow cytometric analysis as detailed in chapter 3. The non-BAL specimens were also prepared in the same way. Briefly, this involved centrifugation, filtering and a further centrifugation step before the cell pellets were resuspended up to a volume of 1 ml in phosphate buffered saline (PBS).

5.2.6 Flow Cytometry

Flow cytometry was performed following staining with CD45 FITC and CD15 PE on both the prepared BAL samples as well as peripheral blood as detailed in chapter 3. In
addition, a second panel containing CD4-FITC (Royal Free Hospital), CD8-PE (Royal Free Hospital) and CD3-PEcy5 (Dako, Ely, UK) was also used to assess the CD4/CD8 ratios as described in chapter 4. The samples were run on a Cytoron flow cytometer using an absolute counting protocol. Gating strategies using a CD45 panleukogate and a CD15+ granulocyte gate were performed and the lymphocyte, macrophage/monocyte and granulocyte proportions calculated as detailed previously in chapter 3. The CD4/CD8 ratios were determined as detailed in chapter 4.

5.2.7 Statistics
Data on leukocyte differentials and CD4/CD8 ratios was not normally distributed and therefore median values and interquartile ranges were quoted in the text. Comparison between data sets was performed using Mann-Whitney analysis.

5.3 Results
5.3.1 General characteristics of BAL
The median volume of saline instilled during BAL was 180ml (IQR: 180-200ml) and the median return was 50% (IQR: 40%-55.6%), Table 5.1. Following removal of aliquots for routine analysis, the remaining BAL (normally more than 25ml) was left for analysis. When the BAL sample had been washed, filtered and the pellet resuspended up to a volume of 1 ml, only 50μl was used for the leukocyte differentials and 50μl for the CD4/CD8 ratios. The remaining 0.9ml of BAL was reserved for further phenotypic and functional analysis in the HIV-infected group and those with suspected tuberculosis. Therefore, the number of events acquired following staining with CD45 and CD15 represented only 5% of the available sample. The median total number of CD45+ leukocytes acquired from the 50μl sample even after resuspension up to 1ml was 8312 (IQR: 4867-17440).

These findings confirm that routine clinical BAL specimens provide more than adequate cells for the simple flow cytometric analysis described here. The majority of samples were of good quality as determined by the percentage of CD45+ leukocytes in the total number of events acquired by FCM. A median of 82.0% (IQR: 57.4-92.3%) of all events fell within the panleukogate. The non-leukocyte cells were a mixture of epithelial cells and non-cellular debris.
Since an absolute counting FCM was used (CytoronAbsolute), the absolute number of events in the total (1 ml) BAL sample could be calculated. The median number of CD45+ BAL leukocytes was $7.5 \times 10^6$ (IQR: 3.6-14.4 X 10^6).

### Table 5.1 Characteristics of BAL from study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median value</th>
<th>Inter-quartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline instilled at BAL (ml)</td>
<td>180</td>
<td>180-200</td>
</tr>
<tr>
<td>% return of saline</td>
<td>50%</td>
<td>40-55.6%</td>
</tr>
<tr>
<td>Number of leukocytes acquired by FCM</td>
<td>8312</td>
<td>4867-17440</td>
</tr>
<tr>
<td>% Leukocytes in total number of BAL events</td>
<td>82.0 %</td>
<td>57.4-92.3%</td>
</tr>
<tr>
<td>Total number of leukocytes in BAL sample</td>
<td>$7.5 \times 10^6$</td>
<td>3.6-14.4 X 10^6</td>
</tr>
</tbody>
</table>

### 5.3.2 Diagnoses in patients undergoing BAL

162 patients underwent BAL of whom 70 (43.2%) were HIV+, reflecting the particular cohort of respiratory patients seen at the Royal Free Hospital. A pathological result was obtained from the BAL, or endobronchial or transbronchial biopsies in 99 (61.1%), table 5.2. In several patients with sarcoidosis, the BAL provided additional diagnostic information in terms of the lymphocyte percentages and CD4/CD8 ratio, even though the endobronchial or transbronchial specimens were non-diagnostic.

The most common BAL diagnosis in both HIV+ and HIV- patients was tuberculosis, accounting for 51 (31.5%) of all BAL. TB was diagnosed in 12 (17.1%) of BAL from HIV+ patients and 39 (42.4%) of those that were HIV-. Sarcoidosis was diagnosed in 15 patients. Bacterial organisms other than mycobacteria were only cultured in seven patients (4.3%). The low frequency of bacterial culture positivity may in part have reflected prior antibiotic usage. Nevertheless, a number of patients in whom a bacterial infection was suspected, but not proven had a marked increase in BAL neutrophil count. Two HIV- individuals with *Pneumocystis carinii* pneumonia (PCP) were immunocompromised on therapy for lymphoma and of the two HIV- patients with
Table 5.2 Main BAL diagnoses In HIV- and HIV+ patients

<table>
<thead>
<tr>
<th>HIV status</th>
<th>BAL Diagnosis1</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV- (n=95)</td>
<td>No pathogen</td>
<td>25</td>
</tr>
<tr>
<td>HIV+ (n=70)</td>
<td>Mycobacterium tuberculosis</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>sarcoid</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Bacterial infection</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Atypical mycobacteria</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Pneumocystis carinii</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cytomegalovirus</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>other</td>
<td>6</td>
</tr>
<tr>
<td>HIV- (n=95)</td>
<td>Total</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>No pathogen</td>
<td>41</td>
</tr>
<tr>
<td>HIV+ (n=70)</td>
<td>Mycobacterium tuberculosis</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Pneumocystis carinii</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Bacterial infection</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Cytomegalovirus</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>other</td>
<td>4</td>
</tr>
<tr>
<td>HIV- (n=95)</td>
<td>Total</td>
<td>72(^2)</td>
</tr>
</tbody>
</table>

Footnotes
1. Diagnosis determined by pathological investigation of BAL (see methods).
2. One HIV+ subject was co-infected with cytomegalovirus and TB.

cytomegalovirus infection, one was a bone marrow recipient and the other had chronic renal failure. For further analysis, the BAL differentials were only considered in those in whom a firm clinical diagnosis was determined in order to assess their diagnostic relevance. The major clinical disease groups studied were those with sarcoidosis, tuberculosis and HIV.
5.3.3 Sarcoidosis

15 patients were diagnosed with pulmonary sarcoidosis. The demographic, diagnostic and clinical features together with the BAL leukocyte differentials and CD4/CD8 ratios are displayed in table 5.3. Patients were divided dependent on the radiological staging of their disease into those with stage 1, stage 2 or stage 3 pulmonary disease [17]. No patients had stage 4 disease. A computed tomogram (CT) of the chest was performed in all these patients. Stage 1 pulmonary sarcoidosis included those with bilateral hilar lymphadenopathy without evidence of interstitial or parenchymal disease. Patients with Stage 2 disease had mediastinal and/or hilar lymphadenopathy with evidence of pulmonary involvement. Stage 3 disease comprised those with interstitial or parenchymal disease without lymphadenopathy and stage 4 was those with irreversible pulmonary fibrosis. The diagnosis was supported by endobronchial or transbronchial biopsy at bronchoscopy in six patients (37.5%). For a further two patients characteristic histological features were also determined by mediastinal lymph node biopsy following inconclusive bronchial biopsies. All BAL and biopsy specimens were sent for mycobacterial culture and all were negative. In seven cases, the biopsies were not helpful and a combination of high clinical suspicion, a raised serum angiotensin converting enzyme (SACE) level and an abnormal BAL lymphocyte profile were supportive of the diagnosis in most cases (table 5.3). Patient 15 had skin lesions from which histology demonstrated characteristic features of sarcoidosis.

When analysed together, the BAL leukocyte differentials in the sarcoidosis patients demonstrated a striking lymphocytosis (median 65.7%, IQR: 46.4-77.0%). When the patients were divided into the different stages of pulmonary disease (figure 5.1), the median percentage lymphocytosis was higher in those with stage 1 (74.4%) than those with stage 2 disease (51.2%). This difference was not significant (p=0.11). In the single patient with stage 3 disease the BAL lymphocyte percentage was 35.3%.

The median BAL CD4/CD8 ratio for the whole group was 5.1 (IQR: 3.9-9.5), but as with the BAL lymphocyte percentages, the CD4/CD8 ratio was higher in stage 1 (median 8.8) than stage 2 sarcoidosis (median 4.4), figure 5.2. The difference between the two was not significant (p=0.15). In the single case with stage 3 disease, the BAL CD4/CD8 ratio was 4.2.
Table 5.3 Demographic and diagnostic features of patients with sarcoid.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age</th>
<th>Ethnicity</th>
<th>Histology</th>
<th>Stage</th>
<th>SACE</th>
<th>BAL leukocyte %</th>
<th>CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph</td>
<td>Mac</td>
<td>Neut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M 34</td>
<td>C</td>
<td>Non-diagnostic</td>
<td>1 (BHL + EN)</td>
<td>40</td>
<td>78.0</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>F 29</td>
<td>C</td>
<td>Non-diagnostic</td>
<td>1 (BHL + EN)</td>
<td>58</td>
<td>70.7</td>
<td>27.8</td>
</tr>
<tr>
<td>3</td>
<td>M 30</td>
<td>C</td>
<td>Non-diagnostic</td>
<td>1 (BHL + EN)</td>
<td>112</td>
<td>83.8</td>
<td>15.7</td>
</tr>
<tr>
<td>4</td>
<td>F 42</td>
<td>C</td>
<td>Supportive (med LN)</td>
<td>1 (BHL,ML)</td>
<td>89</td>
<td>65.7</td>
<td>31.6</td>
</tr>
<tr>
<td>5</td>
<td>F 41</td>
<td>C</td>
<td>Non-diagnostic</td>
<td>1 (BHL + EN)</td>
<td>85</td>
<td>53.3</td>
<td>45.5</td>
</tr>
<tr>
<td>6</td>
<td>M 36</td>
<td>A</td>
<td>Supportive (med LN)</td>
<td>1 (BHL)</td>
<td>80</td>
<td>81.9</td>
<td>17.9</td>
</tr>
<tr>
<td>7</td>
<td>F 49</td>
<td>BAC</td>
<td>Supportive (EB)</td>
<td>2 (BHL,ML + nodules)</td>
<td>135</td>
<td>44.8</td>
<td>53.4</td>
</tr>
<tr>
<td>8</td>
<td>F 71</td>
<td>O</td>
<td>Supportive (EB+TB)</td>
<td>2 (BHL,ML + nodules)</td>
<td>169</td>
<td>82.0</td>
<td>17.5</td>
</tr>
<tr>
<td>9</td>
<td>F 40</td>
<td>C</td>
<td>Supportive (EB+TB)</td>
<td>2 (BHL + nodules)</td>
<td>61</td>
<td>54.3</td>
<td>44.0</td>
</tr>
<tr>
<td>10</td>
<td>M 40</td>
<td>BA</td>
<td>Supportive (LN+Liver)</td>
<td>2 (BHL,ML + nodules)</td>
<td>254</td>
<td>68.4</td>
<td>24.7</td>
</tr>
<tr>
<td>11</td>
<td>M 33</td>
<td>C</td>
<td>Non-diagnostic</td>
<td>2 (BHL,ML + nodules)</td>
<td>47</td>
<td>76.0</td>
<td>23.3</td>
</tr>
<tr>
<td>12</td>
<td>M 29</td>
<td>BA</td>
<td>Supportive (EB)</td>
<td>2 (BHL,ML + nodules)</td>
<td>120</td>
<td>48.0</td>
<td>50.9</td>
</tr>
<tr>
<td>13</td>
<td>F 35</td>
<td>C</td>
<td>Supportive (EB)</td>
<td>2 (BHL + nodules)</td>
<td>88</td>
<td>26.8</td>
<td>71.0</td>
</tr>
<tr>
<td>14</td>
<td>M 45</td>
<td>O</td>
<td>Non-diagnostic</td>
<td>2 (BHL + nodules)</td>
<td>40</td>
<td>39.6</td>
<td>59.4</td>
</tr>
<tr>
<td>15</td>
<td>F 52</td>
<td>BA</td>
<td>Non-diagnostic</td>
<td>3 (Reticulo-nodular)</td>
<td>112</td>
<td>35.3</td>
<td>64.6</td>
</tr>
</tbody>
</table>

Footnotes
1. Ethnicity; C= Caucasian, A= Asian, BAC= black Afro-Caribbean, BA= black African, O= other.
3. Staging of pulmonary sarcoidosis into stage 1, 2 or 3 disease. BHL = bilateral hilar lymphadenopathy, EN = erythema nodosum, ML = mediastinal lymphadenopathy.
4. SACE = serum angiotensin converting enzyme. The normal range at the Royal Free Hospital is < 50.
5. The BAL CD4/CD8 ratio determined by FCM.
Figure 5.1
Percentage of BAL lymphocytes by flow cytometry in patients with sarcoidosis according to the stage of their pulmonary disease. The bars represent the median values for each group. The differences between the groups were not statistically significant.

Figure 5.2
BAL CD4/CD8 ratio determined by FCM in patients with sarcoidosis according to the stage of their disease. The bars represent median values.
5.3.4 Tuberculosis

51 patients were diagnosed with tuberculosis of which 39 were HIV seronegative and 12 HIV seropositive. The leukocyte differentials and CD4/CD8 ratios were further considered in HIV negative group, the majority of whom had pulmonary disease (31, 79.5%). Of the patients with non-pulmonary disease, two had tuberculous lymphadenopathy, one spinal and one pharyngeal disease. These patients had normal chest radiographs with failure to culture the organism from BAL. Four patients were diagnosed with disseminated TB in which there was both pulmonary and extra-pulmonary involvement. In patient 5 the predominant clinical manifestation was cerebral tuberculomas. Patient 6 had miliary TB, whilst patients 7 and 8 had predominantly lymph node disease.

Mycobacterium tuberculosis was confirmed by culture in all but three patients. In patient 4 with pharyngeal TB, acid fast bacilli were seen within granulomas of a pharyngeal biopsy, but the specimen was not sent for culture. The pharyngeal mass resolved on treatment. Patients 33 and 34 in whom a clinical diagnosis of pulmonary TB was made had suggestive respiratory symptoms and chest radiographic abnormalities both of which resolved on anti-tuberculous therapy. *M. tuberculosis* was cultured from the appropriate tissue biopsies of the patients with non-pulmonary TB, other than patient 4.

In most patients a polymerase chain reaction (PCR) test was also performed on the BAL. The demographic and diagnostic characteristics as well as the BAL FCM findings are detailed in table 5.3. The BAL leukocyte differentials demonstrated a lymphocytosis in many, but not all patients with TB. The BAL lymphocyte percentages were compared with those from the sarcoidosis patients and five healthy control subjects (figure 5.3). The difference in the median BAL lymphocyte percentage between the patients with TB and those with sarcoidosis was highly significant (*p*< 0.0001). Nevertheless, there was still considerable overlap in the BAL lymphocyte percentages between these two groups suggesting that a raised lymphocyte percentage alone would not be a very good discriminating marker. When compared to the control subjects, there was no significant difference in the BAL lymphocyte percentages in those with TB (*p* = 0.07).

