Mechanisms of Resolution of Inflammation in Paediatric Neutrophilic Lung Disease

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Submitted for the degree of MD (Res)

January 2015

For my family

"There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes."

George Bernard Shaw 1856-1950: *The Doctor's Dilemma 1906*

Abstract

Many paediatric airway diseases are characterised by persistent neutrophilic inflammation, which can lead to damage to the airways and lung parenchyma. One possible mechanism for the persistence of neutrophilic inflammation is failure of the normal active resolution of the inflammatory process. There is limited published literature on the role of inflammatory resolution in paediatric inflammatory lung disease. It is possible that targeting inflammatory resolution mechanisms and the ability to "switch off" inflammation may provide therapeutic targets in the future for these diseases.

This thesis investigates the hypotheses that failure of mechanisms terminating acute inflammation are important in the pathophysiology of infective and inflammatory lung disease; and that the differences in prognosis between childhood inflammatory lung diseases: cystic fibrosis (CF) (both established and newly diagnosed by newborn screening (CF NBS)), bronchiectasis, primary ciliary dyskinesia (PCD) and persistent bacterial bronchitis (PBB), are related to the ability to resolve inflammation in each disease.

A number of mechanisms and mediators important for inflammatory resolution are described in a cross-sectional study of bronchoalveolar lavage (BAL) and endobronchial biopsies (EBB): BAL CD25+FoxP3+ T regulatory cells by flow cytometry; annexin A1 (AnxA1) and its receptor ALX by RT-PCR of BAL and RT-PCR and immunofluorescent staining of EBB; the transcription factor Lung Krüppel-Like Factor by immunofluorescent staining of EBB; BAL lipid mediators by liquid chromatography – mass spectrometry and the lipid enzyme 15-lipoxygenase by immunofluorescent staining of EBB. Findings were related to underlying diagnosis, clinical status and airway inflammatory status (BAL neutrophils, CXCL8 and IL-10).

The main abnormality found was in the AnxA1 axis, where BAL AnxA1 mRNA levels were lower in neutrophilic lung disease as compared to controls. However this was related to disease severity rather than the CFTR defect. The ratio of BAL CXCL8: IL-10 was higher in CF as compared to other neutrophilic lung diseases and thus there was

evidence that the ability of IL-10 to resolve CXCL8 mediated inflammation was reduced in CF. Therefore there was some evidence for the importance of inflammatory resolution mechanisms studied in the paediatric neutrophilic airway, and limited evidence to suggest that the anti-inflammatory function of IL-10 is impaired in CF.

Acknowledgments

I am grateful to my three supervisors, Professor Clare Lloyd, Professor Andy Bush and Professor Jane Davies for giving me the opportunity to perform this research. They each provided support, enthusiasm and encouragement throughout.

Within Professor Lloyd's laboratory I was fortunate to work with a number of excellent researchers. In particular I wish to thank Sara Mathie and Lisa Gregory for general support and guidance, Jenna Murdoch and Carla Pereira Jones da Silva for flow cytometry supervision and Gaynor Campbell for assistance with RT-PCR analysis. I would also like to thank Martin Spitalier, Facility for Imaging by Light Microscopy, for his help with analysis of immunofluorescent staining of endobronchial biopsies.

In addition I was privileged to be able to work within Professor Mauro Perretti's laboratory, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, where they kindly provided the annexin A1 antibody and facilities for RT-PCR analysis. I am grateful to Jesmond Dalli and Karin Greco for their patience in teaching me how to perform RT-PCR.

I am also grateful to Professor Andy Bush, Professor Jane Davies, Dr Ian Balfour-Lynn, Dr Mark Rosenthal, Dr Sejal Saglani and Dr Clare Hogg for obtaining bronchoalveolar lavage and endobronchial biopsy samples for this research during the bronchoscopies they performed.

Particular thanks go to all the children and their families who agreed to take part in the clinical study. Finally I would like to express my gratitude to my family who supported me throughout, especially Isobel, without whom this thesis would have been written a whole lot sooner!

Declaration of originality

I confirm that all experimental work in this thesis was carried out by me, with the exception of:

Cutting of endobronchial biopsies was performed by Nikki Newman, Department of Gene Therapy.

Lipid analysis of bronchoalveolar lavage was performed by Professor Valerie O'Donnell's laboratory, Institute of Infection and Immunity, Cardiff University School of Medicine.

Luminex analysis of bronchoalveolar lavage cytokines was performed by Dr Rebecca Thursfield, National Heart and Lung Institute, Royal Brompton Hospital.

Sarah M N Brown
January 2015

Publications arising from the work in this thesis

Novel keto-phospholipids are generated by monocytes and macrophages, detected in cystic fibrosis, and activate peroxisome proliferator-activated receptor-gamma
Victoria J Hammond, Alwena H Morgan, Christopher P Thomas, Sarah Brown, Bruce A Freeman, Clare M Lloyd, Andrew Bush, Jane Davies, Anna-Lisa Levonen, Emilia Kansanen, Luis Villacorta-Perez, Y Eugene Chen, Francois J Schopfer, Valerie B O'Donnell. J Biol.Chem 2012; 287(50): 41651-41666

Abstracts

The inflammatory resolution protein annexin A1 and the paediatric CF airway
Brown SM, Dalli J, Perretti M, Bush A, Davies JC, Lloyd CM
Paediatric pulmonology ISSN:8755-6863; pages 265-266

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Abbreviations

AA	Arachidonic acid
AnxA1	Annexin A1
ALX	Annexin receptor
APC	Allophycocyanin
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CF NBS	Cystic fibrosis newborn screened
COX	Cycloxygenase
CRS	Chronic respiratory symptoms
CSLD	Chronic suppurative lung disease
CT	Computerised tomography
CCL	Chemokine (C-C motif) ligand
CXCL	Chemokine (C-X-C motif) ligand
CYP	Cytochrome P450
DC	Dendritic cell
eNaC	Epithelial sodium channel
EBB	Endobronchial biopsy
ELISA	Enzyme linked immunosorbant assay
ESR	Erythrocyte sedimentation rate
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate

FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Haematoxylin and eosin
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
ICS	Inhaled corticosteroid
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IQR	Interquartile range
IV	Intravenous
LC/MS/MS	Liquid chromatography / mass spectrometry
LKLF	Lung krüpple-like factor
LLD	Lower limit of detection
LOX	Lipoxygenase
LTB_4	Leukotriene B_4
LXA_4	Lipoxin A_4
MAPK	Mitogen activated protein kinases
MCC	Mucociliary clearance
NAC	N acetylycysteine
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
PA	Pseudomonas aeruginosa
PBB	Persistent bacterial bronchitis

PCD	Primary ciliary dyskinesia
PD_1	Protectin D1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PG	Prostaglandin
RIA	Radio immunosorbant assay
RT-PCR	Real-time polymerise chain reaction
RvD_1	Resolvin D1
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
Tregs	T regulatory cells
Tris EDTA	Trishydroxymethylaminomethane ethylenediaminetetraacetic acid
TxB_2	Thromboxane B_2

Chapter 1

Introduction: Inflammation and its resolution in neutrophilic airway diseases

1.1 Introduction

The classical acute inflammatory response of the airway to an infective or irritant stimulus is characterised by neutrophilic infiltration [1]. The inflammatory process serves primarily as a host defence mechanism; identifying and destroying invading organisms. There are at least three possible sequelae of acute inflammation. There may be complete resolution of inflammation such as is seen in acute lobar pneumonia. This may be considered as the "normal" response. Alternatively a chronic inflammatory infiltrate may develop, characterised by macrophagic and lymphocytic infiltration. This is seen in tuberculosis due to persistence of the infecting organism, and can also be considered a normal response. Finally, there may be continuous and unrelenting acute neutrophilic inflammation – a highly abnormal response which is found in many paediatric airway diseases [2], including cystic fibrosis (CF) [3], bronchiectasis [4], primary ciliary dyskinesia (PCD) [5] and persistent bacterial bronchitis (PBB) [6]. Persistent neutrophilic inflammation can lead to irreversible damage to the airways and lung parenchyma via release of the many cytotoxic neutrophil products, with subsequent decline in respiratory function and ultimately may result in respiratory failure. Paediatric airway diseases characterised by neutrophilic inflammation differ in their overall morbidity and mortality, with CF having the most severe phenotype. This may partly be due to their underlying pathophysiology, but may be related to an inability to resolve acute neutrophilic inflammation.

There are several possible proposed mechanisms for the persistence of acute neutrophilic inflammation. These include:

- An inherently pro-inflammatory airway epithelium
- An overwhelming and ongoing pro-inflammatory stimulus
- An excessive inflammatory response by the airway and the immune system to a given stimulus
- Failure of resolution of the acute inflammatory process

These mechanisms are not necessarily mutually exclusive and persistent neutrophilic inflammation may be multifactorial.

The mechanism by which persistent neutrophilic inflammation leads to airway damage was proposed by Cole in 1986, and termed the "vicious cycle hypothesis" [7]. Cole suggested that an initial event such as bacterial infection leads to compromised mucociliary clearance (MCC) and resulting airway infection. The invading pathogens themselves then cause inflammation, which further impairs MCC, leading to further bacterial infection and ongoing inflammation. Thus a vicious cycle is established leading to progressive lung damage and bronchiectasis (figure 1.1).

It had previously been suggested that the differences in prognosis and inflammatory status between paediatric inflammatory lung disorders were solely related to differences in neutrophil chemoattractant activity. However, this is now not thought to be the case. There is evidence that patients with PCD have higher levels of pro-inflammatory cytokines than patients with CF (a disease with a more severe clinical phenotype than PCD), and so clearly pro-inflammatory cytokine levels cannot exclusively be responsible for inflammatory differences between these conditions [8, 9]. This has led to the hypothesis that anti-inflammatory or pro-resolution mechanisms may be important in paediatric neutrophilic lung disease and targeting such mechanisms may potentially lead to new therapies. Proof of concept of the damaging effects of inflammation has been shown by seemingly paradoxical studies examining the role of anti-inflammatory therapies in patients with CF. Such treatments have included corticosteroids and the non-steroidal anti-inflammatory drug ibuprofen, reviewed in more detail later in this chapter. Although both these treatments have led to an improvement in the respiratory status of patients with CF, they are associated with an unacceptable level of side-effects, particularly in children [10-12]. This has therefore precluded their widespread use in clinical practice, at least in the UK. They have however generated interest in the possibility of targeting other anti-inflammatory mechanisms, including the host's ability to "switch off" inflammation. It may be that acute inflammation is beneficial early on by clearing infection, and may only be damaging at a later stage. The purpose of delineating these mechanisms would ultimately be to develop novel anti-inflammatory therapies with fewer adverse effects.