Several patients with TB were noted to have very low BAL lymphocyte percentages, and these were usually associated with a co-existing BAL granulocytosis. The site of the BAL was detailed in this subgroup by referring to the chest radiographic and/or computed tomographic findings in addition to the bronchoscopy report.
Table 5.4 Demographic, diagnostic and BAL FCM data of patients with TB

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age</th>
<th>Ethnicity</th>
<th>TB diagnosis</th>
<th>BAL findings</th>
<th>BAL Leukocyte %</th>
<th>BAL CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AFB PCR cult</td>
<td>Lymph Mac Neut</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F 16</td>
<td>BA</td>
<td>Spinal TB</td>
<td>- - -</td>
<td>42.8 57.1 0.1 2.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M 28</td>
<td>BA</td>
<td>Lymph TB</td>
<td>- - -</td>
<td>32.0 65.6 2.4 1.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F 42</td>
<td>BA</td>
<td>Lymph TB</td>
<td>- - -</td>
<td>29.0 18.1 52.8 2.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F 39</td>
<td>BUK</td>
<td>Pharyngeal TB</td>
<td>- - -</td>
<td>46.2 52.6 1.2 3.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M 32</td>
<td>BA</td>
<td>Disseminated TB</td>
<td>- + +</td>
<td>43.6 55.3 1.1 7.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M 21</td>
<td>BA</td>
<td>Disseminated TB</td>
<td>- + +</td>
<td>44.6 53.5 1.9 2.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F 32</td>
<td>O</td>
<td>Disseminated TB</td>
<td>- ND³+</td>
<td>20.2 77.9 2.1 2.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F 41</td>
<td>BUK</td>
<td>Disseminated TB</td>
<td>- + +</td>
<td>27.6 70.2 2.2 4.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F 24</td>
<td>C</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>33.0 30.2 36.8 2.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M 24</td>
<td>A</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>62.0 37.1 0.9 0.4</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F 21</td>
<td>BA</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>20.8 6.3 72.9 11.7</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>M 18</td>
<td>BA</td>
<td>Pulmonary TB</td>
<td>- + +</td>
<td>33.5 63.7 2.8 2.0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M 24</td>
<td>A</td>
<td>Pulmonary TB</td>
<td>- + +</td>
<td>70.4 27.6 2.0 2.6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M 27</td>
<td>C</td>
<td>Pulmonary TB</td>
<td>- + +</td>
<td>15.7 82.4 1.9 2.4</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>M 37</td>
<td>A</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>41.3 7.2 11.5 1.7</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>M 35</td>
<td>C</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>4.6 27.9 67.5 1.2</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>M 51</td>
<td>C</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>15.2 79.7 5.1 1.1</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>F 26</td>
<td>BA</td>
<td>Pulmonary TB</td>
<td>+ + +</td>
<td>46.8 46.7 7 5.9</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>F 27</td>
<td>BA</td>
<td>Pulmonary TB</td>
<td>- + +</td>
<td>10.3 19.5 70.2 1.1</td>
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<tr>
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<td>- + +</td>
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<td>7.1 78.7 14.2 1.7</td>
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</tr>
<tr>
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<td>F 35</td>
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<td>- - +</td>
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<td>Pulmonary TB</td>
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<td>13.1 86.1 0.8 1.0</td>
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<td>+ ND³+</td>
<td>35.8 63.3 0.9 1.7</td>
<td></td>
</tr>
</tbody>
</table>

Footnotes

1. Ethnicity. BA=black African, A=Asian, C=Caucasian, O=other
2. Acid fast bacilli (AFB), polymerase chain reaction (PCR) and culture results for *M. tuberculosis* from BAL. Patients 1-3 had positive cultures from tissue biopsies. Patients 33 and 34 were clinical diagnoses and patient 4 was a combination of pathological and clinical diagnosis but without culture of the organism.
3. ND=not done
Figure 5.3

The BAL lymphocyte percentages in patients with tuberculosis, sarcoidosis and in healthy control subjects. The bars represent median values.

Figure 5.4

The percentage of BAL lymphocytes in patients with pulmonary tuberculosis. The first column contains the lymphocyte percentages when washings were taken from areas of radiologically abnormal lung that were not cavities. In the second column washings were taken from cavities and in the third column, washings were taken from radiologically normal lung.
In cases where washings were performed from a pulmonary cavity, the predominant leukocytes were granulocytes with a corresponding reduction in the lymphocyte percentage. In several cases (patients 22 and 31), frank pus was aspirated at BAL. The BAL lymphocyte percentages were then compared between those in whom washings were taken from cavities, those with non-cavitatory radiographic abnormalities and those with normal chest radiographs (figure 5.4). This latter population consisted of those with non-pulmonary disease. A reanalysis of the BAL lymphocyte percentages following the separation of those with tuberculosis into different groups based on the type of disease revealed several interesting features.

First, those with non-pulmonary disease all had a BAL lymphocytosis, a finding that is of relevance for the antigen-specific analyses detailed in chapter 8. Second, advanced pulmonary TB with cavitation was characterized by a granulocytosis (neutrophila) with corresponding low lymphocyte percentages. Nevertheless, even after those with cavitation were separated from the main group with TB, two patients were noted to have low BAL lymphocyte percentages, (patients 20 and 26, table 5.3). Interestingly, these two individuals were entirely asymptomatic, but were referred to the infectious diseases team at this hospital following the discovery of abnormal chest radiographs on arrival into the United Kingdom by air. These findings are consistent with the hypothesis that the immune response plays a primary role in the symptomatology of TB and that in the early phase of mycobacterial proliferation, patients might be expected to have low BAL lymphocyte percentages as these cells are actively recruited to the site of infection.

Finally, the BAL lymphocyte percentages were compared in eight patients with TB in whom washings were simultaneously taken from radiologically normal and abnormal lung (table 5.4). In two of these subjects (patients 19 and 21, table 4.3), washings were taken from apical cavities as well as radiologically unaffected lobes. Taken together with the BAL lymphocyte differentials from the patients with non-pulmonary disease, these findings demonstrate that a generalised BAL lymphocytosis is a common feature in patients with pulmonary and non-pulmonary TB. The two caveats are that washings should not be taken from cavities and also that patients should be symptomatic.

A reanalysis of the BAL lymphocyte percentages following the exclusion of the two patients with asymptomatic disease and those in whom washings were taken from cavities demonstrated a significant difference when compared to the healthy controls (P=0.01), figure 5.5.
Table 5.5  BAL lymphocyte percentages from washings taken from radiologically abnormal and normal areas in patients with pulmonary tuberculosis

<table>
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<th>Patient</th>
<th>Radiologically abnormal</th>
<th>Radiologically normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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</tr>
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<tr>
<td>7</td>
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<td>68.5%</td>
</tr>
<tr>
<td>8</td>
<td>29.7%</td>
<td>16.3%</td>
</tr>
</tbody>
</table>

Footnotes

1. Washings were taken from apical cavities from patients 1 and 2.

Figure 5.5
Scatter plot demonstrating the BAL lymphocyte percentages in all TB patients, in symptomatic TB patients when washings were not taken from cavities and in healthy controls.
It should be noted both that the number of normal controls was small and also that two non-smoking subjects had rather high BAL lymphocyte percentages of 22.2% and 23.3% respectively. The lymphocyte percentages in healthy subjects generally lie within the range of 4-18% in non-smokers and 3-8% in smokers [11]. However, it has also been demonstrated that the lymphocyte percentages in healthy individuals may fluctuate considerably [18]. The comparison between the BAL lymphocyte differentials in the TB patients and the controls may reach more statistical significance with a larger control group.

The CD4/CD8 ratios were also examined in all patients with TB and compared with the values obtained in patients with sarcoidosis and in control subjects (figure 5.6). When compared to the patients with sarcoidosis, the BAL CD4/CD8 ratios in patients with TB were significantly lower ($p=0.001$). There was no statistical difference in the CD4/CD8 ratios between the patients with TB and the controls ($p=0.44$).

![Figure 5.6](image)

**Figure 5.6**

BAL CD4/CD8 ratios derived by flow cytometry in patients with tuberculosis, sarcoidosis and in control subjects.
5.3.5 HIV

The final major group to be considered in terms of the BAL leukocyte differentials and CD4/CD8 ratio were the HIV+ patients. Of the 70 total HIV+ patients in whom BAL was performed, a diagnosis was achieved in 29 (41.4%). As expected, the CD4 counts were lower in the patients with respiratory pathogens (median 47 cells/µl IQR: 11-109) than those in whom no pathogens were found (median 133 cells/µl IQR: 36-261). In the HIV+ group with respiratory disease, different BAL pathogens occurred at varying levels of immunosuppression. In patients with bacterial infections, the median blood CD4 count was 107 cells/µl (IQR: 104-176), in those with tuberculosis it was lower (median 60 cells/µl, IQR: 51-244) whilst the HIV+ group with PCP, CMV, MAI, cryptococcus and invasive strongyloides had the lowest CD4 counts (median 14 cells/µl IQR: 7-31) (figure 5.7). Since the hallmark of HIV infection is the depletion of CD4 lymphocytes, both the leukocyte differentials, and more especially the CD4/CD8 ratios would therefore be expected to be disturbed in HIV+ individuals.

Figure 5.7

Blood CD4 count in HIV+ patients according to pathogens determined in BAL. The group denoted ‘other diagnoses’ had Pneumocystis carinii, Cytomegalovirus, Mycobacterium avium intracellulari, cryptococcus or strongyloides isolated from the BAL. All data points and median values are shown.
In view of this a decision was taken to focus the analysis on BAL from patients in whom no respiratory pathogens were identified since the effect of any lung pathogens would be difficult to interpret in the context of co-infection with HIV. Although there were 41 patients in whom no BAL diagnosis was made, a number of these subjects had very high granulocyte percentages by FCM suggestive of a bacterial infection, despite failure to culture an organism. Seven patients had a BAL granulocyte percentage of greater than 40% and were therefore excluded, leaving 34 available for analysis.

The remaining 34 patients were categorized according to their blood CD4 count into three groups; those with a CD4 count of <100 cells/μl, those with 101-200 cells/μl and those with >201 cells/μl. There was a tendency for an increasing BAL lymphocytosis in those with lower blood CD4 counts (figure 5.8), although the differences between the BAL lymphocyte percentages in the highest and lowest CD4 groups did not reach statistical significance (p=0.06).

**Figure 5.8**

Comparison of BAL lymphocyte percentages in HIV+ patients without respiratory pathogens grouped according to their blood CD4 counts. Mean values and standard error of the mean are shown.
More interesting was a comparison between the CD4/CD8 ratios in blood and BAL for each of these CD4 categories. As expected, the CD4/CD8 ratios in both blood and BAL declined in parallel with a decline in the blood CD4 count. However, for each blood CD4 category, the CD4/CD8 ratio in BAL was lower than that in blood (figure 5.9). The differences between BAL and blood CD4/CD8 ratios reached statistical significance for those with a CD4 count <100 and 101-200 cells/μl (p=0.009 and 0.01 respectively), but not for the highest CD4 group (p=0.22).

![Figure 5.9](image)

**Figure 5.9**

Comparison of the CD4/CD8 ratios in blood (hatched bars) and BAL (black bars) in HIV+ patients without respiratory pathogens according to their blood CD4 counts. Means and standard error of the means are shown.

Taken together, the findings of a declining CD4/CD8 ratio, but an increasing lymphocytosis in BAL in patients with increasing immunodeficiency suggests that a BAL CD8 lymphocytosis may be a feature of advanced HIV disease.

In order to directly investigate whether this was the case, this data was also analysed by separately determining the percentage of CD8 and CD4 cells from the total CD3+ T cell pool in BAL and blood for each of the blood CD4 categories. CD4 lymphocytes accounted for fewer of the total number of T cells in BAL than blood for each of the three CD4 categories and the difference between these two sites was statistically significant.
for each category (figure 5.10). When this analysis was performed on CD8 lymphocytes, no statistically significant difference was detected in the proportion of CD8 lymphocytes between BAL and blood for each CD4 category (figure 5.11).

**Figure 5.10**

Box and whisker plots comparing the percentage of CD4 lymphocytes (figure 5.10) and CD8 lymphocytes (figure 5.11) from the total T cell compartment in BAL (black) and blood (red) according to blood CD4 categories in HIV+ patients without respiratory disease. The p values indicate the significance between the T cell subset percentages in BAL with those in blood for each CD4 category.

**Figures 5.10 and 5.11**

Box and whisker plots comparing the percentage of CD4 lymphocytes (figure 5.10) and CD8 lymphocytes (figure 5.11) from the total T cell compartment in BAL (black) and blood (red) according to blood CD4 categories in HIV+ patients without respiratory disease. The p values indicate the significance between the T cell subset percentages in BAL with those in blood for each CD4 category.
When the CD8 proportions in BAL alone were considered, there was a significant increase in this lymphocyte subset between those in the lowest and the highest blood CD4 categories (p=0.002). These findings confirm that there is a more profound CD4 lymphopoenia in BAL than blood and that a CD8 lymphocytosis is a characteristic feature of advanced HIV in both blood and BAL. Since the proportion of CD3+ events that were neither CD4+ nor CD8+ did not change with declining CD4 count (approximately 10% in BAL), it is reasonable to attribute the increasing total lymphocyte percentage in BAL in HIV+ subjects to a CD8 lymphocytosis. Nevertheless, CD3-lymphocytes such as natural killer cells and B cells could have contributed to the lymphocytosis and these were not measured here.

5.4 Analysis of leukocyte differentials in non-BAL fluids

Leukocyte differentials and CD4/CD8 ratios were performed by flow cytometry in tissue fluids other than BAL in several cases. The samples investigated included pleural fluid from five patients and ascitic and cerebrospinal fluid from one patient each.

Figure 5.12

FCM dotplots of leukocyte discrimination and lymphocyte T cell subset analysis in pleural fluid (a), ascitic fluid (b) and cerebrospinal fluid (c).
As demonstrated in figure 5.12, CD45 directed panleukogating allowed the discrimination of leukocytes from non-leukocytes and within the leukocyte gate lymphocytes were easily distinguished in all samples by their low side scatter. The discrimination of CD15+ granulocytes and CD4/CD8 T cell subsets was also achieved by FCM in the same manner as with BAL.

5.5 Discussion

This chapter has examined the BAL leukocyte differentials determined by flow cytometry in patients with sarcoidosis, tuberculosis and HIV. Although many previous studies have investigated these cellular features in similar patients using cytopin technology, the published data using FCM has been limited mainly to determining BAL CD4/CD8 ratios in sarcoidosis. The data presented here demonstrates broad agreement with previous cytopin studies. This finding, together with fact that FCM enables the rapid and precise enumeration of the CD4/CD8 ratios by FCM when compared to cumbersome immunofluorescent techniques required for cytopin preparations supports the conclusion of chapter 3 that FCM is the optimum technique for BAL leukocyte analysis. Moreover, preliminary data presented here has demonstrated that a simple CD45 directed gating strategy together with CD15 allows the differentiation of the leukocyte populations in tissue fluids other than BAL. Likewise, the CD4/CD8 ratios in these fluids can be readily determined by FCM exactly as in BAL.

Aside from this broad conclusion regarding the suitability of FCM for tissue fluid analysis, the results from this chapter have also stimulated a critical appraisal of the published literature on the diagnostic relevance of leukocyte differentials in different respiratory diseases.

Sarcoidosis is the most widely examined disease in terms of BAL leukocyte differentials. Many investigators have demonstrated that a BAL lymphocytosis and increased CD4/CD8 ratio are supportive of the diagnosis [2-6, 19]. However, others have questioned the diagnostic relevance of raised BAL CD4/CD8 ratios [20, 21]. One problem with the analysis of these BAL parameters is that sarcoidosis is an evolving disease and therefore patients presenting with early disease may have profoundly different BAL differentials than those with late pulmonary fibrosis. Therefore studies that have considered all stages of the disease together may be misleading. For example, Kantrow et al analysed the CD4/CD8 ratio in 86 patients with sarcoidosis and found that only 42% of these had BAL CD4/CD8 ratios >4 [20]. However, in their sample 46% had
stage 2 and 14% stage 3 sarcoidosis. These authors did not distinguish the T cell subset ratios with different stage of disease presentation. By contrast, the findings recorded in this thesis support those of other investigators that stage 1 disease is more likely to be associated with both a BAL lymphocytosis and a raised CD4/CD8 ratio [2, 4].

The conclusion, therefore is that in early sarcoidosis BAL is most likely to be a helpful diagnostic test. This finding is of significance since in stage 1 disease endobronchial and transbronchial biopsies are less likely to reveal characteristic non-caseating granulomata than with stage 2 or 3 disease [22, 23]. The data presented in this thesis supports these findings since none of the five patients with stage 1 sarcoidosis had diagnostic endobronchial or transbronchial biopsies and two of these went on to have mediastinal lymph node biopsies. The value of undertaking endobronchial and transbronchial biopsies may therefore be questioned in those with presumed stage 1 sarcoidosis in whom *Mycobacterium tuberculosis* culture and skin tests are negative and the BAL differentials are characteristic. In particular, it has been suggested that the risks of performing mediastinal lymph node biopsy outweigh the potential diagnostic benefits in this setting [24].

When patients with tuberculosis rather than sarcoidosis were examined, similar BAL features were noted as with previous published data. In particular, a predominant BAL lymphocytosis but with a CD4/CD8 ratio within the normal range has been described [8, 25]. The data presented in this thesis confirm that a BAL lymphocytosis is a common feature in tuberculosis. It has also been demonstrated here that a raised BAL lymphocyte percentage is a hallmark of non-pulmonary and disseminated TB.

When washings were performed from tuberculous cavities, the predominant cell types were often granulocytes with corresponding decreases in the lymphocyte percentages. Nevertheless, when washings were performed simultaneously from both radiologically unaffected and affected areas, a lymphocytosis was noted from the unaffected lung in all cases. This finding is contrary to that from previous investigators where lymphocyte percentages in washings from radiologically normal lung were similar to those seen in control subjects [9].

The demonstration of a BAL granulocytosis when washings were taken from tuberculous cavities as compared to a lymphocytosis from radiologically normal or non-cavitatory areas may reflect different outcomes in the battle between pathogen and host response. It has been known from both murine and human studies that T cell responses, in particular those involved in the production of type-1 cytokines such as IFN-γ and TNF-
α are crucial for the formation of protective granulomas and the control of infection [26-33]. In the light of these findings, the demonstration of large proportions of granulocytes and corresponding low percentages of lymphocytes from tuberculous cavities may represent failure of protective immune responses [34].

The data presented here on the BAL differentials from patients with both sarcoidosis and tuberculosis is largely in agreement with previously published data. Only a few healthy control subjects underwent bronchoscopy and cell differential analysis in this thesis. Two control subjects had relatively high BAL lymphocyte percentages (22.5 and 23%). Most large studies report the range of lymphocyte percentages to be between 3-18% in non-smoking subjects and 3-8% in smokers [11]. Nevertheless, wide fluctuations have been noted in the lymphocyte proportions in healthy subjects who had serial BAL's [18].

A large proportion of the BAL samples were obtained from HIV+ patients, reflecting the mix of patients seen at this institution. As expected, opportunistic infections with pathogens such as *Pneumocystis carinii*, *cytomegalovirus*, *Mycobacterium avium intracellulare* and *Cryptococcus* occurred at low CD4 counts whereas tuberculosis and other bacterial infections occurred with better preserved CD4 cell counts. The findings of a relative CD4 lymphopenia in BAL when compared to blood in patients in whom no pathogens were determined in the BAL is of interest. Several investigators have demonstrated that HIV is present in BAL [35-39]. The differences in CD4 lymphocyte percentages between lung and blood could reflect differences in the HIV viral load between the two sites. The CD4 lymphopenia may be a function of either direct HIV mediated cell death or alternatively of immune activated cell death. Some evidence from simian models exists that immune activation may be the most important factor in the local depletion of CD4 lymphocytes since macrophage tropic SIV viral strains predominated in the lung whilst lymphotropic strains were dominant in the blood [35]. The role of immune activation in HIV pathogenesis is explored further in chapter 6.

These studies into the leukocyte differentials and lymphocyte subset ratios in BAL demonstrate the difference between the lung and the blood in a variety of different disease states and provide tantalizing clues to the nature of disease pathogenesis. Nevertheless, such parameters are crude measurements of processes that are undoubtedly subtle and complex. What is clear, however is two points: first that the lung is the relevant investigative site in patients with respiratory disease and second that a
powerful and precise tool such as FCM will be instrumental in exploring the nature of these immune responses.

5.6 References


Chapter 6

Increased Proportions of Activated and Proliferating Memory CD8$^+$ T Lymphocytes in both Blood and Lung are Associated with Blood HIV Viral Load.
6.1 Introduction

In recent years there has been an intense debate about the nature of HIV pathogenesis. It has been argued that CD4 depletion is primarily a result of infection and subsequent cell death caused by HIV virions that homeostatic mechanisms are eventually unable to correct [1, 2]. Others have emphasised that whilst HIV does infect some CD4 lymphocytes, its primary mechanism of pathogenesis is by causing immune activation of both CD4 and CD8 cells resulting in apoptosis of this activated population [3-5]. Evidence in support of the latter theory has been provided by measuring the proliferation of cells in vivo using radioactive labels such as Bromodeoxyuridine (brdU) [5] or deuterated glucose [6]. These studies demonstrated reductions in not only CD4, but also CD8 cell proliferation in HIV-infected subjects when the HIV viral load was reduced by drug therapy. Further support for the importance of immune activation in HIV pathogenesis has been provided by the study of SIV in different Simian species. Sooty Mangabeys and African Green Monkeys, which are the natural hosts of SIV tolerate high SIV viral loads yet maintain relatively normal CD4 counts and live normal lifespans [7, 8]. By contrast, Macaques infected with SIV undergo a course of infection similar to that in humans with high viral loads resulting in CD4 cell depletion, illness and death. brDU labeling studies have demonstrated that the Sooty Mangabeys have low rates of both CD4 and CD8 cell turnover when compared to the Macaques [9], suggesting that they have developed mechanisms to avoid immune activated cell death.

Studies in patients infected with HIV have demonstrated that immune activation, determined by the expression of CD38 on CD8+ T lymphocytes is associated with disease progression [10-14] and that effective antiretroviral therapy results in a decline in the expression of this marker in parallel with the fall in HIV viral load [15]. These findings suggest that HIV drives immune activation, although some authors have stressed the role of additional infections that might contribute to HIV-induced activation [16].

One serious drawback of previous studies has been the exclusive examination of blood T lymphocytes. The lung was investigated in this study for two reasons: First, previous studies have demonstrated that the lung is a site of HIV replication [17-20] and therefore the investigation of this organ in addition to the blood may provide a more closely associated picture between viral replicative events and immune activation than the examination of blood alone. Second, the role of additional respiratory pathogens in stimulating immune activation could be assessed by comparison with HIV+ subjects in whom no BAL pathogen was identified.
In this study both the activation and proliferation of CD8+ memory T cells in lung and blood was investigated. Activation was determined by CD38 expression on CD8+ lymphocytes using a simple, reproducible gating strategy. Proliferation was measured in both the CD38bright and CD38dim CD8+ populations using Ki67, a nuclear marker associated with dividing cells [21, 22].

6.2 Methods

6.2.1 Patients
HIV-infected Patients undergoing bronchoscopy for suspected respiratory disease were invited to take part in the study that was approved by the hospital ethics committee. 35 HIV+ patients were tested and a control group of 5 healthy individuals was also examined. The HIV cohort mostly consisted of patients with advanced disease with only five patients on antiretroviral therapy. The median CD4 count of the HIV+ patients was 75 cells/μl (IQR: 11-265) and median HIV viral load was 164,000 copies/ml (IQR: 49,300-413,000) at the time of investigation. The demographic characteristics, CD4 counts, HIV viral loads and BAL diagnoses of the study population are depicted in table 6.1.

6.2.2 Determination of HIV Viral Load
HIV viral loads were determined by the automated amplicor polymerase chain reaction (Cobas Amplicor; Roche Diagnostics, Basel, Switzerland). The minimal level of detection was 50 HIV copies/ml and the upper limit 750,000 copies/ml. Viral load was not determined in BAL for the following reason. The procedure of BAL involves the instillation of large volumes of saline (typically 180-200ml) with the bronchoscope wedged into a subsegmental bronchus. The return of both fluid and cells is highly variable and dependant on operator technique, patient tolerability and the pathological state of the lungs. It is possible to control for these variable factors by measuring the concentration of standard metabolites such as urea that are found in both BAL and blood and adjusting the viral load measurements accordingly, but this was not performed here.

6.2.3 Standard Investigations for Respiratory Pathogens in BAL
All BAL samples from HIV+ individuals were investigated for the presence of respiratory viruses, including influenza, parainfluenza, adenovirus and respiratory syncitial virus
Table 6.1. Demographic, Immunological, Viral and Diagnostic Data of the HIV+ Study Population

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<th>Patient</th>
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Footnotes

1 Ethnicity: C=Caucasian, BA=black African, O=other.
2 HIV viral load copies/ml
3 PCP= pneumocystis carinii pneumonia
4 CMV=cytomegalovirus
(RSV). Cytomegalovirus direct antigen fluorescent foci (DEAFF) test and polymerase chain reaction (PCR) were performed in those with CD4 counts less than 100 cells/μl. Culture of BAL for bacteria, fungi and mycobacteria was undertaken and in addition, a stained specimen was examined by a cytopathologist for the presence of pneumocystis carinii, acid fast bacilli and fungal pathogens.

6.2.4 Bronchoscopy and Sample Preparation
Fibreoptic bronchoscopy was performed according to established methodologies as previously described in chapter 2. Bronchoalveolar lavage was performed from an area of radiologically abnormal lung, otherwise standard right middle lobe lavage was performed. The samples were kept on ice and then divided and aliquots sent to relevant laboratories for pathological investigations. The remaining BAL was kept for immunological analysis. The BAL samples were prepared as described in chapter 2. At the time of bronchoscopy 5ml of EDTA peripheral blood was also taken.

6.2.5 Flow Cytometry and Gating strategies
The proportions of lymphocytes, granulocytes and alveolar macrophages in BAL in addition to the CD4/CD8 ratios were determined as previously described in chapters 3 and 4. These procedures were carried out using a volumetric flow cytometer (cytoron absolute, Raritan, New Jersey, USA) that enabled the absolute counts of CD4 and CD8 lymphocytes to be determined.

An aliquot of BAL containing 1x10⁶ CD8⁺ lymphocytes and a sample of blood containing the same number of cells were then fixed and permeabilised as previously described in chapter 2. No lysis step was included as adequate decanting during the fixation and permeabilisation stage removed nearly all red cells. Following this procedure, the separate samples were stained at 4°C for 30 minutes, followed by a wash step. The following monoclonal antibodies were used in pre-titrated optimal concentrations in a single four-colour panel: Ki67 FITC (Immunotech, Marseilles, France), CD38 PE (Caltag Medsystems, Towcester, UK), CD8 PEcy7 (Caltag Medsystems) and CD45RA APC (Southern Biotechnology, clone sn130, Alabama, USA). Stained blood and BAL specimens were run on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, US). 20,000 CD8⁺ lymphocytes were acquired and the list mode data were analysed using Winlist 4.0 software (Verity inc. Topsham, Virginia, USA). Primary immunological gating of CD8⁺ lymphocytes was performed and
these events that were confirmed to lie within a lymphoid scatter gate. These CD8^+ cells were further investigated in terms of their naïve/memory phenotype by their CD45RA isoform expression (Figure 6.1).

**Figure 6.1**

FCM gating strategy for determining the activation and proliferation status of CD8^+ memory lymphocytes. Primary immunological gating of CD8^+ cells with low side scatter (R1) is performed. These events are then confirmed to fall within a tight lymphoid scatter gate (R2). Events fulfilling both R1 and R2 gating constraints are then confirmed to be CD45RA^- (R3). These CD45^- CD8^+ memory lymphocytes are then scrutinized in terms of their CD38 expression. CD38^{bright} (R4) and CD38^{dim} CD8^+ memory lymphocytes are finally analysed in terms of their expression of the proliferation marker, Ki67.
Since CD45RA\(^+\) memory CD8\(^+\) lymphocytes formed the vast majority of BAL lymphocytes with only a small percentage of CD45RA\(^-\) naïve/revertant cells, the degree of activation in the memory pool in both blood and BAL compartments using CD38 expression was investigated here. Decisions regarding placement of the gate to differentiate between CD38\(^+\) and CD38\(^-\) CD8 cells were determined from staining of control blood. The majority of CD8\(^+\) T cells in healthy individuals were CD38\(^{dim}\) with only a few activated cells, although Natural killer (NK) cells express this marker brightly. Therefore, a decision was taken to draw a gate to differentiate between the predominantly CD38\(^{dim}\) CD8\(^+\) lymphocytes and the CD38\(^{bright}\) NK cells. Using this method on blood from healthy controls that had been fixed and permeabilised, the gate was drawn at log 1.3 mean fluorescence intensity (MFI) CD38 expression (Figure 6.2). The proportions of CD38\(^{bright}\) and CD38\(^{dim}\) CD8\(^+\) cells in BAL and blood from the HIV\(^+\) study population and the normal controls was then determined by the same cut-off.

Lastly, the proportions of memory CD8\(^+\) lymphocytes that expressed the marker of cell proliferation, Ki67 were determined in the CD38\(^{bright}\) and CD38\(^{dim}\) subpopulations (Figure 6.1).

**Figure 6.2**

Demonstration of the CD38 gating strategy by flow cytometry. CD3\(^-\) CD8\(^{dim}\) natural killer (NK) cells (R3) form a CD38\(^{bright}\) population, whereas the CD3\(^+\), CD8\(^+\) T lymphocytes (R2) are predominantly CD38\(^{dim}\) in a healthy subject. The gate to differentiate between CD38\(^+\) and CD38\(^-\) is drawn at log 10\(^{1.3}\) mean fluorescence intensity (MFI) between the NK and the bulk of the CD8 T cell population.
6.2.6 Statistical Analysis
Median values and interquartile ranges were expressed in the text. Non-parametric analysis by the Mann-Whitney method was used to compare the data sets.