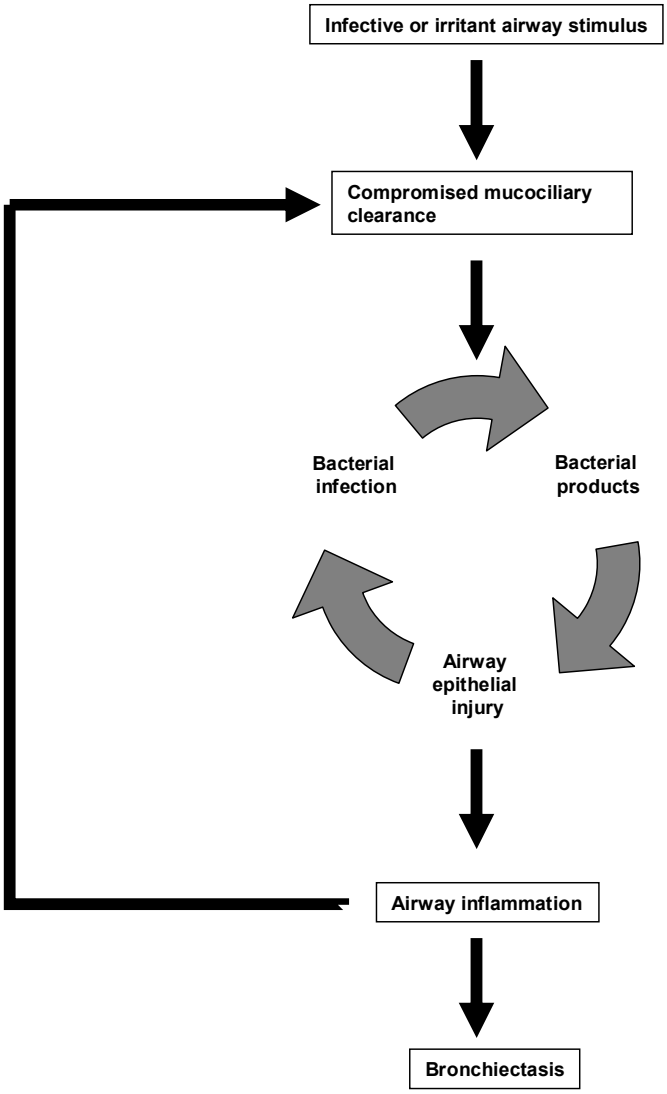


Figure 1.1: The vicious cycle hypothesis. Pathogens within the airway lead to progressive epithelial injury. This in turn leads to impaired airway defence and increased susceptibility to airway infection. Subsequent airway inflammation follows leading to airway damage and ultimately bronchiectasis. Inflammation within the airway also compromises mucociliary clearance, further impairing airway defence [7].

One of the most convincing arguments for this approach came from work examining levels of the anti-inflammatory lipid mediator lipoxin A_4 (LXA_4) [13]. LXA_4 has numerous effects leading to resolution of the acute inflammatory process. These include inhibition of neutrophil chemotaxis, suppression of neutrophil activation and pro-inflammatory cytokine production, and are discussed in more detail in section

1.11.5. Bronchoalveolar lavage (BAL) LXA_4 levels are lower in paediatric CF patients, as compared to children with other inflammatory lung conditions (including pneumonia, interstitial lung disease and reactive airways disease), despite similar BAL interleukin-8 (CXCL8):neutrophil ratios (CXCL8 being an important neutrophil chemoattractant). This suggests that paediatric CF patients may have an impaired ability to resolve neutrophilic inflammation due to lower levels of BAL LXA_4.

Overall while there is extensive literature on the role of pro-inflammatory mechanisms in inflammatory airway disease, the role of anti-inflammatory and pro-resolution mechanisms in the context of neutrophilic inflammatory airway disease is a less well researched area. The majority of published work involves research into CF rather than other neutrophilic lung diseases; and there is little research involving children.

The ideal method for studying the relationship of resolution of airway inflammation in the context of paediatric inflammatory airway disease would be by longitudinal natural history and preferably interventional studies. However, it is likely such studies would have to be invasive as young children are unable to expectorate sputum, thus requiring repeated non-clinically indicated bronchoscopic airway sampling which is ethically unacceptable. In addition at the moment there are insufficient data to perform an intervention study. This thesis therefore aims to investigate the role of resolution of inflammation in children with CF, PCD, bronchiectasis, and PBB compared with control patients (those with isolated upper airway disease, described further below) by the acquisition of BAL and endobronchial biopsies (EBB) from patients undergoing clinically indicated bronchoscopies. These samples provide a means of examining both the airway wall and lumen in patients with neutrophilic lung disease. The aim of this cross-sectional approach is to highlight potential differences between disease groups and control patients, and generate hypotheses for future testing, in particular with interventional studies. It may be difficult to show whether any differences detected are related to a primary defect or secondary to chronic mucus production and bacterial infection, irrespective of the underlying disease, so inevitably this thesis will be hypothesis generating rather than definitive. However, if significant differences are found between disease groups, these may be related to different effects on

inflammatory resolution. Conversely if no differences are seen, then it would be unlikely that any changes seen in the CF group are due to the underlying defective protein in these patients. In addition, with the advent of neonatal screening for CF, the opportunity has arisen to study the lungs of babies with CF before they become infected or develop clinical symptoms (although some have both at diagnosis). This may therefore help to tease out, in CF at least, whether any changes in inflammatory resolution are due to a primary or secondary defect. The next section reviews these paediatric neutrophilic pulmonary diseases in more detail.

1.2 Disease Groups

1.2.1 Cystic fibrosis

Cystic fibrosis (CF) is a multi-system disorder principally affecting the respiratory and gastrointestinal systems. It affects mostly, but not exclusively, the Caucasian population, with a carrier rate of 1:25 and incidence of 1:2500 live births in the UK. The CF gene has been localised to chromosome 7, and encodes for a protein termed CF transmembrane conductance regulator (CFTR). More than 1800 mutations have now been identified, although most have not yet been definitively confirmed as disease causing (see www.cftr2.org). The commonest disease causing genetic defect is Phe508del which accounts for 70% of mutations in the Caucasian CF population [14-16].

CFTR is a cyclic adenosine monophosphate (cAMP) regulated multi-functional protein which is known to be a chloride channel, but also inhibits the epithelial sodium channel (ENaC). The defect in CFTR leads to failure to secrete chloride across the epithelium and increased reabsorption of sodium and water. This causes dehydration of the epithelial surface resulting in viscous secretions, particularly in the airway. In turn this excess reabsorption of salt and water leads to ciliary collapse especially in the face of viral infection and thus impaired ciliary clearance of mucus and bacteria, referred to as the low volume hypothesis [17]. In addition, normal CFTR transports bicarbonate ions, impairment of which causes a reduced pH in the CF airway and impacts normal innate defence protein function. Impaired MCC and mucosal defence result in chronic bacterial infection, particularly with organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and persistent neutrophilic inflammation. Airway infection

with *Pseudomonas aeruginosa* is associated with a poorer prognosis [18, 19]. The disease almost inevitably progresses to bronchiectasis – characterised clinically by a persistent productive cough and radiologically by persistent dilated airways. Management is by a combination of daily physiotherapy to clear respiratory secretions, sometimes combined with mucolytic therapy such as hypertonic saline or rhDNase, optimal nutrition (most patients with CF also have impaired pancreatic exocrine function and therefore malabsorption), exercise and aggressive antibiotic treatment. Life expectancy for patients with CF has improved considerably over the past three decades, and the median life expectancy for a child born today with CF and diagnosed by newborn screening is predicted to be around 40-50 years [15, 20, 21].

1.2.2 CF Newborn Screening:

Newborn screening (NBS) for CF is now available throughout many parts of the world including the UK, with the aim of improving the prognosis of patients with CF through earlier diagnosis [22]. These patients provide an opportunity to assess disease progression in CF and to see how the ability to resolve inflammation may vary between those patients with established disease and those newly diagnosed at birth. The Royal Brompton perform flexible bronchoscopy on all NBS CF babies at around 3 months of age, primarily to look for pathogens (in particular *Pseudomonas aeruginosa*) which would otherwise potentially go undetected and remain untreated [23, 24]. This group of patients provides an opportunity to study the effects of the CFTR mutation and to establish whether babies born with CF have inherent defects in the way that they resolve inflammation, which are primarily due to the underlying genetic defect, or whether delayed resolution of inflammation is a secondary consequence of chronic airway mucus retention and bacterial infection. There is already evidence that pulmonary inflammation (manifested by increased BAL neutrophilia and CXCL8 as compared to controls) is present early in infancy in many children with CF, even in the absence of overt pulmonary disease [25, 26]. This suggests that a primary defect in inflammatory resolution may be important in the pathophysiology of CF. However, this also shows that the airways of CF NBS infants may already be infected with pathogens and so as a group they are not necessarily a true reflection of the CF airway prior to infection and inflammation. It may therefore be challenging teasing out

whether any differences seen in CF patients are primarily due to the CFTR defect. In addition, infants aged 3 months diagnosed by NBS have been found to have abnormal lung function with increased ventilation inhomogeneity, hyperinflation and diminished airway function as compared to healthy controls, despite optimal CF management [27]. Radiological evidence of structural lung disease in the absence of overt clinical disease in CF NBS infants has also been described [28]. Collectively these findings have emphasised the need for close surveillance of CF NBS infants, and have led to interest in interventional trials in these young children.

1.2.3 Primary ciliary dyskinesia

Primary ciliary dyskinesia (PCD) is an autosomal recessive condition in which there are defects in the structure, and function of cilia [29]. The motile cilia on the airway epithelium normally beat in a coordinated manner in order to propel surface mucus, inhaled particles and pathogens towards the upper airway and provide a means of airway clearance. This process is impaired in PCD and leads to reduced MCC. Chronic rhinitis, recurrent otitis media, neonatal respiratory distress and dextrocardia or situs inversus are also phenotypic markers of the disease [30-33].