6.3 Results
6.3.1 Diagnoses in the HIV* patients with respiratory disease and BAL lymphocyte percentages
In 19 HIV* patients a respiratory pathogen was identified in BAL. The diagnoses were; nine culture confirmed tuberculosis (TB), seven pneumocystis carinii pneumonia (PCP), one bacterial pneumonia and one cytomegalovirus (CMV) infection. In addition, one patient had multiple infections with TB, PCP and CMV simultaneously. In 19 HIV* patients, no pathogens were identified in BAL. Three subjects in this group were excluded because FCM demonstrated a marked BAL neutrophilia suggestive of a bacterial lung infection despite failure to culture an organism. 16 HIV* patients were therefore included in this group. The BAL lymphocyte percentages were highly variable (table 6.1). When compared to the control subjects, BAL from the HIV subjects without respiratory disease contained a lymphocytosis (median 26.1% vs 10.2%, p=0.06). This BAL lymphocytosis was more marked in the patients with respiratory disease (median 40.1%, p=0.02 compared to control values).

6.3.2 CD45 Isoform Expression of CD8* T lymphocytes in BAL and blood in HIV* Patients and control subjects
BAL CD8* T lymphocytes from HIV* patients were overwhelmingly of a memory phenotype with a median of 97.5% CD45RA- (IQR: 96.1-97.9%). There was no significant difference in the proportion of CD45RA- phenotype between those patients with respiratory pathogens isolated in BAL (median 97.6%) and those in whom no pathogens were isolated (median 97.1%). In the control patients, slightly fewer (median 91.8% IQR: 86.4-97.2%) of BAL lymphocytes were CD45RA-. Therefore, BAL in both the HIV-infected patients and the control subjects contained predominantly memory CD8* T lymphocytes.

The expression of CD45RA+ does not delineate a naïve CD8 population, however, since some memory CD8 lymphocytes may switch from the CD45RA+/RO+ isoform to CD45RA+/RO-. These cells can be distinguished from true naïve cells by their lack of
CD27 expression [23]. In BAL approximately a quarter of the CD45RA⁺ CD8⁺ T lymphocytes in this HIV⁺ cohort also expressed CD27 and were therefore truly naïve (data shown in chapter 7).

Peripheral blood from the HIV⁺ patients comprised a median value of 56.3% (IQR: 48.5%-73.1%) of CD45RA⁻ CD8⁺ T lymphocytes. The corresponding value in blood from the control patients was 36.9% (IQR: 32.1-42.0%).

6.3.3 CD38 expression in CD45RA⁺ CD8⁺ lymphocytes from BAL and blood of HIV⁺ patients and control subjects

The proportion CD38⁺⁺⁺ CD8⁺ T lymphocytes were examined in both blood and BAL. The results were stratified according to the blood HIV viral load between the following categories: undetectable to 1000 copies/ml (low viral load group), 1000 to 100,000 copies/ml (medium viral load) and greater than 100,000 copies/ml (high viral load, figure 6.3). In the first viral load category, three patients had an undetectable HIV viral load with the assay limit of detection at 50 copies/ml. The remaining subjects had 400 and 506 HIV copies/ml. The upper limit of detection of the viral load assay was 750,000 copies/ml and four patients had unspecified HIV viral loads above this level. Higher percentages of CD38⁺⁺⁺ CD8⁺ T lymphocytes in both blood and BAL were associated with higher blood HIV viral loads (figure 6.3). For the patients in the lowest category of viral load data the median percentage of activated CD8 lymphocytes was 29.8% (IQR: 17.8-37.7%) in BAL and 24.1% in blood (IQR: 21.9-28.4%). In the medium HIV viral load category, the CD8⁺ lymphocytes were more activated in both blood and BAL compartments with median values of 42.4% (IQR: 35.9-63.8%) and 53.6% (IQR: 24.9-80.7%) respectively. Lastly, in the highest viral load category, the CD8 lymphocytes were most activated, with median values of CD38⁺⁺⁺ CD8⁺ T lymphocytes in BAL of 73.5% (IQR: 45.7-88.7%) and in blood of 74.6% (IQR: 64.8-87.1%). In the control subjects only a minority of BAL (median 4.5%, IQR: 3.2-5.7%) and blood (median 12.3%, IQR: 4.2-14%) of the CD8⁺ lymphocytes were activated. When the control patients were examined, the percentages of activated CD8 lymphocytes were much lower than in the HIV⁺ patients in both BAL (median 4.5% IQR: 3.2-5.7%) and blood (median 12.3% IQR: 4.2-14%).
6.3.4 CD38 expression in CD45RA+ CD8+ lymphocytes from BAL of HIV+ patients with and without Respiratory Pathogens

Since higher viral loads were associated with lower CD4 counts and increased rates of pathogens detected in the lung, the analysis was repeated but this time comparing the proportion of activated CD8+ lymphocytes for the same HIV viral load categories in BAL from HIV+ patients with respiratory disease and those in whom no pathogens were identified in BAL (figure 6.4). The aim for this analysis was to assess the relative contributions of respiratory pathogens and of HIV viral load in inducing CD8 cell activation.
In the patients in whom BAL revealed no pathogens, the median values for CD38bright CD8+ T lymphocytes in BAL were 29.8%, 50.4% and 68.2% for each of the increasing viral load categories. In the HIV+ patients with respiratory pathogens, the median values were 49.9% and 81.0% for the medium and high viral load categories, since no patients with respiratory pathogens had a viral load in the lowest range group. There was no significant difference in the percentage of activated CD8+ lymphocytes between the patients with respiratory disease and those without (p=0.5 in the highest viral load category). This data suggests that HIV viral load is the most significant factor in stimulating CD8 lymphocyte activation.

**Figure 6.4**

Comparison between CD38bright CD8+ T lymphocytes from BAL of HIV+ patients in whom no respiratory pathogens were identified (black) and those in whom respiratory pathogens were found in BAL (blue) according to HIV viral load categories. Median values are shown. There were no patients with respiratory disease in the lowest HIV viral load category.
5.3.5 Expression of Ki67 in activated and unactivated CD8⁺ lymphocytes in lung and blood

The relationship between CD38 activation and CD8 lymphocyte proliferation was next investigated by comparing the proportions of Ki67⁺ CD8⁺ cells in the CD38bright and CD38dim populations in both BAL and blood (figure 6.5). Activated, CD38bright CD8⁺ T lymphocytes were associated with higher percentages of Ki67⁺ cells in BAL (median 2.37, IQR: 1.65-3.98%) than in the CD38dim CD8⁺ lymphocytes (median 1.10%, IQR: 0.38-1.535). Increased percentages of Ki67⁺ CD8 lymphocytes in the activated cells were also documented in blood (median 1.48%, IQR: 0.77-3.03%) when compared to the unactivated cells (median 0.04%, IQR: 0-0.29%). These differences between the Ki67⁺ populations in the activated and unactivated CD8⁺ lymphocytes were highly significant for both compartments (p=<0.0001). In the control subjects, high proportions of Ki67⁺ CD8⁺ cells were noted amongst the rarer CD38bright population in BAL (median 7.68, IQR: 1.96%-11.5%), whilst in the predominant CD38dim component, Ki67 expression was very low (median 0.12%, IQR: 0-0.54%). Ki67⁺ CD8 cells in blood from the controls were very low both in the CD38bright (median 0.24%) and the CD38dim (median 0.01%) populations.

Figure 6.5

Box and whisker plots determining the percentage of Ki67⁺ CD8⁺ T lymphocytes in the CD38bright and CD38dim populations in both BAL (black) and blood (red).
6.4 Discussion

In this study the relationship between blood HIV viral load and the features of activation and proliferation of memory CD8+ T lymphocytes in both blood and lung was investigated. Early studies have noted that CD38 expression on CD8 lymphocytes was associated with accelerated HIV disease progression [10, 11, 24], a finding that was most clearly documented in the CD45RO+ memory CD8+ pool [12]. This observation can be explained in the light of the immune activation model of HIV pathogenesis by postulating that activated lymphocytes proliferate more rapidly and undergo apoptosis at a much faster rate than unactivated lymphocytes.

Whilst previous studies have demonstrated increased lymphocyte proliferation in HIV-infected humans and SIV-infected animals using Ki67 expression or radiolabelling, this is the first study to investigate directly the role that immune activation plays in cell proliferation in both the blood and a relevant tissue compartment, the lung. In this study it has been demonstrated that activated, CD38bright memory CD8+ cells have significantly higher rates of proliferation as measured by Ki67 expression than CD38dim CD8+ cells in both blood and lung. This finding, together with the demonstration that the CD8 activation status in both compartments was correlated with the blood HIV viral load is direct evidence for HIV in promoting increased proliferation.

Some authors have questioned the extent to which Ki67 expression accurately reflects cell proliferation [14]. This consideration has arisen due to the observation that nearly half of the Ki67+ CD4+ lymphocytes also expressed CTLA-4, a marker for activated cells arrested at the G1 stage of proliferation [25]. However, Ki67 may be a more reliable measure of cell proliferation for CD8 lymphocytes since only 10% of Ki67+ CD8 cells also co-expressed CTLA-4 [14].

The HIV viral load in the lung has not been measured here since the process of bronchoalveolar lavage introduces a highly variable dilution factor rendering quantitative HIV viral loads difficult to interpret. Nevertheless, our data supports the findings of previous investigators that the lung is a site of HIV replication [17-20].

Since this cohort included HIV-infected patients both with and without respiratory disease, it was possible to further investigate the relationship between HIV and CD8 lymphocyte activation and to consider whether co-infections with respiratory pathogens could play an important role in this process. No significant difference between the CD38bright CD8+ lung lymphocytes was documented between the patients with respiratory
pathogens and those without pathogens in each of the three HIV viral load categories. This finding is important additional evidence for the primary role of HIV in driving cellular activation. It cannot be concluded, however, that co-infections may not exacerbate immune activation, since in our cohort most patients had either TB or PCP. It is interesting to note that the patient in whom CMV was the only respiratory pathogen found did not have especially activated BAL CD8+ lymphocytes.

Lastly, this study raises the issue of whether the CD38 activation status of CD8+ lymphocytes could be used as a surrogate marker for HIV viral load that may be applicable in resource-poor settings. Whilst CD38^{bright} CD8 cells were significantly higher in both blood and BAL, for each viral load category there were several outliers suggesting that such a marker may not be reliable in routine clinical practice. Moreover, HIV-infected subjects from resource-poor settings may have increased activation status of their lymphocytes due to a variety of co-factors as previously demonstrated [16].

In summary, a simple, reliable gating strategy for determining the CD38 activation status of CD8^{*} lymphocytes has been developed here. Using such a system it has been shown that CD8 cell activation is related to the blood HIV viral load in both blood and an important tissue site, the lung. The primary role of HIV in stimulating this immune activation is strengthened by the demonstration that respiratory co-infections did not significantly increase the CD8 activation state of lung CD8^{*} lymphocytes when compared to those with matched HIV viral loads without respiratory pathogens. Lastly, it has also been documented that the activated, CD38^{bright} CD8 cells proliferate more than the CD38^{dim} cells. Taken together, these findings are consistent with the model that HIV drives cell activation and proliferation and that this may be the central mechanism of HIV pathogenesis.

6.5 References

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13. Liu Z, Cumberland WG, Hultin LE, *et al.* Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count,


Chapter 7

Memory Phenotype CD8+ T
Lymphocytes Including CD45RA+ CD27-
Revertants Accumulate in the Lung
7.1 Introduction

The pathways of differentiation of CD8+ T lymphocytes following their encounter with antigen are understood in some detail. It is well established that changes in the expression of the CD45RA/RO isoform is now no longer an adequate sole discriminating marker for differentiating naïve and memory CD8 lymphocytes. Several investigators have shown that the expression of the co-stimulatory molecules CD28 and CD27 can be used in conjunction with CD45RA to distinguish subpopulations of memory CD8 lymphocytes [1]. More recently, this analysis has been taken a step further by performing CD8 lymphocyte subset analysis on antigen-specific cells through the use of class-1 tetramers or peptide induced cytokine synthesis methods [2-6]. These studies have produced remarkable insights into the function of subpopulations of memory CD8 lymphocytes in terms of the ability of these cells to synthesise cytotoxic effector molecules such perforin or granzymes and cytokines such as IFN-γ.

However, the vast majority of studies in this field have concentrated on examining lymphocyte responses in blood, with a only few important studies directed towards the responses in lymph nodes [7-9] and other tissues [10]. Thus there is a paucity of information on the function and phenotype of CD8 lymphocytes at tissue sites such as the lung in humans. This is an important omission since activated lymphocyte populations are available for analysis from the lung by simple bronchoalveolar lavage. Indeed, the lung is a crucial primary site for encountering foreign antigen including viral infections and tuberculosis and thus the BAL samples might be expected to contain lymphocytes able to generate responses against various pathogens.

The aim of this chapter was therefore use to investigate CD8+ T lymphocyte subsets using the discriminatory markers CD27 and CD45RA in order to assess the relative accumulation of memory and naïve CD8 T lymphocytes in the lung.

7.2 Materials and Methods

7.2.1 Patients

Patients undergoing bronchoscopy for suspected non-malignant respiratory disease were invited to take part in the study, which was approved by the hospital ethics committee. The majority of patients investigated were HIV seropositive, reflecting the patient population of the respiratory team. A total of 46 patients were investigated, of whom 37 were HIV+. Four subjects had sarcoidosis and six were healthy controls.
without respiratory disease. The demographic characteristics, BAL diagnoses and CD4 counts of the study population are shown in table 7.1.

Table 7.1 Demographic and diagnostic data for patients undergoing CD8 phenotypic analysis in blood and BAL

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Footnotes
1: HIV viral load expressed as number of copies/μl in blood. Lower limit of detection 50 copies/μl and upper limit 750,000 copies/μl.
7.2.2 Bronchoscopy

Fibreoptic bronchoscopy was undertaken as previously described in chapter 2. Bronchoalveolar lavage was performed from an area of radiologically abnormal lung, otherwise a standard right middle lobe lavage was performed. The samples were divided and aliquots sent to relevant laboratories for pathological investigations, and the remaining BAL was kept for immunological analysis. At the time of bronchoscopy 5ml of blood was also taken.

7.2.3 Sample preparation

The BAL samples were collected into a siliconized glass container kept on ice and were analysed within two hours of their acquisition. Aliquots were sent to the relevant laboratories for pathological investigation and the remaining sample was filtered and washed and the absolute CD4 and CD8 counts determined as previously described using the CytoronAbsolute flow cytometer. A sample of BAL containing 1x10^6 CD8 lymphocytes and a sample of blood containing the same number of cells were then stained for 15 minutes at room temperature with the following monoclonal antibodies in pre-titrated optimal concentrations. CD27 FITC (Becton Dickinson), CD8 PE (Cymbus), CD3 PECy7 (Caltag Medsystems) and CD45RA (Southern Biotechnology, Alabama, USA). Following staining, 1ml of lysis buffer was added to the blood sample and left at room temperature for 15 minutes to ensure red cell lysis. Both the blood and BAL samples were then washed and the pellets resuspended in PBS. In further selected BAL samples, staining was performed with both CD45RA and CD45RO in order to confirm that CD8+ T lymphocytes did not co-express both markers. The antibodies used for these samples were: CD3 FITC (Becton Dickinson), CD45RO PE (Southern Biotechnology), CD8 PECy7 (Caltag) and CD45RA (Southern Biotechnology) and staining was performed as described above.