PCD shares a number of characteristics with CF. Both conditions lead to impaired MCC, although via different underlying mechanisms. As a result in both conditions there are retained airway secretions, chronic bacterial infection and neutrophilic inflammation. Airway bacterial infection in PCD involves similar pathogens to CF including *Pseudomonas aeruginosa* – although the acquisition of these pathogens is later in life as compared to CF patients [33]. Management of PCD includes physiotherapy and aggressive antibiotic use and the disease process leads to lung damage and bronchiectasis [34]. However, the prognosis for patients with PCD is better than those with CF, provided aggressive treatment is adhered to in order to preserve pulmonary function [35]. This is despite the fact that the majority of patients with PCD are often diagnosed late when the airway epithelium is already damaged and there has been a decline in pulmonary status [30]. As compared to CF, there is less research into airway inflammation and pathophysiology in PCD, and limited research involving children.

A number of studies have examined the inflammatory profile of the PCD airway in comparison to the CF airway, although varying results have been described. As briefly discussed above, sputum CXCL8 has been shown to be higher in paediatric PCD patients as compared to matched CF controls, which is surprising given the worse prognosis in CF. In addition some CF patients in this study had a respiratory exacerbation at the time the sputum was collected, which makes the findings even more significant [9]. Other work has shown that there are multiple neutrophil chemoattractant factors in CF and CXCL8 may not be the most important one [8]. It is possible that sputum CXCL8 levels in CF patients are affected by other factors, such as binding to DNA and actin, which are plentiful in CF sputum [36]. It is also feasible that raised CXCL8 levels are merely a marker of neutrophilia, and are not of pathophysiological significance. Furthermore these findings imply that strategies to reduce sputum CXCL8 levels as a therapeutic measure may not provide clinical benefit.

A study looking at the spontaneously expectorated sputum from CF and PCD paediatric patients also found that the differences in prognosis between these two groups were unrelated to levels of neutrophil chemoattractants; with levels similar in the two groups, or slightly reduced in CF patients [8]. Again most CF patients, but no PCD patients, in this study were undergoing an acute exacerbation which may have confounded the results. However, this would suggest that the CF patients would have had worse inflammation, which makes the finding that the neutrophil chemoattractant levels were the same in both CF and PCD patients even more striking. Many of the CF patients, but not PCD patients, were also prescribed azithromycin and recombinant human DNase (rhDNase). There have been mixed reports on the effects of rhDNase on CXCL8, although a large study in children prescribed 3 months of rhDNase did not show any changes in sputum CXCL8 [37]. In addition the effects of azithromycin on pro-inflammatory markers and mediators continues to be debated – and is discussed further in section 1.6.3. In one study comparing CF and PCD patients, increased sputum CXCL8 levels were seen in CF patients prescribed azithromycin, although this may have been a self-selecting group with more severe disease [8].

The inflammatory profile of exhaled breath condensates of children with PCD has also been examined. The concentration of 8-isoprostane (a marker of oxidative stress) was significantly increased although there were no differences between the two groups as regards levels of leukotriene B_4 (LTB_4), and CXCL8 was undetectable. No correlation was found between sputum neutrophils or forced expiratory volume in 1 second (FEV_1) and LTB_4 or 8-isoprostane [38].

In summary, the reasons for the difference in prognosis between CF and PCD are likely to be linked to the underlying differences in pathophysiology. It would appear that levels of proinflammatory cytokines and neutrophil chemoattractants are not solely responsible for any differences seen in disease severity or prognosis. Alternative mechanisms may be important and this may include the differing ability to resolve pulmonary neutrophilic inflammation.

1.2.4 Non-CF, non-PCD Bronchiectasis

These children by definition have radiological evidence of bronchiectasis, but not CF or PCD. Worldwide, (in adults and children combined) non-CF bronchiectasis is more prevalent than CF-bronchiectasis. Within the United States alone there are estimated to be 110,000 people with non-CF bronchiectasis, as compared to 30,000 with CF [39]. The incidence and prevalence also increases with age [39, 40]. Despite this, there is limited research into this condition, particularly in children. Diagnosis is often delayed, especially in developing countries and indigenous populations. Within these groups (for example Australian Aboriginal infants [41], New Zealand Maoris [42] and indigenous children from South-West Alaska [43]) bronchiectasis is a significant cause of morbidity and mortality. This may be due to differences in the underlying aetiology of bronchiectasis, nutritional status, frequency of exacerbations, immunisation status and access to healthcare.

Often the underlying cause is unknown despite extensive investigation. Conditions that may lead to bronchiectasis include previous persistent or severe lung infection, tuberculosis, measles, *Bordatella pertussis*, inhaled foreign body, immunodeficiency, gastro-oesophageal reflux disease and aspiration [44].

The main clinical finding is of a persistent wet cough. Radiologically and pathologically bronchiectasis is defined by persistent airway dilatation. In addition there is also some evidence that bronchiectasis (or strictly speaking, airway dilatation) may reverse [45].

The principles of management of non-CF, non-PCD bronchiectasis are the same as in CF or PCD (once a potential remediable underlying cause has been ruled out). With appropriate and aggressive management, life expectancy may be expected to be normal and lung function preserved in most patients within Western countries [46, 47]. Indeed there is some evidence that in developed countries the incidence of bronchiectasis may be falling, possibly because of increased antibiotic use and aggressive treatment [47]. However, the ability to diagnose bronchiectasis has changed over the years, particularly with the advent of high resolution computed tomography (CT) scans and it is therefore difficult to be certain about true changes in epidemiology.

The underlying mechanisms that lead to bronchiectasis from an initial airway insult are not well understood. There may be intrinsic abnormalities of the innate and adaptive immune systems that predispose individuals to airway damage. The vicious cycle hypothesis proposed by Cole (discussed earlier) remains the best explanation to date [7]. MCC can subsequently be impaired by bacteria via a variety of mechanisms. Some bacteria, including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*, release mediators which inhibit ciliary function and damage ciliated epithelium [48]. Similar to patients with CF and PCD, *Pseudomonas aeruginosa* infection is associated with increased age, a more severe phenotype and worse prognosis [49, 50].

In a similar manner to CF, neutrophils invade the airway in these patients in response to pro-inflammatory cytokines (including CXCL8) and other chemoattractants, and release mediators including neutrophil elastase, matrix metalloproteinase and reactive oxygen species. These mediators damage the airway by digesting airway elastin and basement membrane collagen and proteoglycan, thus weakening bronchial wall structure and leading to bronchial dilatation [51].

In addition, a lymphocytic and macrophagic infiltrate is seen in the airway submucosa in patients with bronchiectasis [52]. Macrophages contribute to neutrophil influx and also release proteases. Lymphocytes are associated with increased immunoglobulin production and the formation of immune complexes.

To date, the role of inflammatory pro-resolution mechanisms and mediators has not been studied in children with bronchiectasis.

1.2.5 Persistent bacterial bronchitis (PBB)

Persistent bacterial bronchitis (PBB) is a diagnosis of exclusion in a young child with a continuous wet cough of at least 4 weeks duration, in whom after full investigation there is no alternative diagnosis such as CF, and no evidence of bronchiectasis. These children have bacterial infection, particularly with *Haemophilus influenzae* and *Moraxella catarrhalis*, and BAL neutrophilia [53, 54]. Management involves the administration of prolonged courses of oral antibiotics – sometimes for several months, in order to clear the underlying infection [55]. Life expectancy in children with PBB who are appropriately managed is almost certainly normal, although there are no long term follow up studies [56, 57]. In an otherwise healthy child it is often unclear why bacterial infection has persisted for so long and there is little published research in this area. Indeed, the diagnostic criteria for PBB are controversial, and a wet cough and PBB can co-exist with other conditions such as aspiration. There are a number of alternative names for PBB in the literature, including pre-bronchiectasis. This implies that eventually untreated PBB may progress to bronchiectasis, which highlights the potential for any chronic bacterial infection to lead to lung damage; however, this pathway remains hypothetical at the moment. To date there have been few studies looking at levels of pro or anti-inflammatory cytokines or airway pathophysiology in these patients. Circulating peripheral blood natural killer (NK) cell numbers are higher in children with PBB as compared to those with upper airway disease [54]. However, these findings may not be representative of the airway in these children. In this cohort, peripheral NK cell levels were particularly high in those with adenovirus detected by PCR in BAL. Although these children may not have had active infection at the time of

bronchoscopy, raised NK cell levels are likely to be representative of recent viral infection [54]. Children with PBB have higher levels of BAL CXCL8 and matrix metalloproteinase 9 as compared to controls both with and without other causes of coughing. These patients also have higher levels of BAL Toll-like receptor (TLR)-2 and TLR-4 mRNA (TLRs are fundamental components of the innate immune system), a possible indicator of innate immune activation [58]. It is unclear as to whether children with PBB have differences in their ability to resolve pulmonary neutrophilic inflammation as compared to, for example, those with CF - despite the ongoing bacterial load in the lower airways. However, children with PBB are a group with a much better prognosis than CF, despite airway neutrophilia, and are thus a useful comparator.

The next section reviews current knowledge regarding neutrophilic inflammation in CF (the most serious childhood airway neutrophilic disease) and the concept of compartmentalisation of inflammation in the airway wall and lumen.

1.3 Neutrophilic inflammation in CF

The majority of research into neutrophilic airway diseases involves CF patients. Aside from potential defects in inflammatory resolution pathways, there are a number of hypotheses as to why there is airway neutrophilia in CF.

1.3.1 Defects in neutrophil function

Neutrophils in CF patients may have inherent defects in their function – given that even in the presence of large numbers of neutrophils, there is persistent bacterial infection. Such defects include decreased shedding of L-selectin (a carbohydrate binding molecule -selectin shedding is required to regulate neutrophil rolling and recruitment) altered Cl^- levels, altered pH levels, increased myeloperoxidase activity, increased TLR4 and CXCL8 expression as well as an impaired response to the anti-inflammatory cytokine IL-10 [59-63]. Neutrophil expression of CD11b, an integrin required for neutrophil adhesion, has been shown to be reduced following a course of intravenous antibiotics for pulmonary exacerbations in CF [64].

1.3.2 The role of CFTR in neutrophil function

The question has arisen as to whether altered neutrophilic function seen in CF is due to abnormal CFTR function. Experimental work has shown an increase in inflammatory responses by airway cells treated with a CFTR inhibitor. Likewise a reduction in inflammation can be seen when CF epithelial cells are treated with a CFTR corrector [65]. CFTR is expressed in neutrophils at both an mRNA and protein level [60]. However, as levels of CFTR are low in neutrophils, abnormalities may not be due to a primary CFTR defect, and may be secondary to mucus production and overwhelming bacterial infection. Although evidence directly linking CFTR mutations with defects in neutrophilic function is inconclusive, the gene IFRD1 has been proposed as a modifier of CF lung disease severity. Polymorphisms of this gene have been significantly associated with neutrophil effector function [66].

1.3.3 Pro-inflammatory gene upregulation

At least two genes are upregulated in sputum neutrophils from CF patients; TNF receptor and amphiregulin – an epidermal growth factor receptor ligand. Amphiregulin contributes to TNFα-induced release of CXCL8 from airway epithelial cells, suggesting an upregulated pro-inflammatory state [67]. Both TNFα and CXCL8 in CF BAL are important in the priming and activation of CF neutrophils, leading to the increased production of neutrophil elastase as compared to control subjects, discussed further below [68].

1.3.4 Pro-inflammatory cytokines and increased oxidative stress

The underlying pro-inflammatory mechanisms and overwhelming neutrophilic infiltration seen in the CF airway can by studied using BAL and spontaneously expectorated or induced sputum in children and adults. BAL from CF patients has increased numbers of neutrophils, higher levels of pro-inflammatory cytokines such as CXCL8, and also lower levels of anti-inflammatory cytokines such as IL-10, as compared to the healthy airway [69, 70]. Levels of NFκB are also elevated in BAL from CF patients, although it is not clear whether this is due to the overwhelming bacterial and neutrophilic infiltration, or whether there is an inherent defect in NFκB activation [71]. The ultimate result of this increased activity is greater NFκB expression and pro-

inflammatory cytokine production [71]. A number of studies have also shown increased oxidative stress in the CF airway. This is partly due to a deficiency of inducible nitric oxide synthase in airway epithelial cells, and also a deficiency of the anti-oxidant glutathione in the extracellular fluid [72, 73].

1.3.5 Neutrophil elastase

The neutrophilic load in the airways of CF patients overwhelms the natural airway defences against neutrophil elastase (including α1-antitrypsin and secretory leukoprotease), thus leading to further airway destruction through damage to airway elastin, collagen and proteoglycans [74]. Neutrophil elastase also leads to epithelial cell damage, goblet cell hyperplasia and increased mucus secretion through increasing MUC5AC expression [75]. Elevated levels of neutrophil elastase have been found in BAL from children with CF under 6 months of age, implying neutrophil-mediated pulmonary damage may occur very early in infancy [25].

1.3.6 Persisting bacterial infection

The overwhelming bacterial load and mucus characteristics of the CF airway lead to reduced neutrophil phagocytic ability. Chronic *Pseudomonas aeruginosa* airway infection leads to the development of biofilms and a mucoid phenotype through quorum sensing, thus further impairing innate immune bacterial clearance mechanisms [76].

1.4 Compartmentalisation of inflammation in the CF airway

There is increasing evidence that pulmonary inflammation in CF is compartmentalised – with neutrophilic inflammation predominating in the airway lumen, and lymphocytic inflammation within the airway wall submucosa [77]. Inflammatory changes in the airway wall are fundamental to the pathology of CF. Previous published work has shown that explanted lung tissue from CF patients has a predominant lymphocytosis within the bronchial mucosa, particularly distally in association within more severe tissue damage. In contrast the airway surface epithelium had an accumulation of neutrophils, thought to have migrated towards the airway surface [78]. However, these patients represent those with the most severe CF disease. Further work has shown that in paediatric CF patients, a cohort with generally milder disease, T lymphocytes

predominate in the airway wall submucosa, and these significantly increase during a respiratory exacerbation and with *Pseudomonas aeruginosa* infection. The presence of *Pseudomonas aeruginosa* was not associated with BAL inflammatory cell profile changes [77]. The inflammatory state of the airway is therefore not purely reflected by BAL inflammatory findings.

The following sections introduce the concept of inflammatory resolution, and evidence that reducing airway inflammation may be beneficial in some patients with neutrophilic lung disease.

1.5 Resolution of neutrophilic inflammation

Until recently resolution of acute neutrophilic inflammation was thought to be a passive process, due to the gradual reduction in pro-inflammatory mediators, with the inflammatory response essentially petering out. It is now clear that resolution of inflammation (figure 1.2) is an active process, requiring a number of specific pro-resolution and anti-inflammatory mechanisms to return tissue to normal homeostasis.

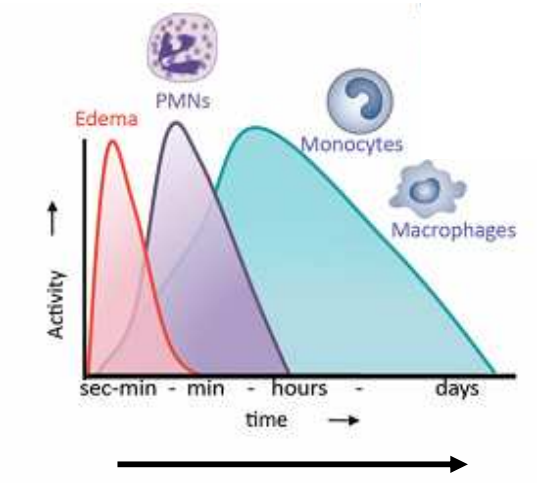


Figure 1.2: The inflammatory response and resolution time course. Reproduced with permission from the American Chemical Society; Chem Rev [79]. Copyright 2011.

There is a difference in the concept of anti-inflammatory mechanisms and pro-resolution mechanisms, although these terms are often used interchangeably. Anti-inflammatory products act by blocking pro-inflammatory mediators, whereas pro-

resolution molecules actively return tissue to homeostasis and re-establish immune defence mechanisms [80]. These include:

- T regulatory cells (CD4+CD25+FoxP3+ and CD4+IL-10+ cells) [81, 82]
- Cytokines such as IL-10, TGFβ and IL-35 [69, 83, 84]
- Arachidonic acid derived lipid mediators (lipoxins, resolvins, protectins and maresins) [79, 85, 86]
- Chemerin (a chemottractant protein) and its receptor Chem R23 [87]
- Transcription factors (for example lung krüppel-like factor) [88]
- Proteins (for example annexin A1) [89]
- Cell surface markers (for example CD200 / CD200R - required for macrophage immune homeostasis) [90]

The inflammatory resolution process is coupled closely with promoters of acute inflammation, thus maintaining the delicate homeostatic balance [91, 92]. Inflammatory resolution involves both the innate and adaptive immune systems. An example of this involves TLRs and T regulatory cells (Tregs). There is evidence that negative-feedback inhibitors of TLRs contribute to inflammatory resolution [93]. *In vitro* work has shown Tregs function to resolve inflammation through interactions with TLRs. TLR2 is expressed on Tregs and macrophages, and these sense TLR2 ligands on invading bacteria. In response to this, Tregs proliferate but remain functionally inhibited. Macrophages however respond by bacterial clearance through phagocytosis. Once the TLR2 ligands have been cleared, the inhibition of Tregs ceases leading to resolution of the acute inflammatory process (figure 1.3) [94, 95]

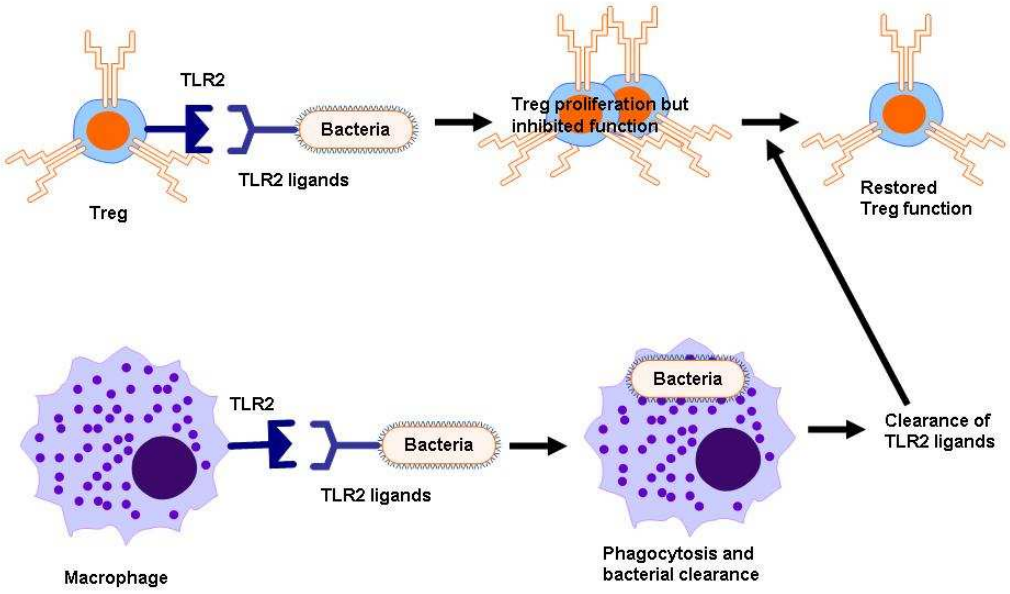


Figure 1.3: The innate and adaptive immune systems are both important for inflammatory resolution. *In vitro* work has shown Tregs function to resolve inflammation through interactions with TLRs.

Resolution of inflammation is aided to some extent by removal of the agent that initially led to the inflammatory process, thus leading to a reduction in pro-inflammatory drive. However, unless the resulting neutrophilic infiltration is curtailed, tissue damage may result. There needs to be a fine balance between the processes of inflammatory resolution, while still ensuring that the original infective or irritant stimulus is effectively removed. This balance of pro-inflammatory and pro-resolution mediators is crucial to maintaining pulmonary homeostasis.

The study of inflammatory resolution mechanisms could lead to the development of treatments that might switch off the inflammatory process. This would potentially be of benefit in those diseases where there is a persistent acute neutrophilic inflammatory state. However, the development of acute neutrophilic inflammation is also crucial for the removal of invading pathogens and therefore inappropriate resolution of inflammation may not be beneficial. This has been demonstrated in a study looking at the effects of steroid therapy in children with CF (discussed in more detail below).