7.2.4 Flow Cytometry and Gating strategies

Phenotypic analysis of the CD8 populations was performed using the FACScalibur (Becton Dickinson). 20,000 CD8+ T lymphocytes were acquired and the list mode data analysed using Winlist 4.0 software (Verity inc. Topsham, Virginia, USA). Primary immunological gating of CD3+ lymphocytes was performed and these events were confirmed to lie within a lymphoid scatter gate. Defining CD8+ T lymphocytes by their co-
expression of CD3 and CD8 ensured that there was no contamination with natural killer (NK) cells that express CD8 dimly, but do not express CD3. CD8+ T cells were then gated from this population and then further investigated in terms of their naïve/memory phenotype by the expression of CD45RA and CD27 (figure 7.1).

Lastly, in the BAL samples in which both CD45 isoforms were stained for simultaneously, the expression of these markers was directly determined on CD8 lymphocytes (figure 7.2).

Figure 7.1

FCM dotplots demonstrating CD8 T lymphocyte naïve and memory subsets in BAL in a patient with HIV infection (a) and sarcoidosis (b). Memory CD8 lymphocytes comprise those cells in the bottom two quadrants and upper left quadrant. CD45RA+ CD27+ naïve CD8 T cells in the upper right quadrant are rare populations in BAL in both patients.
FCM dotplots demonstrating the expression of the CD45 isoforms CD45RA and CD45RO in CD8+ T lymphocytes in BAL. The CD45RA+ population of the CD8+ lineage is mainly represented by the CD45RA+,RO- cell type and not by the rare transitional forms that are CD45RO+,RA+ (upper right quadrant).

7.2.5 Statistical Analysis

Median values and interquartile ranges of the expression of the CD8 phenotypic markers were noted in the text. Non-parametric analysis by the Mann-Whitney method was used to compare the data sets.

7.3 Results

7.3.1 Comparison of the proportion of memory CD8+ T lymphocytes in the total CD8 T cell pool in BAL and blood.

Memory CD8+ T lymphocytes were defined as being either CD45RA- CD27+, CD45RA- CD27- or CD45RA+ and CD27-, the latter population being consistent with a revertant phenotype [11]. True naive CD8+ T cells were defined as cells that were both CD45RA+ and CD27+. In BAL CD8+ T lymphocytes from all subjects were overwhelmingly of a memory phenotype (median 99.5% IQR: 98.8-99.8%, figure 7.3). Peripheral blood from the same patients demonstrated a lower predominance of memory CD8 lymphocytes (median 88.0%, IQR: 78.8-93.7%). When compared to the lung, the difference in memory CD8 lymphocytes in the blood was highly significant (p=<0.001).
This data was then further examined to determine whether there was any difference in the memory CD8 lymphocyte proportions between those with HIV infection and those with sarcoidosis or the healthy control subjects.

Figure 7.3

Box and whisker plots demonstrating the percentages of CD8 T lymphocytes that expressed a memory phenotype for the whole study population in BAL and blood.

In the HIV-infected patients the median BAL CD8 memory proportion was 99.5% (IQR: 98.5-99.9%). In the Sarcoidosis patients the equivalent values were median 99.8%, (IQR: 99.6-99.8%) and for the healthy control subjects they were median 98.5% (IQR: 96.2-99.6%). The proportion of memory CD8+ T cells in BAL did not differ significantly between those with HIV and the healthy control subjects (p=0.23) or between those with HIV and sarcoidosis (p=0.16).

When the blood was compared between these three patient groups, there was a significant difference (p=0.02) between the CD8 memory proportion in those with HIV (median 90.2%, IQR: 83.0-93.9%) and the healthy controls (median 72.7%, IQR: 56.9-80.2%), but not between the HIV+ patients and those with sarcoidosis.
7.3.2 Expression of CD27 and CD45RA in CD8 lymphocyte subpopulations in the lung and blood

Next, the relative proportions of the four subpopulations of CD8 T lymphocytes described by the expression of CD45RA and CD27 were compared in the lung and the blood samples from all patients together (figure 7.4). These subpopulations were described as follows: CD45RA+ CD27+, CD45RA- CD27+, CD45RA- CD27- and CD45RA+ CD27-. Naïve CD8 lymphocytes characterized by the dual expression of CD45RA+ and CD27+ were a rare population in the lung, accounting for a median of 0.7% of all CD8 cells (IQR: 0.1-2.4%). By contrast, naïve cells were far more common in the blood (median 13.4%, IQR: 6.0-32.8%). The difference between the proportions of naïve CD8 cells in these two compartments was highly significant (p=<0.0001).

The second population of CD8 cells were CD45RA- and CD27+. In BAL these contributed to a highly variable proportion of the total CD8 pool but with a low median proportion (4.5%, IQR: 0.8-54.1%). In blood, these cells were more common (median 32.7%, IQR: 18.5-51.8%). The difference in the proportions of these CD8 cells between the two sites was significant (p=0.02). The third population were CD45RA- CD27- and these comprised the majority of the lung CD8 lymphocytes (median 92.6%, IQR: 42.5-97.6%). In the blood, the equivalent cells accounted for a much lower proportion of the total CD8 lymphocyte pool (median 22.7%, IQR: 11.0-40.8%). Again, the difference in the proportions of these CD8 populations between the lung and the blood was significant (p<0.0001).

The final population was that of the CD45RA+ CD27- CD8 cells. It has recently been determined that a proportion of memory CD8 lymphocytes switch their CD45 isoform from CD45R0+/RA- to CD45RO-/RA+. This population can be distinguished from true naïve CD8 lymphocytes since they have short telomeres, indicating a replicative history and do not express CD27 [11]. In BAL these cells were rare (median 2.2%, IQR: 0.9-5.3%), whilst in blood they were much more common (median 31.2%, IQR: 12.4-44.0%). The difference between the proportions of this memory pool in BAL and blood was again significant (p<0.0001).

These findings demonstrate that lung directed CD8 lymphocytes in BAL consist overwhelmingly of a memory phenotype that are CD45RA- and CD27-. The new observation is that we can find in the lung a smaller subset of CD45RA+ CD8+ cells that are also of memory type because of their CD27 negativity. These are known to be mature memory cells (figure 7.4).
Figure 7.4

Pie charts demonstrating the percentages of the CD8 T lymphocyte subpopulations defined by their expression of CD45RA and CD27 in BAL and blood for the whole study population.

7.3.3 Differences in CD8 lymphocyte subpopulations between patients with HIV, sarcoidosis and healthy control subjects

Next, the same CD8 lymphocyte subpopulations were examined in the three different patient groups. This analysis was performed since chronic antigenic stimulation in patients with untreated or inadequately treated HIV infection may result in skewed populations of lymphocytes with increases in the memory subsets. The majority of the
HIV+ patients in this cohort had advanced disease with low CD4 counts (median 85 cells/μl, IQR: 18-175) and high blood HIV viral loads (median 146,000 copies/μl, IQR: 13,800-413,000). Thus HIV itself could act to stimulate the proliferation and differentiation of lymphocytes in these patients.

CD45RA+ CD27+ naïve CD8 lymphocytes were rare populations in all three groups in BAL. In HIV+ subjects they accounted for a median of 0.6% (IQR: 0.1-2.0%), whilst in the sarcoidosis patients the proportions were very similar (median 0.2% (IQR: 0.1-0.5%). In BAL from the healthy control subjects, naïve lymphocytes comprised a slightly higher proportion of the total CD8 pool (median 1.4%, IQR: 0.3-5.4%). The difference between the naïve lymphocyte proportions in BAL between the HIV+ and control subjects and between the sarcoidosis and control subjects was not significant (p=0.24 and 0.11 respectively). In blood there was a significant difference (p=0.02) between the naïve CD8 population in the HIV+ subjects (median 11.6%, IQR: 5.6-29.3%) and the healthy controls (median 33.3%, IQR: 17.0-52.8%).

The next population to be compared was that of the CD45RA- CD27+ memory lymphocytes. In BAL from the HIV+ individuals this subset comprised a median of 3.1% of the CD8 cells with a very wide interquartile range (0.8-61.3%). In the control group a slightly higher proportion (median 14.3%, IQR: 1.1-27.9%) of the CD8 lymphocytes expressed these markers, but the difference between the two was not significant (p=0.76). In BAL from patients with sarcoidosis, similar low values of CD45RA- CD27+ CD8 cells were determined (median 6.8%, IQR: 3.9-9.1%). In blood, the proportions of this subset were very similar (p=0.27) in both the HIV+ subjects (median 33.3%, IQR: 18.8-47.9%) and in the controls (median 29.6%, IQR: 17.0-39.6%).

The third population to be compared between the different patient categories was that of the memory CD45RA- CD27- CD8 lymphocytes. In BAL, these cells comprised the predominant CD8 lymphoid population in the HIV+ patients (median 94.4%, IQR: 36.2-98.0%), with the corresponding proportions in the sarcoid patients being slightly lower (median 85.7%, IQR: 80.4-91.5%) and for the controls lower again (median 76.6%, IQR: 63.3-93.7%). The differences in the proportion of these memory lymphocytes between all three patient groups were not significant (p=0.79 HIV vs control, p=0.95 HIV vs sarcoid and p=0.61 sarcoid vs control). In the blood, the CD45RA- CD27- memory CD8 cells were higher in the HIV patients (median 24.7%, IQR: 14.0-41.7%) than in the healthy controls (median 8.7%, IQR: 4.7-23.2%) and this time the difference was statistically significant (p=0.02).
The final population was that of the CD45RA+ CD27- 'revertant' memory phenotype. In BAL from the HIV+ patients this subset accounted for very few of the total CD8 pool (median 1.9%, IQR: 0.7-4.2%), whilst in the control subjects (median 7.7%, IQR: 2.7-10.5%) and the sarcoidosis patients (median 7.3%, IQR: 4.1-11.0%) the equivalent cells were more common. The differences were significant between the HIV and control group (p=0.01) and the HIV and sarcoid group (p=0.008), but not between the controls and those with sarcoidosis (p=0.76). By contrast, the proportion of CD45RA+ CD27-memory CD8 lymphocytes in the blood of the HIV patients (median 30.4%, IQR: 13.1-
42.8%) and the control subjects (median 28.4%, IQR: 10.8-45.1%) was very similar (p=0.75). The differences in the proportions of these CD8 lymphocyte subsets in both BAL and blood from the HIV+ patients and the healthy controls are graphically represented in pie charts (figure 7.5).

**7.4 Discussion**

This study demonstrates that CD8 lymphocytes recovered from the alveolar space are overwhelmingly of a memory phenotype. Very few published reports have investigated the differentiation and migration patterns of lymphocytes to tissue sites such as the lung in humans [12, 13] or animal models [14, 15] and none of these have considered the CD8 lymphocyte subsets in the lung using an optimum combination of discriminatory markers such as CD45RA or RO and CD27.

Here it has been shown that this memory CD8 cell accumulation in the lung is predominantly of a mature effector phenotype in which the cells do not express the co-stimulatory marker CD27. These cells are CD45RA- and in a selective series of tests their CD45RO positivity has been documented. Only very few transitional CD45RA+ RO+ doubles were found in the lungs. Therefore the CD45RA- phenotype is synonymous with CD45RO positivity.

Several investigators have demonstrated that CD27- CD45RA- CD8 lymphocytes express high levels of the cytotoxic molecule perforin when compared to the CD27+ CD45RA- CD8 cells in blood [2, 5, 6] and it has therefore been suggested that the CD27+ CD45RA- phenotype may represent an intermediate stage between CD45RA+ CD27- and CD45RA- CD27- cells. It is of interest that despite the fact that low median values for the proportion of CD45RA- CD27+ CD8 cells were obtained for all patient groups, there were many patients in whom this population comprised a significant minority of the total BAL CD8 pool, particularly in the HIV+ population.

This finding is of interest since it has been established that HIV results in a failure of maturation of HIV-specific CD8 effector lymphocytes [3, 16, 17]. It would therefore be important to investigate the antigen specificity of the BAL CD8 lymphocytes in such patients to determine the relative proportions of HIV-specific cells within the CD27+ and CD27- pools.

A further significant finding in this study is the discovery of small percentages of CD45RA revertants among the CD27- memory population. This population of memory CD8 lymphocytes has been demonstrated to contain perforin and granzyme, to
synthesise both interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) [1] and also to express high quantities of the anti-apoptotic molecules Bcl-2 and Bcl-xL [11, 18]. Although initially thought to be terminally differentiated [19], it has recently been demonstrated that these cells can proliferate in response to antigens and show strong cytotoxic activity [4, 11, 18]. In view of these findings, this subset of CD8 lymphocytes has been termed an effector memory population and has been implicated in the control of chronic viral illness [20]. In the control subjects, these CD45RA+ revertants comprised 7.7% of the total BAL CD8 lymphocytes, whilst in the HIV+ subjects the same population accounted for only 1.9%. Failure of differentiation of CD8 lymphocytes in BAL to this mature effector phenotype may represent a specific defect induced by the HIV virus. It would be interesting to determine whether this defect is restricted to the HIV-specific CD8 lymphocytes, or whether it occurs across a broad range of different antigen-specific responses, a factor that would help to account for the markedly increased rates of respiratory infections in this population.

Nevertheless, these findings do indicate that the lung is equipped to maintain long-term immunity. Indeed, such specific responses to challenge are well documented in the fast stimulation induced by purified protein derivative (PPD) in the BAL of patients with tuberculosis (explored in chapter 8).

Lastly, it has been demonstrated here that naïve CD8+ T lymphocytes, characterized by their co-expression of CD45RA and CD27 are also found in BAL. This subset accounted for only approximately 1% of the total CD8 pool in BAL in all three patient groups, in contrast to the higher proportions found in the blood. A small percentage of these cells designated as memory lymphocytes may not be true CD45RA+ CD27+ lymphocytes since it has been demonstrated that approximately 10% of the CD45RA+ lymphocytes in BAL are transitional forms that co-express the CD45 isoform R0+. Nevertheless, the conclusion can still be made that naïve CD8 lymphocytes can be found in the alveolar space at low frequencies.

In summary, this study has demonstrated that a focused investigation of lymphocytes using good discriminatory markers reveals important differences between the lung and the blood, in direct conflict to the findings of previous investigators [21]. Here it has been shown that there is a preferential accumulation of memory CD8 lymphocytes in the lung, the majority of which displayed a mature effector phenotype characterized by the lack of expression of the co-stimulatory molecule CD27. Importantly, it has also been demonstrated that revertant effector memory CD8 cells are also detected in BAL and it is
suggested that these may play a vital role in the immune response against pathogens. Lastly, differences in the relative proportions of these lymphocytes have been shown between the HIV infected and normal control subjects. Specifically, the reduction in the proportion of revertant CD8 cells in BAL in addition to the demonstration of significant numbers of CD27+ memory cells may indicate a specific defect in the maturation of CD8 lymphocytes induced by HIV.