Only those children that were chronically infected with *Pseudomonas aeruginosa* had an improvement in FEV_1 with steroid therapy, implying resolution of inflammation in CF patients may only be beneficial under certain circumstances [10].

Current knowledge of inflammatory resolution in CF and therapies targeting the inflammatory process are discussed in the next section.

1.6 Inflammatory resolution in CF and anti-inflammatory therapies

There has been limited research on the role of inflammatory resolution in CF patients. However, levels of the pro-resolution lipid mediator, lipoxin A_4, and the pro-resolution protein, annexin A1, are reduced in BAL from adult CF patients [13, 96, 97] . In addition levels of IL-10 (traditionally thought to be anti-inflammatory), produced from bronchial epithelial cells, are lower in the airways of CF patients [69]. IL-10 deficient mice, generated by gene targeting, and infected with *Pseudomonas aeruginosa* have prolonged airway neutrophilia and inflammatory cytokine levels, despite adequate pathogen clearance, suggesting that IL-10 deficiency may contribute to prolonged inflammatory responses in CF [98]. Together, this literature suggests that there may be an imbalance between inflammatory and pro-resolution mediators in patients with CF. If this discrepancy is of more significance in patients with CF than other inflammatory airway diseases then it could contribute to the worse prognosis seen in CF. If there is failure of resolution of inflammation in CF, this may be due to a CFTR-related fundamental defect in the resolution process, or possibly the fact that the persistent neutrophilic inflammation is so great that it overwhelms a normal pro-resolution process. There have also been suggestions that the neutrophil apoptotic process that normally leads to the termination of acute inflammation may be abnormal in the CF airway, with apoptosis replaced by the less favourable process of neutrophil necrosis, which leads to the release of cytotoxic enzymes from the necrotic neutrophil [64].

Attempts at reducing inflammation in the CF airway have not always had a good outcome. A phase 2 trial in CF of a LTB_4 receptor antagonist led to an increase in pulmonary exacerbations in the treatment group [99]. This highlights the delicate balance of pro- and anti-inflammatory mediators in maintaining tissue homeostasis, and the fact

that in most situations, and in some aspects of CF, neutrophilic inflammation is needed to clear infective agents and is therefore not always a negative consequence of pathogen invasion. The next sections review current knowledge regarding anti-inflammatory drug therapy in CF.

1.6.1 Glucocorticoids

Glucocorticoids have beneficial effects on reducing inflammation and improving lung function in some patients with CF. In many respects this is counter-intuitive given the fact that steroids promote neutrophil survival and in other situations can actually promote increased and more severe infections [100, 101]. A number of steroid studies have been conducted in CF patients (table 1.1). The earliest study to show the beneficial effects of steroids in CF was performed in 21 children aged 1-12 years of age with mild to moderate lung disease. Patients received either alternate day prednisolone or placebo and were followed up over a four year period. At the end of four years those children treated with prednisolone had better growth and lung function parameters as well as fewer admissions to hospital. No steroid-induced side-effects were reported [12]. However, as is well known, and has been shown in a subsequent larger multi-centre study, glucocorticoids have a number of side-effects (including effects on growth, cataracts and diabetes) which limit their use, particularly in children [10]. This next study compared two alternate day dosing regimens of prednisolone (1mg/kg and 2mg/kg) with placebo over a 4 year period. 285 children with CF and mild to moderate lung disease aged 6 to 14 years enrolled in the study. Although beneficial effects on pulmonary function were described, side-effects were documented as above. Indeed, the 2mg/kg group was withdrawn after two years due to side-effect concerns. In addition, in this larger study, there was evidence that pulmonary inflammation may only be detrimental at certain stages of the disease. Only those that were chronically infected with *Pseudomonas aeruginosa* had an improved FEV_1 with steroid therapy throughout the 48 month study period [10].

The effect of shorter steroid courses has also been investigated in CF. In one study, 24 children were randomised to receive either placebo or a 2mg/kg dose of prednisolone – initially daily for 2 weeks and then reduced to 1mg/kg on alternate days for 10 weeks.

The prednisolone treatment group had improved FEV_1 and forced vital capacity (FVC) at 2 weeks as compared to placebo, although this improvement was reduced by the end of the study period. The treatment group also had reduced levels of immunoglobulin G (IgG) and pro-inflammatory cytokines including IL-1α and soluble IL-2 receptor [102]. Other studies have looked at the role of short pulses of steroids to treat acute pulmonary exacerbations. An initial pilot study, investigating adding 5 days of prednisolone treatment to standard therapy for acute pulmonary exacerbations in CF, did not find a significant improvement in lung function or sputum inflammatory markers [103]. A similar study, examining the effects of adding intravenous hydrocortisone to conventional intravenous antibiotics for an acute CF respiratory exacerbation in infants, found that steroid treatment did not lead to clinical improvements at the end of the study period. However there were improvements in lung function 1-2 months later in the steroid treatment group [104]. A further small study reported clinical improvement following a 3 day course of methlyprednisolone in 4 children with CF admitted with acute respiratory distress presumed due to severe inflammation of the small airways [105].

Collectively these studies provide evidence that reducing inflammation in CF patients with glucocorticoids may be beneficial for some patients under certain circumstances, although long-term steroid therapy is undesirable due to adverse effects.

The use of inhaled corticosteroids (ICS) in CF patients has been examined in a number of studies, with the hypothesis that inhaled steroids may have clinical benefits similar to systemic steroid therapy, but without significant side-effects. Inhaled fluticasone propionate has been shown to reduce lipopolysaccharide-induced release of IL-6 and CXCL8 in CF tracheal xenografts implanted in nude mice [106]. In a separate paper using freshly harvested CF bronchial epithelial cells, inhaled fluticasone reduced levels of IkappaB-α / beta-kinases, leading to decreased NFκB and thus reduced airway inflammation [106]. Most of the dosing regimens in ICS trials in CF patients are extrapolated from asthma. It is therefore possible that the steroid doses used are not high enough to penetrate mucus in the CF airway, although high doses carry an increased risk of systemic side-effects. However a recent Cochrane review concluded that evidence was lacking from a number of trials to conclude that ICS were beneficial

in CF patients, and that there are some concerns regarding effects on growth [107]. There is also some evidence that potent topical corticosteroids may increase the risk of infection [108].

1.6.2 Ibuprofen

Another anti-inflammatory treatment that has been utilised in the context of CF is the non-steroidal anti-inflammatory drug ibuprofen. Ibuprofen has anti-inflammatory effects through its actions on prostaglandin synthesis and potential positive benefits in CF were initially demonstrated in an animal model [109]. Studies by Konstan *et al* in adult and paediatric CF patients have shown that prolonged treatment with high-dose ibuprofen leads to a slower decline in pulmonary function – particularly in younger patients with milder disease [11, 110]. High dose ibuprofen induces the expression of heat shock protein 70, an inhibitor of pro-inflammatory cytokine production and of NFκB activation [111, 112]. Further work demonstrated that the positive effects of limiting neutrophil migration into the airways were highly dependent on the achievement of appropriate plasma concentrations and hence pharmacokinetics. Indeed, low plasma levels were associated with an increase in neutrophil migration into the lung [113]. In addition there are concerns regarding potential gastrointestinal bleeding and renal toxicity which have made many wary about its use in clinical practice [114].

1st author ref	Year	Number of CF patients	Age of CF patients	CF severity	Study design	Steroid dose	Results	Side effects
Auerbach [12]	1985	45	1-12 years	Mild to moderate lung disease – mean FEV_1 > 85%	Randomised, double-blind, placebo controlled. Alternate day prednisolone or placebo. Follow-up over 4 years	Prednisolone 2mg/kg alternate days.	Improved growth FEV_1 & FVC. Improvement in IgG and ESR. Fewer hospital admissions	None reported
Eigen [10]	1995	285	6-14 years	Mild to moderate lung disease - mean FEV_1 > 80%	Randomised, double-blind, placebo controlled. Two alternate day prednisolone dosing regimens or placebo. Follow-up over 4 years.	Alternate day 1mg/kg or 2mg/kg prednisolone	2mg/kg group terminated early due to side effects. Improvement in FVC only seen in chronic PA infection. Reduced IgG.	Abnormal glucose metabolism, cataracts, growth retardation
Pantin [115]	1986	20	17-51 years	Severe airflow obstruction – median FEV_1 27%	3 weeks placebo then 3 weeks prednisolone	Mean daily prednisolone 0.48mg/kg	No improvement in lung function, although lung function fell significantly on stopping steroids	2 patients developed pneumothoraces while on steroids, but not known whether due to drug
Greally [102]	1994	24	5.5-19.5 years	Mean FEV_1 54%	Randomised, double-blind, placebo controlled. Daily prednisolone for 2 weeks, then lower dose alternate days for 10 weeks.	Prednisolone daily 2mg/kg for 2 weeks, then 1mg/kg alternate days for 10 weeks	Increased FEV_1 and FVC at 2 weeks, but effect lost by end of study. Reduced IgG and inflammatory cytokines	1 patient transient hyperglycaemia

1st author ref	Year	Number of CF patients	Age of CF patients	CF severity	Study design	Steroid dose	Results	Side effects
Dovey [103]	2007	24	Mean 21 years	Mean FEV_1 70%. Acute exacerbation	Randomised placebo controlled. 5 days of prednisolone added to standard treatment for acute infection	Prednisolone 2mg/kg for 5 days	No significant improvement in FEV_1 or sputum inflammatory markers	6 patients glycosuria. 2 patients hyperglycaemia
Tepper [104]	1997	20	Mean 8.8 months	Acute exacerbation	Randomised, placebo controlled. IV hydrocortisone (added to course of IV antibiotics)	IV hydrocortisone 10mg/kg/day	No improvement in lung function at end of study period, but improved lung function 1-2 months later	None reported
Ghdifan [105]	2010	4	6 weeks – 6 months	Severe pulmonary exacerbation	3 days methylprednisolone	IV methylprednisolone $1g/1.73m^2$/day	Significant clinical improvement	None reported

Table 1.1: Summary of the main studies using oral or intravenous steroids to reduce inflammation in CF. The overall findings suggest that steroids may be helpful in improving lung function in some patients under certain circumstances. However there are prevailing concerns regarding side-effects and any positive effects seen may not be sustained on cessation of treatment. FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, IgG = immunoglobulin G, ESR = erythrocyte sedimentation rate, PA = *Pseudomonas aeruginosa*, IV = intravenous.