7.5 References


Chapter 8

Antigen-Specific Responses in the Lung in Patients with Pulmonary and Non-Pulmonary Tuberculosis
8.1 Introduction

It is estimated that one third of the world’s population is infected with *mycobacterium tuberculosis* and that there are eight million new cases of TB and nearly three million deaths each year [1]. The most common route of infection is through the inhalation of droplets carrying the mycobacterium. This results in a local lung immune response that generally contains the infection. However, re-infection, or reactivation may occur resulting predominantly in apical lung disease [2].

The gold standard diagnostic test for tuberculosis remains the visualisation of acid-alcohol fast bacilli (AFB) by Ziehl-Neelsen or auramine staining, with confirmatory culture of the organism. This can take up to eight weeks using solid culture medium. However, only 54% of all cases of TB and 61% of those with pulmonary TB were culture positive in 1999 in the UK [3]. Although the true figures for culture positive TB may be higher due to underreporting, there still remains a large proportion of TB diagnoses that are made on clinical grounds alone. More recently, DNA amplification techniques have been employed as a rapid diagnostic test in suspected TB cases [4-6]. However, the sensitivity of DNA amplification tests may be reduced in those with smear negative disease [7, 8].

An alternative diagnostic strategy is suggested by the discovery that antigen-specific cells can be identified by a variety of different techniques [9-11]. Nevertheless, these studies have so far been almost exclusively directed towards examining responses in the blood. We reasoned that in infectious lung diseases antigen-specific responses of lymphocytes recovered from BAL might prove to be clinically more relevant. We used flow cytometry (FCM) to detect CD4 lymphocyte cytokine production in response to PPD in short-term cultures of blood and BAL in patients with suspected TB.

8.2 Methods

8.2.1 Patients
The hospital ethics committee approved this study to obtain BAL and blood from patients with suspected or proven TB. Of the 60 patients included in this study, 34 were diagnosed with mycobacterium tuberculosis infection (TB). Of these, 29 were confirmed by culture (table 8.1), including from aspirates of bone (patient 1) and lymph node (patients 3 and 4). TB was not cultured from the remaining five patients, but acid fast bacilli were identified in caseating granulomas from tissue specimens in two patients (2
### Table 8.1 Demographic and diagnostic results in patients with TB

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<td>1.1</td>
<td>31.3</td>
<td>0.21</td>
</tr>
<tr>
<td>20</td>
<td>F 26</td>
<td>BA+</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
<td>46.8</td>
<td>5.9</td>
<td>21.8</td>
<td>0.02</td>
</tr>
<tr>
<td>21</td>
<td>F 27</td>
<td>BA+</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
<td>20.3</td>
<td>1.3</td>
<td>5.71</td>
<td>0.92</td>
</tr>
<tr>
<td>22</td>
<td>M 28</td>
<td>A+</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
<td>7.0</td>
<td>1.3</td>
<td>1.98</td>
<td>0.08</td>
</tr>
<tr>
<td>23</td>
<td>M 31</td>
<td>C+</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
<td>3.2</td>
<td>3.0</td>
<td>31.6</td>
<td>0.07</td>
</tr>
<tr>
<td>24</td>
<td>M 50</td>
<td>BUK-</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
<td>0.7</td>
<td>2.9</td>
<td>3.01</td>
<td>0.03</td>
</tr>
<tr>
<td>25</td>
<td>M 19</td>
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<td>Pulmonary TB</td>
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<td>28.4</td>
<td>0.26</td>
</tr>
<tr>
<td>26</td>
<td>F 25</td>
<td>A+</td>
<td>Pulmonary TB</td>
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<td>BA+</td>
<td>Pulmonary TB</td>
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<td>BA-</td>
<td>Pulmonary TB</td>
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<td>Pulmonary TB</td>
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<td>0.10</td>
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<tr>
<td>31</td>
<td>M 55</td>
<td>O-</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
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<td>5.2</td>
<td>3.51</td>
<td>0.06</td>
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<tr>
<td>32</td>
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<td>C+</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
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<td>1.0</td>
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</tr>
<tr>
<td>33</td>
<td>M 32</td>
<td>C-</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
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<td>34.0</td>
<td>0.02</td>
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<tr>
<td>34</td>
<td>M 37</td>
<td>O+</td>
<td>Pulmonary TB</td>
<td>AFB - PCR +</td>
<td>40.6</td>
<td>11.3</td>
<td>2.07</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Footnotes**

1. Ethnicity; BA= black African, BUK= black UK born, A=Asian, C= Caucasian, O=other. BCG vaccinated (+) or not (-).

2. Diagnosis of TB from BAL by smear (AFB), PCR and culture. In patients 1, 3 and 4 tissue culture confirmed TB. In patients 2 and 5 caseating granulomas and AFB were seen on biopsy.

3. Percentage of BAL CD4 lymphocytes synthesizing IFN-γ.

and 5, table 8.1). Three further subjects had clinical diagnoses of TB of whom two had resolution of chest radiographic abnormalities on therapy and the final patient had a
positive tuberculin skin test and cerebrospinal fluid changes supportive of tuberculosis meningitis. All of these five patients responded to anti-tuberculous therapy.

25 of the patients with tuberculosis had pulmonary disease, three had tuberculous lymphadenopathy, five disseminated disease and one each had spinal and pharyngeal TB. The patients with disseminated TB had predominantly cerebro-spinal disease (patients 8 and 9) and lymph node disease (patients 6 and 7). The remaining patient had classical miliary chest radiographic changes. All patients with TB with the exception of patient 9 were HIV tested and were seronegative.

The control group comprised of 26 patients with a variety of conditions requiring a bronchoscopy in which TB was considered in the differential diagnosis (table 8.2). Of these, eight were diagnosed with sarcoidosis. This diagnosis was made on the basis of computed tomograms (CT) of the chest demonstrating bilateral hilar lymphadenopathy in all cases, with or without lung parenchymal nodules in addition to a failure to culture TB. Supportive lung histology was present in five of the eight patients and additional diagnostic clues were provided by the BAL lymphocyte percentage and CD4/CD8 ratios, the serum angiotensin converting enzyme (SACE) levels and gallium scanning.

Seven patients presenting with respiratory symptoms were confirmed on culture to have mycobacterial infection other than TB (MOTT), including three with *M. fortuitum*, and two with *M. kansasii* and one each with *M. chelonei*, and *M avium intracellulare*. Five of these patients responded to appropriate anti-mycobacterial therapy. The patient with *M. avium intracellulare* (MAI) had previously been given a course of anti-mycobacterial drugs for culture proven MAI that had failed to sterilise the infection and she was not treated again and a patient with *M. Kansasi* was noted treated. Two of these patients were confirmed to be HIV negative and the others were not tested. The remaining 11 patients with non-tuberculous respiratory disease had a variety of different diagnoses (table 8.2). Two patients were confirmed to have cytomegalovirus (CMV) infection of whom one had chronic renal failure and the other was a bone marrow recipient. In four patients no diagnosis was determined from the bronchoscopy, but their symptoms resolved.

Patients 1,2,7,14,17,18,19 and 20, (table 8.2) were HIV negative and the remainder were not tested. These patients were generally older and felt not to be in a high HIV risk group. Moreover, absolute blood CD4 counts measured from these patients as part of the study methods were all greater than 500 cells/μl). In addition, blood was taken from 20 BCG-vaccinated control subjects who were nursing, medical and laboratory staff at
the Royal Free Hospital. Nine of this control group were females and the median age was 36 years.

Table 8.2 Demographic and diagnostic results of patients with non-tuberculous respiratory disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex and age</th>
<th>Ethnicity1</th>
<th>Diagnosis</th>
<th>BAL Lymph %</th>
<th>BAL CD4/CD8</th>
<th>BAL CD4</th>
<th>Blood CD4</th>
<th>IFN-γ</th>
<th>IFN-γ</th>
</tr>
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<tr>
<td>1</td>
<td>M 40</td>
<td>BA-</td>
<td>Sarcoidosis</td>
<td>68.4</td>
<td>9.4</td>
<td>15.2</td>
<td>0.08</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>M 33</td>
<td>BA+</td>
<td>Sarcoidosis</td>
<td>31.4</td>
<td>3.0</td>
<td>13.7</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F 35</td>
<td>C+</td>
<td>Sarcoidosis</td>
<td>26.8</td>
<td>2.0</td>
<td>13.9</td>
<td>0.01</td>
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<td></td>
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<td>Sarcoidosis</td>
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<td>5</td>
<td>F 41</td>
<td>C+</td>
<td>Sarcoidosis</td>
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<td>0.02</td>
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<td>M 33</td>
<td>C+</td>
<td>Sarcoidosis</td>
<td>76.0</td>
<td>43.4</td>
<td>0.14</td>
<td>-</td>
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<td>Sarcoidosis</td>
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<td>5.1</td>
<td>2.99</td>
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<td></td>
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<td>8</td>
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<td>C+</td>
<td>Sarcoidosis</td>
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<td>4.6</td>
<td>4.78</td>
<td>0.07</td>
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<td>9</td>
<td>F 56</td>
<td>C-</td>
<td>M fortuitum</td>
<td>48.1</td>
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<td>0.55</td>
<td>0.01</td>
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<td>M 38</td>
<td>A-</td>
<td>M fortuitum</td>
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<td>0.39</td>
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<td></td>
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<td>M 66</td>
<td>A-</td>
<td>M fortuitum</td>
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<td>8.21</td>
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<td>C-</td>
<td>MAI</td>
<td>34.5</td>
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<td>2.70</td>
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<tr>
<td>13</td>
<td>M 72</td>
<td>C-</td>
<td>M kansasii</td>
<td>3.3</td>
<td>21.5</td>
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<td>0.17</td>
<td></td>
<td></td>
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<tr>
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<td>M chelonei</td>
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<td>15</td>
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<td>C-</td>
<td>M kansasii</td>
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<td>2.87</td>
<td>0.05</td>
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<tr>
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<td>F 71</td>
<td>O-</td>
<td>Carcinoma</td>
<td>74.5</td>
<td>6.2</td>
<td>0.0</td>
<td>0.01</td>
<td></td>
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<td>17</td>
<td>M 26</td>
<td>A-</td>
<td>Cytomegalovirus</td>
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<td>1.12</td>
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<td>O-</td>
<td>Cytomegalovirus</td>
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<td>0.35</td>
<td>0.06</td>
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<td>2.7</td>
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<td>0.06</td>
<td></td>
<td></td>
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<tr>
<td>20</td>
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<td>C-</td>
<td>Lymphoma</td>
<td>21.4</td>
<td>1.7</td>
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<td>0.02</td>
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<td>C+</td>
<td>Bronchiectasis</td>
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<td>Lung fibrosis</td>
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<td>1.0</td>
<td>0.45</td>
<td>0.0</td>
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<td>23</td>
<td>F 66</td>
<td>A-</td>
<td>No diagnosis</td>
<td>17.9</td>
<td>1.7</td>
<td>0.16</td>
<td>0.02</td>
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<tr>
<td>24</td>
<td>F 71</td>
<td>O-</td>
<td>Carcinoma</td>
<td>74.5</td>
<td>6.2</td>
<td>0.0</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
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<td>A-</td>
<td>No diagnosis</td>
<td>3.2</td>
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<td>0.07</td>
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<tr>
<td>26</td>
<td>F 70</td>
<td>C-</td>
<td>No diagnosis</td>
<td>32.6</td>
<td>7.2</td>
<td>0.83</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Footnotes
1. Ethnicity and BCG status as defined in table 8.1 above.

8.2.2 Bronchoalveolar lavage

BAL was undertaken by standard technique as described in chapter 2. An area of radiologically affected lung was washed otherwise the right middle lobe was used. In five patients that already had a prior microbiological diagnosis of TB, bronchoscopies were performed within two weeks of commencing TB therapy. In the remaining cases, TB therapy was commenced following bronchoscopy.
8.2.3 Sample preparation

The fresh BAL samples were prepared as described in chapter 2 and the washed and filtered BAL was resuspended in culture medium before FCM analysis to assess the BAL leukocyte components, CD4/CD8 ratio and the absolute CD4 count as described previously.

8.2.4 PPD stimulation and FCM analysis

Aliquots of the BAL suspension containing $1 \times 10^8$ CD4+ lymphocytes in 1 ml of culture medium were placed into two sterile 5 ml polystyrene tubes (Thermo Life Sciences, UK). In addition, 1 ml of peripheral blood from the same patient collected into lithium heparin tubes was also placed into two polystyrene tubes. To one of the BAL and blood samples, 10 μg of PPD (Statens Serum Institute, Copenhagen, Denmark) was added. The other tubes were unstimulated control samples. In selected cases, 0.08 IU of tetanus toxoid (Pasteur Merieux, Lyon, France) was added to a third BAL sample as a control antigen. The samples were incubated for two hours at 37°C and 5% CO₂, after which time 5 μg of Brefeldin A (Epicentre Technologies, Cambridge, UK) was added and the samples incubated for a further 14 hours.

Following incubation, the samples were vortexed vigorously to detach cells from the walls of the tube. First, lymphocyte surface markers were stained using CD4-FITC (Royal Free Hospital) and CD3-PerCP (Becton Dickinson) for 15 minutes in the dark at room temperature followed by a wash step. Fixation and permeabilisation of the cells was performed as previously described using Fix-and-Perm (An-Der-Grub, Kaumberg, Austria) [12]. Following this, IFN-γ-PE (Caltag Laboratories, Towcester, UK) and TNF-α-APC (Becton Dickinson) were added and the samples stained at 4°C for 30 minutes, followed by a final wash step. Analysis of the stained preparations was performed on FACSCalibur (Becton Dickinson). 40,000 CD4+ events were acquired and the proportion of IFN-γ and TNF-α staining cells within the total lymphocyte and CD4+ T cell populations were analysed using WINMDI software (Version 4.2a; M Trotter) in both the PPD activated and control cultures. The responses attributable to specific PPD effects were calculated by subtracting the responses in the control tubes from those in the PPD activated tubes (figure 8.1).
8.2.5 Statistics

Median values and interquartile ranges were recorded in the text and non-parametric statistical analysis was performed with the Mann-Whitney test to determine any difference between data sets.

**Figure 8.1**

FCM dotplots demonstrating the proportion of CD3+ lymphocytes producing IFN-γ in response to PPD in BAL (a) and blood (d) from a patient with TB. The TNF-α response to PPD in BAL (b) and the IFN-γ response in BAL when no antigen is added (c) are also shown. BAL CD4 responses are high (upper left quadrants) with limited responses in the CD3+ CD4- CD8 cells (lower left quadrants). The responses in the BAL control (c) and blood sample (d) are low.
8.3 Results

8.3.1 Comparison of IFN-γ and TNF-α responses to PPD in BAL between TB-infected and uninfected individuals

The median percentage of BAL CD4 lymphocytes producing IFN-γ in response to PPD in patients with TB was high (25.2%; IQR 17.1-33.2%). Even higher BAL TNF-α CD4 responses were noted in these patients (median response 34.5%; IQR: 18.6-37.1%). FCM analysis determined that both cytokines were mostly produced by the same activated CD4 lymphocyte population, with the higher mean fluorescence intensity of TNFα expression indicating synthesis of more molecules of this cytokine in the antigen-stimulated CD4 lymphocytes than IFN-γ (figure 8.1). When compared to BAL, the most striking feature in the TB patients was the very low IFN-γ and TNF-α synthetic responses in the blood CD4 lymphocytes (median 0.11% and 0.22% respectively).