1.6.3 Macrolides

The benefits of macrolides were first described in patients with diffuse panbronchiolitis in Japan [116]. Many of these patients develop bronchiectasis, have a high mortality and are chronically infected with mucoid *Pseudomonas aeruginosa.* Azithromycin – an azalide traditionally used as an antibiotic, has also been shown to have anti-inflammatory effects and to improve the respiratory status of CF patients. Benefits described include a reduction in frequency of intravenous antibiotics, increased weight gain, improved quality of life and a slower decline in lung function [117-119]. Compared to traditional macrolides, azithromycin has improved tolerability and a longer half-life requiring an infrequent dosing regimen. There are however concerns regarding long-term use and macrolide resistance in particular with non-tuberculous mycobacteria [120]. However, although macrolide monotherapy in the context of mycobacterial infection may lead to resistance, a recent epidemiological study has shown that long term treatment with azithromcyin does not lead to an increased risk of mycobacterial infection in CF patients [121]. Despite the documented clinical benefits, the mode of action of azithromycin remains unclear. *In vitro* studies have shown that macrolides inhibit neutrophil migration and reduce the production of neutrophil elastase and pro-inflammatory mediators including TNFα, IL-1β IL-6 and CXCL8 possibly by inhibiting transcription factors that regulate cytokine production [122-124]. However the effects on CXCL8 have not been backed up by an *in vivo* study [117]. A double-blind randomised placebo controlled trial of azithromycin in children with CF aged 6-18 years uninfected with *Pseudomonas aeruginosa*, showed that those treated with azithromycin had lower serum neutrophil and inflammatory marker (C-reactive protein, myeloperoxidase, serum amyloid A and calprotectin) levels. Levels of C-reactive protein, calprotectin and neutrophil counts also correlated negatively with FEV_1. However, serum markers can only act as a proxy for the airway inflammatory process, most of the patients in this study had baseline markers within the normal range and what constitutes a clinically meaningful change in these inflammatory parameters is unknown [125].

1.6.4 Antioxidants

Antioxidants have anti-inflammatory properties in areas of oxidative stress, including the CF airway. Such treatments include N-acetylcysteine (NAC) and glutathione.

NAC inhibits hydrogen peroxide and increases glutathione levels. High dose NAC in children aged > 11 years with FEV_1 greater than 40% has been shown to increase whole blood and neutrophil glutathione levels, and reduce sputum neutrophils, CXCL8 and elastase. However lung function remained unchanged [126]. Studies looking at the effect of inhaled glutathione therapy have shown improvements in FEV_1, reduction in airway prostaglandin E_2 and increases in airway lymphocytes, but not markers of oxidative stress [127-129]. Further studies are needed to delineate these effects further. Oral supplementation with antioxidants such as vitamin A, vitamin E and β-carotene has shown only limited ability to improve the long-term pulmonary status of CF patients [130].

The search therefore continues for novel treatments that will have a beneficial effect on resolving inflammation, while maintaining tissue homeostasis and minimising side-effects. Targeting pro-resolution rather than anti-inflammatory mediators and mechanisms may be better tolerated as this would potentially mimic a more physiological process by promoting the return to tissue homeostasis. The next sections focus in more detail at specific inflammatory resolution mechanisms and mediators which were investigated in this thesis. The specific areas discussed are T regulatory cells, the protein annexin A1, the transcription factor Lung Krüppel-Like Factor and lipid mediators. Within each section the reason for choosing to examine these mediators is discussed in detail.

1.7 T Regulatory Cells

CD4+ Tregs are a T cell subgroup important for peripheral tolerance, inflammatory regulation and immune homeostasis (figure 1.4). Tregs are able to suppress the activation, proliferation and effector functions of a number of other immune cells, including CD4+ and CD8+ T cells, B cells and NK cells [131]. Tregs are therefore crucial in preventing autoimmunity and persistent inflammation. Defects in Tregs are implicated in the development of autoimmune diseases such as type 1 diabetes and myasthenia gravis, allergic disorders, multiple sclerosis and limiting the inflammatory response in persistent inflammatory conditions [132]. However, they also have less favourable properties such as limiting anti-tumour immunity and thus promoting

tumour development [133]. Furthermore, their effects on limiting the inflammatory response may also prevent effective pathogen clearance. The role of Tregs in human disease is likely to be complex, and there is considerable interest in the role of Tregs in the pathogenesis of a number of autoimmune and inflammatory conditions.

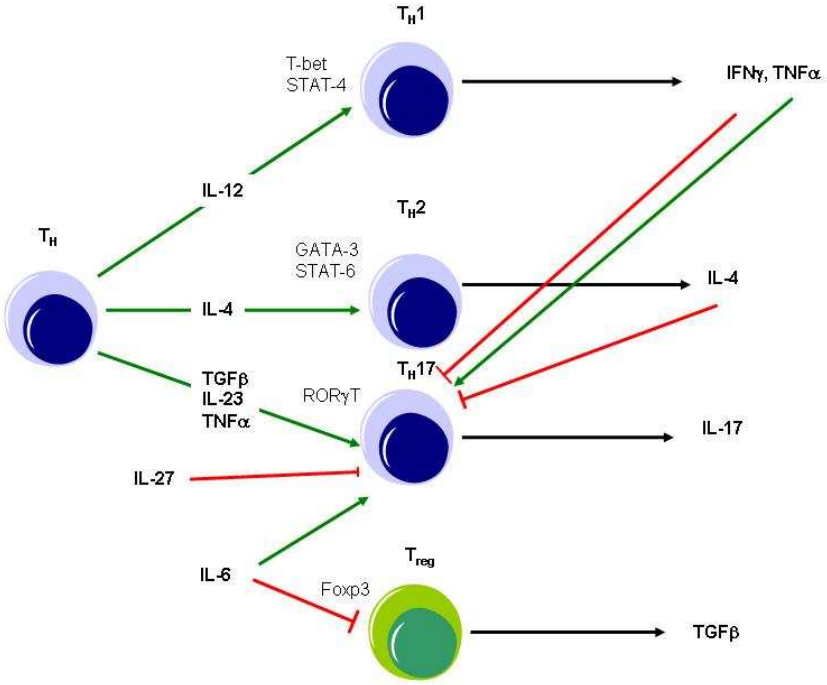


Figure 1.4: T helper cell differentiation from naive T cells and their regulation. Green arrows indicate upregulation, red lines indicate inhibition. Transcription factors for the various T helper subsets are listed beside each cell. Adapted with permission from Macmillan Publishers Ltd; Nature Medicine [134] Copyright 2007.

The transcription factor forkhead box P3 (FoxP3) is central to Treg cell development [135]. Although FoxP3 is important for Treg development and function (particularly in mice), there is now evidence that it is not the only transcription factor in human Tregs, and the development of Tregs may be dependent on a host of interacting transcription factors as well as FoxP3 [136]. There are also FoxP3-independent suppressive Tregs. These include those that secrete IL-10 (Tr1 cells) and TGFβ (Th3 cells) [81]. FoxP3 is also expressed on some non-regulatory lymphocyte groups, and thus FoxP3 expression alone may not be enough to confer a regulatory phenotype [137, 138]. The expression of

other surface markers also differentiates Tregs. This includes expression of CD25 (IL-2Rα) [139] and the downregulated expression of CD127 [139].

There are two main groups of Tregs; naturally occurring Tregs and inducible Tregs. Naturally occurring Tregs develop in the thymus and are CD4+CD25+FoxP3+ cells [82]. Inducible Tregs develop in the periphery from effector T cells, and may or may not express FoxP3 (figure. 1.5). Their development is usually in response to a number of mediators released in the pro-inflammatory state – in particular cytokines such as TGFβ1 and IL-10 [82]. In addition a reciprocal relationship between Th17 and Tregs has been proposed, and IL-6 may inhibit the production of Tregs induced by TGFβ [140]. Clearly for immune homeostasis to be maintained, balance must be preserved between these two T cell subsets.

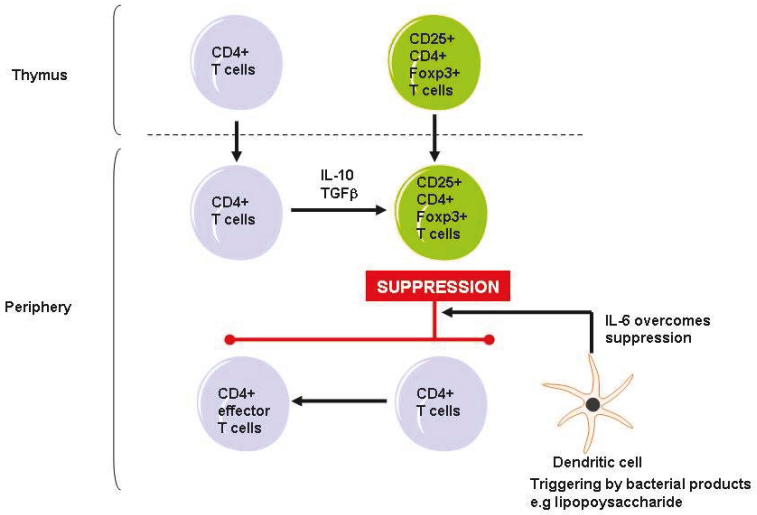


Figure 1.5: The development of T regulatory cells. Tregs expressing FoxP3 are produced in the thymus, but $CD4^+$ cells in the periphery may also acquire regulatory function. Tregs inhibit CD4+ activation and effector function. This suppressive activity can be overcome by triggering of dendritic cells by bacterial products.

1.8 Mechanisms of action of T regulatory cells

There are a variety of possible mechanisms by which Tregs may exert their regulatory and suppressive functions. These can broadly be divided into suppression by cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by

modulation of dendritic cell (DC) function. Suppression by cytokines is discussed in more detail.

1.8.1 Suppression by cytokines

IL-10, IL-35 and TGFβ induce the production of Tregs in the periphery, as well as enhancing Treg function [141-143]. The role of IL-10 and TGFβ in the production and action of natural thymus-derived Tregs is less clear with *in vitro* work suggesting these cytokines are not needed for Treg function [144, 145]. However other *in vivo* studies have suggested that both these cytokines are important for natural Treg activity [146, 147].