By contrast, the BAL CD4 IFN-γ production in patients with non-TB respiratory disease was low in most subjects (median 0.55%; IQR 0.31-2.99%; figure 8.2). The differences in the CD4 IFN-γ responses between the patients with TB and those with non-tuberculous respiratory disease were highly statistically significantly (p<0.0001).

![Figure 8.2](image)

**Figure 8.2**

Scatter plot demonstrating the percentage of BAL CD4 lymphocytes synthesising IFN-γ following incubation with PPD in the TB patients and those with sarcoidosis, mycobacterium other than TB (MOTT) and a heterogenous group with a variety of respiratory diagnoses other than TB (controls).
CD4 TNF-α responses to PPD in BAL were also low in the non-TB control patients (median 2.08% IQR: 0.72-4.07%). The BAL cytokine synthetic responses were low in the majority of patients regardless of whether they had been BCG-vaccinated (n=10) or not (n=15). However, when this group were examined more carefully, high type-1 cytokine responses were noted in several, but not all patients with sarcoidosis (figure 8.2). These were confirmed to be genuine PPD-specific responses since BAL from each of these patients was also stimulated with a control antigen, tetanus toxoid, which failed to elicit a response.

Moderate cytokine responses were also demonstrated in several of the patients with MOTT. Nevertheless, PPD stimulated lower IFN-γ and TNF-α synthesis in these patients than in the majority of the TB patients (p=0.0007).

8.3.3 Type-1 cytokine responses in PPD-stimulated CD4 lymphocytes in BAL in patients with pulmonary and non-pulmonary TB

Intriguingly, high CD4 IFN-γ and TNF-α responses to PPD in BAL were demonstrated in TB patients with non-pulmonary disease (median 25.4% and 41.7% respectively). The median values for the CD4 cytokine responses in the patients with pulmonary and non-pulmonary disease were virtually indistinguishable (figure 8.3).

![Figure 8.3](image-url)

**Figure 8.3**

Percentage of BAL CD4 lymphocytes synthesising IFN-γ following incubation with PPD in patients with pulmonary and non-pulmonary TB.
Importantly, every patient with non-pulmonary TB had high CD4 cytokine responses in BAL, suggesting that such a test may be of particular diagnostic benefit in this group.

8.3.4 Type-1 cytokine synthetic responses to PPD in BAL CD4 and CD8 lymphocytes in patients with TB

In the short incubation period of this assay, PPD was demonstrated to mainly activate CD4 lymphocytes (median IFN-γ 25.2%, IQR: 17.1-33.2%) and not CD8 T lymphocytes (median IFN-γ 4.09%, IQR: 1.57-7.25%). Similarly, the TNF-α responses were low in the CD8 lymphocytes (median 3.87%, IQR: 2.26-6.81%) when compared to the CD4 cells (median 34.5%, IQR: 18.6-37.1%). In this analysis, CD8 lymphocytes were identified as being CD3+ and CD4-. This approach to defining CD8 lymphocytes rather than primary immunological gating of CD8+ events was performed since it ensured that NK cells, that express CD8 dimly, but do not express CD3 were excluded. However, it is possible that γδ T lymphocytes that also express CD3 may have been erroneously included as responding CD8 lymphocyte in this analysis. The characterisation of CD4 lymphocyte cytokine synthesis was the primary objective of this study since it was expected that a complex antigen such as PPD would primarily activate CD4, rather than CD8 lymphocytes. The use of CD4 alone to determine CD4 cells was not sufficient as this marker was variably downregulated in culture (figure 2.7), whereas this was not a feature of CD3 staining. A more precise definition of CD4 and CD8 lymphocytes and their synthesised cytokines will be possible with five or more colour flow cytometry.

8.3.5 Persistence of type-1 cytokine synthetic responses to PPD in BAL following initiation of treatment for TB

The PPD-activated CD4 populations in the lung remained high in patients who had been on anti-tuberculous therapy for up to two weeks (median seven days) prior to BAL (patients 1,3,8,9, 13,19 and 23, table 1). In addition, steroid therapy did not significantly attenuate the response in patient 8 who was treated with corticosteroids for ten days before BAL. Two patients underwent a repeat BAL and PPD stimulation assay within two months of completion of their TB therapy (table 8.3). In one subject, the responses returned to low values, but in the other, they did not. More extensive investigation will be
required to determine the duration of persistence of type-1 cytokine responses following episodes of treated TB.

Table 8.3 BAL lymphocyte percentages and CD4 type-1 cytokine responses in two patients at diagnosis of TB and following completion of TB therapy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>BAL lymph %</th>
<th>BAL CD4 IFN-γ</th>
<th>BAL CD4 TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>At diagnosis</td>
<td>33.0</td>
<td>15.4</td>
<td>21.3</td>
</tr>
<tr>
<td>After TB therapy</td>
<td>35.7</td>
<td>0.17</td>
<td>0.3</td>
</tr>
<tr>
<td>At diagnosis</td>
<td>22.4</td>
<td>31.1</td>
<td>35.5</td>
</tr>
<tr>
<td>After TB therapy</td>
<td>28.1</td>
<td>25.9</td>
<td>21.8</td>
</tr>
</tbody>
</table>

8.3.6 Type-1 cytokine synthetic responses to PPD in BAL from radiologically normal and abnormal areas of lung in patients with TB

High cytokine responses to PPD were present throughout the lung and not just localised to radiologically abnormal areas in patients with TB. In patients with non-pulmonary TB, BAL was performed from the radiologically unaffected right middle lobe and in each case high PPD responses were noted (table 8.1). In addition, comparative washings from areas of radiologically affected and unaffected lung were performed on selected patients with pulmonary TB. In each case, the CD4 cytokine responses elicited in BAL taken from radiologically normal lung (usually the right middle lobe) were equivalent to, and in several cases, higher than the responses in BAL taken from radiologically affected areas (table 8.4). The conclusion from these findings is that powerful type-1 cytokine responses in patients with TB are pan-pulmonary and not specifically directed against areas of pathology such as cavitation defined by chest radiography.

8.3.7 Comparison of IFN-γ and TNF-α responses in the blood of TB patients with BCG-vaccinated controls

Finally, the low level CD4 responses to PPD detected in the blood of the patients with TB were compared with those in healthy, BCG-vaccinated control subjects and with non-BCG vaccinated subjects with respiratory disease other than TB (figure 8.4). Patients
with sarcoidosis and MOTT were excluded from this latter group control since the BAL analysis suggested that both of these conditions could result in a cytokine synthetic response to PPD.

Table 8.4 Comparison of IFN-\(\gamma\) synthesis following stimulation with PPD in BAL from radiologically affected and unaffected lung in patients with TB

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chest radiograph</th>
<th>% CD4 IFN-(\gamma) response from abnormal lung</th>
<th>% CD4 IFN-(\gamma) response from normal lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Upper zone changes</td>
<td>15.4</td>
<td>13.3</td>
</tr>
<tr>
<td>2</td>
<td>Upper zone changes</td>
<td>34.0</td>
<td>18.4</td>
</tr>
<tr>
<td>3</td>
<td>Upper zone cavity</td>
<td>31.6</td>
<td>56.2</td>
</tr>
<tr>
<td>4</td>
<td>Upper zone cavity</td>
<td>1.56</td>
<td>5.71</td>
</tr>
<tr>
<td>5</td>
<td>Mid/upper zone changes</td>
<td>28.4</td>
<td>32.9</td>
</tr>
<tr>
<td>6</td>
<td>Upper zone changes</td>
<td>21.1</td>
<td>25.2</td>
</tr>
<tr>
<td>7</td>
<td>Upper zone changes</td>
<td>42.5</td>
<td>26.7</td>
</tr>
<tr>
<td>8</td>
<td>Upper zone changes</td>
<td>51.6</td>
<td>55.9</td>
</tr>
</tbody>
</table>

Figure 8.4

Percentage of blood CD4 lymphocytes synthesising IFN-\(\gamma\) following incubation with PPD in patients with TB, BCG-vaccinated healthy controls and non-BCG vaccinated patients with respiratory disease other than TB, MOTT or sarcoidosis.
The median frequency of IFN-γ producing CD4+ T lymphocytes in response to PPD in the TB patients was only 0.11% (IQR: 0.04-0.38%). This value was lower than in the BCG vaccinated controls (median 0.14%; IQR: 0.08-0.27%). Similarly, the blood CD4 TNF-α responses in the TB patients were low (median 0.22, IQR: 0.10-0.57%) as were those from the BCG vaccinated controls (median 0.32%, IQR: 0.18-1.23%). The blood IFN-γ responses in the non-BCG vaccinated patients with respiratory disease other than TB were very low (median 0.02%, IQR: 0.001-0.03%).

8.4 Discussion

The demonstration of high CD4 IFN-γ and TNF-α synthetic responses to PPD in BAL when compared to the low proportions in peripheral blood is powerful evidence for the dominance of lung immune responses and for the active recruitment of TB-specific CD4+ T lymphocytes to the lung during TB infection. Previous human [13-15] and murine [16, 17] studies have also indicated that lung immune responses predominate during TB infection in different experimental systems. In two of these human studies [13, 14], T cells were separated from the BAL leukocytes and incubated with peripheral blood mononuclear cells (PBMC) and TB antigens, before investigating [³H] thymidine incorporation. These studies used complex experimental techniques due to concerns regarding the possible suppressive effects of alveolar macrophages and their unsuitability as antigen presenting cells (APC). The impressive antigen-specific CD4 lymphocyte cytokine responses demonstrated in our simplified system supports the findings of previous investigators that BAL contains effective APC [18].

The main conclusion of our paper is that the PPD activated CD4+ T cell type-1 cytokine response, measured by IFN-γ or TNF-α synthesis is a useful diagnostic test for active TB in HIV uninfected individuals. Importantly, high CD4 type-1 cytokine responses were present not only in those with pulmonary disease, but also in patients with non-pulmonary and disseminated TB in whom extra-pulmonary manifestations dominated the clinical picture. This finding has particularly significant diagnostic implications as BAL may be both an easier and safer procedure than the biopsy of other tissues in patients investigated for occult TB.

The other significant development inherent in this assay is that the results were available within 24 hours of acquisition of the BAL sample. It is relevant that in this TB cohort 63% of patients were smear negative, 46% both smear and PCR negative and in
14% *M. tuberculosis* was not cultured. The rapid results achieved by the immunodiagnostic test were therefore of considerable diagnostic interest.

The BAL type-1 cytokine synthetic responses following PPD stimulation in several patients with sarcoidosis is another significant finding and resurrects the fascinating and enduring question of the pathogenesis of this disease. The predominance of lung disease, together with the pathological findings of granulomas have long been held to be evidence that this disease may be a response to mycobacterial antigens [19]. However, attempts to convincingly document the presence of mycobacteria, or mycobacterial nucleic acid from tissue specimens of patients with sarcoidosis has resulted in conflicting evidence [20-23]. Here, it has been demonstrated that some, but not all patients with this disease have significant BAL CD4 responses following incubation with PPD, but not with a control antigen. Further investigation in this field is certainly warranted and it will be interesting to determine which specific mycobacterial antigens optimally stimulate these responses.

Several cases with TB had a low (<10%) BAL CD4 cytokine responses to PPD. It is interesting that all but one of these patients were both smear and PCR negative, and that TB was only confirmed by culture. Three patients were investigated on the basis of abnormal chest radiographs, but without any of the clinical features of TB such as weight loss, fever and prolonged cough. The remaining case with a low BAL CD4 cytokine response had advanced TB with cavitation (patient 24, table 8.1). The BAL from an apical cavity in this subject revealed pus with only 0.7% of the recovered cells being lymphocytes. These findings suggest some important limitations of this immunodiagnostic test. First, BAL CD4 responses may be low in patients with asymptomatic disease, prior to the recruitment of TB-specific lymphocytes to the site of disease and the initiation of the potent inflammatory responses. Second, that in advanced cavitatory TB, characterised by a neutrophilia, lymphocytes may be a scarce population. It is likely that the development of cavitation reflects a failure of the protective lymphocyteresponse to control mycobacterial proliferation. Nevertheless, this limitation may be circumvented as it has been demonstrated here that high CD4 cytokine responses to PPD are present in BAL taken from radiologically unaffected lung even in patients with cavitatory disease.

A corollary of these observations is that testing the CD4 responses to PPD in blood alone is inadequate to diagnose active TB. The blood lymphocyte responses from TB patients remained indistinguishable from the responses seen in BCG vaccinated control
subjects (figure 8.4). The most likely explanation for the low blood responses seen in the TB patients is that these antigen-specific lymphocytes are actively recruited to the site of infection in the lung resulting in their relative depletion in the blood. Furthermore, it has been demonstrated that BCG vaccinated subjects develop cytokine responses to PPD in an ELISPOT system [24]. Therefore, PPD which shares epitopes with both Mycobacterium tuberculosis and BCG is not a discriminating stimulatory antigen when measuring blood lymphocyte responses. Interestingly, BCG vaccination did not provoke a significant BAL cytokine response to PPD as no difference was demonstrated between the BCG vaccinated and unvaccinated control patients (table 1).

One could argue that TB infection and BCG vaccination can be better discriminated by using antigens specific for TB, such as the TB early secretory antigen, ESAT-6. Overlapping peptides of this antigen have been demonstrated to elicit IFN-γ responses in peripheral blood mononuclear cells (PBMC) in patients with TB, but not in BCG vaccinated subjects when measured by a sensitive ELISPOT technique [11]. However, this test did not distinguish between active and latent disease. Furthermore, the use of ESAT-6 does not solve the problem of impaired cytokine responses in blood from patients with severe TB [25, 26]. These pitfalls are avoided by examining lung immune responses where high cytokine responses by TB antigen-recruited cells in addition to a generalised lymphocytosis are supportive of active TB.

The flow cytometric assay used in our study has a considerable advantage over other methods to determine antigen-specific responses such as the ELISPOT system for three reasons. First, FCM provides a rapid and precise quantification of the total BAL lymphocyte percentage together with the proportions and absolute counts of the responding cell types, CD4 or CD8. Second, different cytokine responses, in our study IFN-γ and TNF-α can be investigated from the same activated lymphocytes. Third, the new generation of cheap red diode laser flow cytometers will render this FCM technology an accessible and affordable option [27].