1.8.1.a IL-10

IL-10 is generally reported to be an anti-inflammatory cytokine, although this may be an oversimplification of its functions. It is produced by a variety of cells, including B cells, macrophages, DCs, mast cells, eosinophils and many T cell subsets (including Tregs and Th17 cells). Following binding to its receptor, IL-10 activates two tyrosine kinases (Jak1 and Tyk2) leading to inhibition of downstream signalling [148, 149]. IL-10 production by T effector cells may be important in limiting their inflammatory potential, by blocking CD2, CD28 and inducible co-stimulatory molecules and, under favourable conditions, also inhibiting T cell proliferation. One of the major effects of IL-10 is the inhibition of antigen presentation, which therefore inhibits T cell activation. IL-10 production by innate cells and T cells in the respiratory tract limits the inflammatory response to bacteria and viruses [150, 151]. Il-10 also inhibits the production of cytokines important for Th1 (IL-2 and IFNγ) and Th2 (IL-4 and IL-5) function. Importantly, IL-10 inhibits the production of a number of pro-inflammatory cytokines including IL-1β, TNFα, IL-6, CXCL8 and IL-12. In addition IL-10 increases production of an IL-1 receptor antagonist [152]. IL-10 may have both positive and negative functions in human disease. IL-10 mediated inflammatory suppression is thought to be, at least in part, responsible for Treg activity in allergic airways disease [153, 154]. However, IL-10 is also involved in the function of Tregs in infections such as tuberculosis, which may contribute to persisting infection due to inhibition of effector T cell functions [155, 156]. Glucocorticoid treatment enhances IL-10 production and FoxP3

expression in patients with a variety of conditions. This is therefore one proposed mechanism whereby glucocorticoids can exert their anti-inflammatory actions [157].

As well as the well-documented anti-inflammatory properties of IL-10, there is some evidence to suggest that under certain circumstances IL-10 has immunostimulatory and therefore pro-inflammatory effects. IL-10 stimulates proliferation and differentiation of activated human B cells *in vitro* [158] and preincubation of resting $CD4^+$ lymphocytes with IL-10 enhances their capacity to produce cytokines (IL-2, IL-4, TNFα and IFNγ) after activation [159]. In addition IL-10 enhances IL-2-driven proliferation of preactivated human-purified $CD8^+$ T cells [160]. Addition of IL-10 to mouse splenic NK cell cultures stimulated with IL-12 and IL-18 results in enhanced IFNγ production [161]. The administration of IL-10 in human experimental endotoxaemia potentiates systemic levels of IFNγ and does not inhibit serum TNFα, thus promoting inflammation [162]. This suggests that high-dose IL-10 treatment in patients with inflammatory disorders may be associated with unwanted proinflammatory effects. Indeed a number of other studies examining the effects of treating patients with IL-10 have been published [162, 163]. Collectively these showed that contrary to expectations, treatment with endogenous IL-10 led to increased inflammation.

1.8.1.b TGFβ

TGFβ1 is the prototype of the TGFβ superfamily. TGFβ regulates lymphocyte homeostasis and inhibits Th1 and Th2 responses. As discussed above, dependent on the presence of other cytokines, TGFβ is involved in the production of Th17 cells as well as Tregs, which is in contrast to its suppressive activity. In addition TGFβ is involved in airway remodelling, contributing to the pathogenesis of airway diseases such as asthma [164].

1.8.1.c IL-35

IL-35 is a relatively newly described inhibitory cytokine that appears to be important for the development and function of Tregs. IL-35 is a member of the IL-12 family of cytokines, and is formed by two sub-components: Epstein-Barr virus-induced gene 3 (Ebi3) and p35 (also known as IL-12a). Both Ebi3 and p35 are expressed by activated

mouse FoxP3+ Treg cells and are not expressed by effector T cells [84]. *In vitro* work has shown that a population of Treg cells express and require IL-35 to mediate their suppressive actions. These Tregs do not express Foxp3 and do not require the actions of IL-10 or TGFβ to mediate their suppressive functions. The effect of IL-35 on other cell types is not fully understood, and indeed the range of functions of IL-35 is yet to be fully described [84, 165].

1.8.2 The importance of Tregs in maintaining pulmonary homeostasis

There is strong evidence that Tregs are critical in maintaining pulmonary homeostasis. Much of the work on the role of Tregs in human airway pathology has focused on asthma and the response of the airway to inhaled antigens in developing tolerance or Th2 responses. Repeated exposure of mice to low-dose allergen promotes the development of Tregs expressing membrane-bound FoxP3 and TGFβ. Transfer of these cells to naïve mice prevents the development of allergic sensitisation [166].

Alveolar macrophages are broadly divided into M1 and M2 macrophages - phenotypes based on signals they receive from their environment. M1 macrophages are known as classically activated macrophages induced by IFNγ and TNFα, and M2 are known as alternatively activated macrophages induced by IL-4 and IL-13. Recent work has been published highlighting the role of alveolar macrophages in Treg development and the maintenance of pulmonary homeostasis. Alveolar macrophages and alveolar macrophage-conditioned media from mice and humans induced FoxP3 expression in naïve CD4+ T cells in *vitro*, through the actions of retinoic acid and TGF-β1 [167].

There is one previous study examining BAL Tregs in paediatric airway disease. $CD4+CD25^{hi}$ T cells (used in this instance as markers of Tregs) are reduced in BAL from asthmatic children, as compared to those with chronic cough or control subjects. ICS treatment increased the numbers of BAL $CD4+CD25^{hi}$ in these asthmatic children. $CD4+CD25^{hi}$ cells isolated from non-asthmatic subjects led to suppressed proliferation and cytokine production by CD4+CD25- responder T cells. $CD4+CD25^{hi}$ cells from asthmatic patients failed to lead to a similar pattern of suppression of responder T cells, but this was restored after the use of ICS [168].

Although there has been considerable research into the role of Tregs in allergic airways disease, there has been limited research into their actions in pulmonary neutrophilic lung disease both in adults or children, and their role is uncertain. Whether, despite inflammatory compartmentalisation, there are differences in the levels of these cells affecting the inflammatory resolution process in BAL from different neutrophilic lung diseases, remains to be established. From the paediatric asthmatic data described above, BAL Tregs may also be reduced in neutrophilic lung disease, as compared to controls. In addition levels may be lower in CF as compared to other neutrophilic airway diseases due to the increased disease severity in CF. If this is the case this may contribute to an impaired inflammatory resolution capability in these patients. Given there has been no previous research in these patients this is hypothesis generating research.

1.9 Annexin A1

Annexin A1 (AnxA1) – initially called macrocortin and then lipocortin-1, is a member of the superfamily of annexin proteins, which are characterised by their ability to bind phospholipids through calcium-dependent mechanisms. There are twelve annexins in vertebrates. These generally contain four homologous repeating domains and are differentiated by a unique N-terminus. The annexin proteins have a variety of biological functions. AnxA1 is found in a number of cell types, particularly haematopoietic cells including neutrophils, monocytes, macrophages, mast cells and T cells. AnxA1 is also found in epithelial and endothelial cells, fibroblasts and synovial cells [89]. Expression on the vascular endothelium appears to be upregulated during the inflammatory process [169]. AnxA1 is known to have anti-inflammatory actions through its effect on neutrophils, discussed further below.

In resting cells, AnxA1 is mostly intracellular – although the exact intracellular location varies between cell types and may also depend on the activation state of the cell. Intracellular AnxA1 can form up to 4% of the cytoplasm [170]. AnxA1 can act intracellularly, however it can also be exported and act on target cells – a mechanism by which it exerts most of its anti-inflammatory actions [171, 172]. Mobilisation of AnxA1 to the cell surface occurs upon cell activation, for example through adhesion to the

endothelium, or by the action of cytokines or glucocorticoids (discussed below). The process of externalising AnxA1 to the cell surface is not fully understood and varies with the cell-type involved. In neutrophils a large proportion of AnxA1 is stored in cytoplasmic gelatinase granules [177]. On activation, neutrophils mobilise their granules to the cell surface leading to release of AnxA1 [173].

Once AnxA1 is externalised it binds to its receptor, ALX, in an autocrine, paracrine or juxtacrine (involving cell to cell contact) manner, where it acts in a negative regulatory fashion [174]. However, there is now some emerging evidence that this may be an overly simplistic view. ALX is a G protein-coupled receptor that is able to bind both protein ligands such as AnxA1, and lipid ligands such as LxA_4, as first described in 2000 [175]. It has a higher affinity for LxA_4, an arachidonic acid inflammatory pro-resolution lipid mediator. ALX is found on the surface of neutrophils, moncytes, macrophages, endothelial and epithelial cells [176, 177]. Binding of AnxA1 to ALX leads to a cascade of signalling via phospolipase A2, phospholipase D and mitogen-activated protein kinases (MAPK) such as ERK, Akt, p38 and JNK [182]. Activation of these pathways has different functions in different cells. In neutrophils activation of ERK leads to apoptosis [178].

The action of AnxA1 on ALX is terminated after a few minutes by enzymatic cleavage of AnxA1, in particular by proteinase 3 [179]. Other proteinases, including elastases, also mediate this process; indeed higher levels of BAL neutrophils are associated with lower BAL AnxA1 levels in human subjects [180, 181]. This therefore may be an important mechanism whereby AnxA1 levels may be lower in overwhelming neutrophilic inflammation, and thus potentially no longer able to regulate the inflammatory process.