From an immunological perspective this study highlights several issues. The importance of the type 1 cytokine axis in the control of TB has been documented in both murine experiments [17, 28, 29] and in patients with inherited defects in the interleukin-12 and IFN-γ receptors [30-32]. Other investigators using murine TB models have demonstrated that TNF-α is essential for the generation of protective granulomas, without which TB control is insufficient [29, 33]. Our study gives direct support for both IFN-γ and TNF-α in mediating anti-TB responses in the lung. It is intriguing that powerful
cytokine responses to PPD in BAL are generated in patients with non-pulmonary TB. These findings are suggestive of a lymphocyte recirculation pathway to the lung, presumably reflecting the fact that even in these patients the origin of post-primary disease was the lung.

Finally, the data presented here demonstrate the limitations of exclusively measuring CD4 lymphocyte responses. In patients co-infected with HIV, CD4 lymphopenia may limit the applicability of this CD4 IFN-γ test. In order to develop a reliable immunodiagnostic TB test in this important group of patients, antigen-specific CD8 responses to TB antigens will need to be explored in the lung. Furthermore, the specificity of this method for identifying active TB will also need to be confirmed by examining the responses to PPD in BAL from patients with treated disease.

In conclusion, a novel method for diagnosing TB by measuring intracellular cytokine responses to PPD in BAL by a simple and rapid flow cytometric technique has been described here. High responses were demonstrated in BAL from patients with both pulmonary and non-pulmonary TB. In patients with non-tuberculous respiratory disease, low responses were recorded except in some patients with sarcoidosis, perhaps reflecting a mycobacterial origin of this disease. This test therefore appears to be a promising diagnostic resource, particularly in those with non-pulmonary TB in whom achieving a culture diagnosis may be both difficult and hazardous.

8.5 References


Chapter 9

Discussion
9.1 Discussion

Amongst many researchers there is an idea that FCM is an 'aristocratic' technique. Its use, primarily by immunologists has been to develop ever more intricate ways of investigating the extraordinary complexity of immune responses, culminating in the impressive, but bewildering 11-colour, 13-parameter FCM analysis of lymphocytes [1]. Although FCM has the capability to determine not only cluster differentiation (CD) antigens on the surface of cells, it can also analyse intracellular antigens, such as cytokines, or nuclear markers of cellular proliferation to provide a highly complex analysis of cellular functions. Therefore, this technology has been widely perceived as being appropriate only for complex research applications, primarily in the field of immunology. This view is unjustified since it is the very precision of this technology that lends itself equally well to the simple differentiation of leukocytes as to more complex leukocyte phenotyping studies.

The precision of FCM stems from a number of factors. First, the development of highly specific monoclonal antibodies has enabled subsets of cells or other analytes to be labelled. Second the discovery of different fluorochromes with which the antibodies can be conjugated and which offer minimal spectral overlap following laser excitation has made differentiation feasible by FCM. Third, the fact that large numbers of events are analysed during flow cytometric acquisition of samples has ensured that statistical variability is minimised.

These benefits offered by flow cytometry have long been recognised as an important solution to the inherent limitations provided by manual counting of leukocyte differentials using a microscope [2]. At the present time, automated haematology analysers generally perform leukocyte differential counting using several techniques. However, there is a growing acceptance that FCM is an alternative technique that is precise and offers the advantage of being able to provide absolute cell counts. Recently, the acceptance of CD45 panleukogating in simplifying the protocols for leukocyte and lymphocyte differential analysis has been acknowledged [3, 4]. These developments have heralded a new era for FCM and widened its application from complex immunological research tool to that of a 'workhorse' capable of the routine evaluation of white blood cell differentials. New flow cytometers that work on volumetric sample acquisition and therefore provide absolute cell counts without the need for expensive bead systems are eminently suited to this task [5]. It is particularly exciting that a volumetric cytometer may be able to perform absolute cell counts, simple leukocyte
differentials and also to undertake multiplex analysis of samples to detect the presence of either antigens, or antibodies to a wide variety of different pathogens. This multiplexing facility offers enormous potential for the differential diagnosis of infectious disease [6] and is the sort of technology that could revolutionise laboratory diagnosis in both resource rich and poor settings.

Unfortunately, there has been a reluctance to adopt these exciting new technologies for the analysis of tissue fluid samples such as BAL. Although several investigators have demonstrated that FCM is better suited to performing CD4/CD8 differentials than the time consuming and cumbersome immunofluoresence or immunoperoxidase techniques [7, 8] , it has yet to be adopted as a routine diagnostic tool.

This study has therefore attempted to redress this imbalance by demonstrating that FCM is the appropriate technology for BAL analysis. The first problem was to demonstrate that BAL leukocyte differentials and lymphocyte subset ratios could be reliably performed by FCM. Although previous investigators had already recognised the value of CD45 directed gating [8, 9] , their interest was primarily in determining the lymphocyte component in BAL. Such a focus on the lymphocytes neglected the clinically relevant granulocyte populations in BAL and therefore did not provide adequate evidence of the benefits of FCM over conventional cytospin methods. Here, the value of CD45 panleukogating for distinguishing leukocytes from epithelial cells and debris in BAL has been confirmed. In addition, it has been demonstrated that BAL lymphocyte gating using a combination of CD45 expression and low side scatter is an adequate method for determining the lymphocyte percentages in BAL. When this method was compared with a lymphosum method that gated the individual lymphocyte components (T cells, B cells and NK cells), the results were virtually indistinguishable.

The more difficult problem was to distinguish macrophages from granulocytes. Rather than attempt to directly distinguish macrophages by FCM, a notoriously difficult project due to their autofluorescence, heterogenous intrinsic characterisitcs and the lack of an adequate surface marker, a different approach was adopted here. Characterisation of granulocytes using CD15 allowed the macrophage pool to be derived as those CD45+ events remaining after subtraction of the lymohocytes and granulocytes. When compared with cytospin preparations in which 500 BAL leukocytes were counted by a highly experienced cytologist, this flow cytometric method showed good agreement for determining the BAL leukocyte differentials. As expected, the coefficient of variation for differential analysis by FCM was considerably lower than by cytospin. The conclusion of
this initial study was that the use of only two monoclonal antibodies, CD45 and CD15 was adequate to demonstrate the major clinically relevant leukocyte populations in BAL by FCM. Additional discrimination of granulocytes into eosinophils and neutrophils was achieved by the addition of an antibody against the IgE receptor, CD23.

The value of BAL lymphocyte subset analysis, in particular the CD4/CD8 ratio has also been demonstrated to be of diagnostic value in diseases such as sarcoidosis by many investigators and therefore it was felt to be important to include such discrimination in a single four-colour panel together with CD45 and CD15. A simplified method by which lymphocytes were gated on the basis of CD45 and side scatter and then directly differentiated into CD4 and CD8 components offered no loss of precision when compared to a more sophisticated panel including the T cell marker, CD3. The final conclusion of this initial investigation is that a single four-colour panel combining CD45/CD15/CD4 and CD8 can rapidly provide most of the clinically relevant information required for the differential diagnosis of various respiratory diseases. Moreover, it has also been demonstrated that such a system is equally applicable for the analysis of other tissue fluid samples such as pleural fluid, cerebrospinal fluid and ascitic fluid.

One of the most significant findings of early studies investigating the lung as a site of pathological and diagnostic interest was the discovery that the responses demonstrated at the site of disease activity in the lung were not reflected by the responses demonstrated in the blood. This has held true not only for the simple lymphocyte differentials in patients with sarcoidosis, but also for the demonstration of cytokine responses in the lung in patients with TB [10] and sarcoidosis [11]. These findings have emphasised the importance of focusing on the immune responses at the site of infection, rather than in the blood. Such an approach has also been informative in patients presenting with respiratory illness in unusual circumstances. Here, an HIV-infected man was identified who developed a pneumonic illness following the institution of highly active antiretroviral therapy (HAART) after an episode of treated PCP. Analysis of the CD8 lymphocyte phenotype demonstrated the predominance of proliferating (Ki67+), perforin producing cells in BAL, that declined considerably following steroid therapy and were not seen in the blood [12]. In this case it was argued that the highly atypical proliferating CD8 lymphocytes may have represented an immune reconstitution disorder to *P. carinii* antigen after vigorous immune restoration on HAART. Nevertheless, although interesting and informative, studies such as these are not proof of the concept.
since the antigen-specific responses were not measured. The most informative investigations of tissue fluid analysis will therefore be those that focus on determining the pathogen-specific lymphocyte responses.

In the light of its clinical importance, together with an emphasis on pulmonary presentation, TB has emerged as the most obvious candidate for the investigation of tissue immune responses. Several investigators have sought to determine the TB-specific responses in the lung in both human infection and animal models, but these early studies have generally been hampered by complex techniques that are not appropriate for routine clinical investigation.

Several of the early studies used lymphocytes that were separated from BAL and then incubated with peripheral blood mononuclear cells (PBMC's) from the same patients before measuring proliferative responses to TB antigens [10, 13]. In these two studies the investigators presumably felt that BAL either did not contain adequate numbers of dendritic cells (DC) or that alveolar macrophages may suppress this process and so consequently PBMC's were used as a source of antigen presenting cells. Nevertheless, DC's have been identified in BAL and more importantly, these cells have been demonstrated to be increased following TB infection in both humans [14] and animals [15, 16]. Therefore, unmanipulated BAL from patients with TB should contain sufficient dendritic cells for antigen presentation. This assumption has opened the way for simple, rapid analysis of antigen-specific responses either using ELISPOT or FCM.

IFN-γ synthesis in BAL has been reported both in patients with TB [10], and also in their household contacts [17] in response to TB antigens when measured by ELISPOT. However, it has been argued here that ELISPOT is not the optimum method for determining antigen-specific responses in BAL since the proportion of lymphocytes and the CD4 and CD8 subset ratios are highly variable in the lung during infectious episodes. These facts are relevant because the TB antigens that are used in these assays, such as PPD, Ag85, and ESAT-6 are generally large and predominantly presented to CD4 lymphocytes. By contrast, CD8 responses may be preferentially stimulated by smaller peptides of 9 amino acids.

Therefore, flow cytometry, which can rapidly determine the cytokine synthetic responses specifically in either the CD4 or the CD8 lymphocyte subset is likely to be far more sensitive than the ELISPOT system. For example, in one paper in which ELISPOT was used to determine the IFN-γ secretion following incubation with PPD [10], the median number of spot forming colonies/10^5 BAL cells was 400 in a small subset of
patients with pulmonary TB, giving a response rate of 0.4%. In the study presented in this thesis, the median IFN-γ response rate in the BAL CD4 lymphocytes was 25.2% by flow cytometry. This huge discrepancy may explain why these authors did not find IFN-γ secretion in BAL taken from the unaffected lung in these patients in contrast to the data presented here.

Another important advantage of FCM over ELISPOT is that multiple cytokine responses can be determined in the same cognate T lymphocytes. The demonstration of greater TNF-α synthesis than IFN-γ in BAL CD4 cells from patients with TB is especially interesting. The analysis of multiple cytokine synthetic capabilities by antigen-specific cells may be particularly important in certain situations such as HIV infection, where there may be a relative defect in one of the responses. It would be interesting to note whether the increased presentation of non-pulmonary TB in HIV infected individuals is not only explained by CD4 lymphopenia, but also by a loss of TNF-α synthesis, which has been demonstrated to be vital for protective granuloma formation in animal models.

Although PPD was used as the stimulatory antigen in this thesis, it would be interesting to continue this work by examining the responses to other antigens that are specific for TB and that do not share epitopes with BCG, such as ESAT-6. Since it has been demonstrated here that prior BCG vaccination does not result in type-1 cytokine synthesis following incubation with PPD in the lung, in contrast to the findings in peripheral blood, it is unlikely that ESAT-6, or similar peptides will prove more sensitive for TB diagnosis in BAL. The problem with using specific peptides will be that the responses demonstrated will be almost certainly much smaller than with PPD. However, it will be extremely interesting to investigate which antigens stimulate the optimum cytokine responses in BAL from patients with sarcoidosis. The tantalising discovery of responses to PPD in these patients has opened the old debate as to whether sarcoidosis is indeed a response to mycobacterial antigens, although these may be environmental mycobacteria rather than *M. tuberculosis*.

This simple, rapid flow cytometric method is also applicable for the study of other clinically relevant antigen-specific responses in BAL. A significant CD4 IFN-γ synthetic response to cytomegalovirus (CMV) viral lysate was demonstrated in a bone marrow transplant patient who developed a febrile respiratory illness. Analysis of BAL confirmed the presence of CMV nucleic acid by PCR that was not present in the blood where the CD4 cytokine response was correspondingly low (submitted for publication).
In summary, the work presented in this thesis has demonstrated the value of a focused investigation of BAL and highlighted the role of flow cytometry in this process. FCM has been shown to be applicable both for the routine analysis of simple variables such as the leukocyte differentials and CD4/CD8 ratios in BAL as well as demonstrating the presence of antigen-specific responses to clinically relevant pathogens such as *M. tuberculosis*. These findings have therefore brought flow cytometry firmly into the realms of diagnostic investigation in the fields of respiratory medicine and infectious disease.

### 9.2 References


Glossary of Abreviations

AFB  acid fast bacillus
APC  antigen presenting cell
BAL  bronchoalveolar lavage
BMT  bone marrow transplantation
CD   cluster differentiation
CMV  cytomegalovirus
DEAFF direct early antigen fluorescent foci
DNA  deoxyribonucleic acid
EDTA ethylenediamine tetracetate
ELISA enzyme-linked immunoabsorbant assay
ESAT-6 early secretory antigen of tuberculosis-6
FCM  flow cytometry
HAART highly active antiretroviral therapy
HIV  human immunodeficiency virus
IFN-γ Interferon-γ
MFI  mean fluorescence intensity
MAI  mycobacterium avium intracellulare
MOTT mycobacterium other than TB
NK   natural killer cell
PBMC peripheral blood mononuclear cell
PBS  phosphate buffered saline
PCP  pneumocystis carinii pneumonia
PCR  polymerase chain reaction
PPD  purified protein derivative
RSV  respiratory syncital virus
SACE serum angiotensin converting enzyme
SEB  staphylococcal enterotoxin B
SIV  simian immunodeficiency virus
TB   tuberculosis
TNF-α tumour necrosis factor-α
ZN   Ziehl neelson
Appendix 1

Presentation of results of flow cytometric analysis of BAL leukocyte differentials and CD4/CD8 ratios given to clinicians

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London NW3 2PF
HIV Immunology Unit
Prof G Janossy, MD, PhD, FRCPath, DSc
RFH ex 3745

BAL Leukocyte Differential Analysis by FCM

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Lymphocytes (R2)= 83.8%
Granulocytes (R3)= 0.5%
Macrophages = 15.7%

CD4/CD8 ratio: 15.8

Conclusion
Massive lymphocytosis with greatly increased CD4/CD8 Ratio strongly supportive of sarcoidosis.

Dr S Barry
Appendix 2

Example of results of cytokine synthesis IN BAL following PPD stimulation in patients with suspected TB presented to clinicians.

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HIV Immunology Unit
Prof G Janossy, MD, PhD, FRCPath, DSc
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Diagnostic cytokine production assay in patients with suspected tuberculosis.

Conclusion: There is a very large BAL CD4 response to PPD This is supportive of active TB.

Dr S Barry Bleep 425