A number of mechanisms have been proposed as to how AnxA1 may mediate its anti-inflammatory effects. AnxA1 has potent inhibitory effects on neutrophil and monocyte migration, demonstrated in a number of experimental models [182, 183]. AnxA1 also promotes neutrophil apoptosis, possibly through changes in intracellular calcium levels, and phagocytosis by macrophages [89, 184, 185]. Promotion of phagocytosis of apoptotic cells limits the ongoing pro-inflammatory response that would persist in the presence of

these cells, and in particular if they become necrotic. AnxA1 limits pro-inflammatory cytokine production from macrophages that have engulfed apoptotic cells – thus curtailing the inflammatory process [186]. AnxA1 is also known to inhibit phospholipase A_2 activity and to therefore inhibit inflammatory eicosanoid synthesis – potentially contributing to inflammatory resolution [187]. Levels of IL-10 have also been shown to be increased by AnxA1 [188]. Together this evidence suggests a significant role for AnxA1 in the resolution of neutrophilic inflammation and potentially in the return of pulmonary homeostasis. It has also been proposed that the anti-inflammatory effects of AnxA1 on neutrophils may also be due to down-regulation of ALX, which implies a completely different function of AnxA1 in inflammation. Indeed it may be that AnxA1 has differing roles in both promoting and resolving inflammation in order to deal effectively and appropriately with invading pathogens [189]. There is also an N-terminal cleaved form of AnxA1 that is probably generated during inflammation. This appears to promote leukocyte migration and increases ICAM1 clustering around adherent neutrophils to anchor them to the endothelium and promote transmigration [190]. The function of AnxA1 therefore appears to be multifaceted and an evolving area of inflammatory research.

1.9.1 AnxA1 and glucocorticoids

Glucocorticoids are fundamental to the activity and release of AnxA1 [191]. Rats treated with dexamethasone produce AnxA1 in peritoneal lavage fluid, which can dampen inflammation when administered to rats in a pro-inflammatory state [192]. Initially following glucocorticoid exposure AnxA1 is excreted from cells – a process that occurs within 5-10 minutes of steroid contact and then persists for 30-90 minutes, or until the internal pool of AnxA1 in the cell is depleted. There is then a second phase of AnxA1 release due to upregulation of AnxA1 mRNA production and subsequently the release of newly synthesised AnxA1 protein. This phase can last up to 24 hours post glucocorticoid exposure [193, 194]. However, this process is not seen in all cells and in T cells glucocorticoid exposure leads to a reduction in AnxA1 mRNA and subsequently protein synthesis [195]. There are therefore contrasting effects of glucocorticoids on AnxA1 in the innate and adaptive immune systems.

Despite the strong effect of glucocorticoids on AnxA1 activity, there are few glucocorticoid response elements. A number of other possible regulatory sites have been proposed including a nuclear factor IL-6 site and four GATA-binding protein-3 sites [89, 196]. The pro-inflammatory cytokine IL-6 has been linked to induction of AnxA1 and translocation to the cell membrane, which may therefore be one method of limiting the pro-inflammatory immune response [89, 197]. As well as glucocorticoids and cytokines, bacterial products including LPS lead to upregulation of AnxA1 production [89].

1.9.2 AnxA1 and neutrophilic lung disease

As regards neutrophilic lung disease, AnxA1 has been investigated in adults with CF. However, the role of AnxA1 has not been established in other neutrophilic lung diseases or in children. AnxA1 was undetectable in lung and pancreatic tissue in *cftr* $^{-/-}$ mice, as compared to wild-type mice. Interestingly in wild-type mice, AnxA1 and CFTR are co-expressed at the apical surface of airway cells and alveoli. Expression of AnxA1 shown by immunofluorescence is reduced in human adult CF nasal epithelial cells, as compared to controls, acquired from nasal brushings. The same group found that in patients homozygous for the CFTR nonsense mutation p.Tyr122X, AnxA1 staining was markedly reduced or absent as compared to control patients. The same analysis of nasal brushings from Phe508del homozygous patients revealed differential expression of AnxA1, which appeared to be related to disease severity and phenotype, although this was not further characterised [96]. Levels of intact AnxA1 in BAL are reduced in adult CF patients as compared to controls and also patients with interstitial lung disease (where neutrophilic inflammation may not be as significant). Interestingly, in the few CF patients where AnxA1 appeared to be detectable, there was little neutrophil elastase activity, possibly implying that in the majority of CF patients, levels of AnxA1 were reduced in BAL due to cleavage by neutrophil elastase, resulting in diminished AnxA1 functional activity; however, this is very speculative and requires further work. In addition these patients had increased levels of N-terminal cleaved AnxA1 in BAL, and thus the pro-inflammatory form of AnxA1 as discussed above [97].

Recently CFTR inhibition has been linked to an imbalance in AnxA1 expression in mice. Mice treated with a CFTR inhibitor had an augmented inflammatory response to zymosan-induced peritonitis associated with reduced levels of AnxA1 in inflammatory cells within the peritoneum. The administration of AnxA1 to *cftr* $^{-/-}$ mice, or those treated with a CFTR inhibitor corrected the increased neutrophilic infiltration with a reduction in inflammation [198].

BAL neutrophils from adult CF lung transplant recipients with airway inflammation have reduced or absent AnxA1 when compared to healthy controls. In contrast airway macrophages from these patients have the same level of AnxA1 as healthy subjects. However these patients are phenotypically different to paediatric CF patients, and the effect of immunosuppressive therapies on AnxA1 expression is uncertain [199].

Given these findings and the link between AnxA1 and glucocorticoids, AnxA1 and ALX remain possible candidates as therapeutic targets in the future for a variety of inflammatory conditions, and particularly those characterised by neutrophilic inflammation. Further analysis of the role of AnxA1 and ALX in paediatric neutrophilic lung disease is indicated. Firstly, to ascertain whether changes in AnxA1 and ALX are specific to CF, or are related to levels of neutrophilic inflammation, irrespective of the underlying disease. Secondly, studies involving adult patients with CF generally involve subjects with more severe and advanced disease. The role of AnxA1 and ALX in children and therefore milder disease phenotypes is yet to be fully established. However, the data reviewed above make this axis a promising candidate in inflammatory resolution in patients with CF.

1.10 Lung Krüppel-Like Factor

Lung Krüppel-Like Factor is one member of a group of transcription factors characterised by a DNA binding domain consisting of three zinc fingers [200]. The Krüppel-like factors have a variety of biological functions – including cell growth, development, differentiation and activation.

Lung Krüppel-like factor (LKLF), so named because of its high expression in the lung, was first described in 1995 [200]. As well as being expressed in the lungs, LKLF is also expressed at lower levels in other tissues including the spleen, skeletal muscle, testis, myocardium and uterus, as well as endothelial cells, lymphocytes and monocytes [201]. LKLF appears to have a variety of functions. It is known to have a role in blood vessel and lung development [202]. LKLF knock-out mice die *in utero* from pulmonary haemorrhage [203]. Furthermore, expression of LKLF mRNA is reduced in a mouse model of pulmonary hypoplasia, implying that deficiency of LKLF may be important in the development of pulmonary hypoplasia in conditions such as congenital diaphragmatic hernia [204].

LKLF is also involved in the development and function of a number of cells in both the innate and adaptive immune systems. There is also evidence emerging that LKLF may be an important anti-inflammatory factor, and may play a role in inflammatory resolution.

1.10.1 LKLF and monocytes

LKLF is highly expressed in monocytes where it appears to have an anti-inflammatory role. However when monocytes differentiate into tissue macrophages, levels of LKLF fall. In patients with chronic inflammation mediated by activated monocytes (for example atherosclerosis), levels of LKLF are reduced as compared to healthy controls. Over expression of LKLF in monocytes reduces the production of pro-inflammatory mediators and cytokines such as IL-1β, macrophage inflammatory proteins, CXCL8 and TNF-α, probably through effects on NF-κB and AP-1. Equally, knock-down of LKLF by small interfering RNA (siRNA) leads to increased pro-inflammatory cytokine production. LKLF appears to increase monocyte recruitment to sites of infection but also to inhibit monocyte phagocytosis. LKLF has also been shown to reduce carrageenan-induced inflammation and paw oedema in a mouse model of inflammation. Together this evidence suggests that LKLF has a role in regulating and reducing inflammation. However despite the fact that LKLF appears to promote monocyte recruitment, the associated inhibition of phagocytosis suggests that the actions of LKLF

may not always be beneficial, given that the primary reason for inflammation is to remove invading pathogens [205].

1.10.2 LKLF and T lymphocytes

There is evidence that LKLF inhibits T cell proliferation and is important for promoting a T cell quiescent phenotype - characterised by decreased proliferation, reduced cell size and protein synthesis and decreased surface expression of activation markers [206, 88]. LKLF mRNA is expressed in naïve and memory T cells and is downregulated on TCR stimulation [207]. IL-7 has been shown to promote LKLF re-expression in antigen-stimulated, but not naïve, T cells [208]. IL-2 can also promote LKLF re-expression in activated T cells, and LKLF expression has been linked to the development of a memory T cell phenotype [207]. CD4+ T cells that are deficient in LKLF exhibit increased expression of pro-inflammatory chemokine receptors, such as CXCR3 [209]. These cells also appear to be able to increase expression of these receptors on neighbouring cells through the actions of IL-4 [210]. In addition, dipeptidyl peptidase 2 (DPP2) activity is essential for the maintenance of quiescent lymphocytes and its activity has been linked to the actions of LKLF [211].

1.10.3 LKLF and neutrophilic lung disease

LKLF expression is reduced in the airways of adult patients with airway disease including CF. LKLF inhibits NFκB-driven transcription in the presence of *Pseudomonas aeruginosa*, but is downregulated by TNFα and activated neutrophils. Equally, LKLF expression is enhanced by inhibition of TNFα. LKLF also inhibits the release of CXCL8. It is therefore probable that reduced levels of LKLF in neutrophilic inflammatory airway diseases lead to increased pro-inflammatory cytokine production and further neutrophilic infiltration, thus impairing inflammatory resolution and the return to pulmonary homeostasis [212]. However, the role of LKLF in paediatric neutrophilic lung disease has not been examined, and it may be that these patients, who generally have milder disease phenotypes, may have different responses to LKLF in the context of inflammatory resolution. It is also possible that targeting LKLF for therapeutic purposes in these diseases may not be beneficial given that there may also be negative effects on macrophage phagocytosis and pathogen removal.

1.11 Lipid mediators

There has been increasing interest in the role of inflammatory pro-resolution lipid mediators. The action of arachidonic acid (AA) derived pro-inflammatory lipid mediators in the acute inflammatory response, including leukotrienes and prostaglandins, derived from omega-6 (ω-6) AA, has been known for some time. These lipid mediators promote vasodilation and neutrophil influx into tissues, as well as having a wide variety of other biological functions. As AA contains 20 carbon atoms, the derived lipid mediators are collectively known as eicosanoids. Macrophages are an important source of eicosanoids. There are other groups of inflammatory pro-resolution lipid mediators, which are needed in order to resolve acute inflammation and promote tissue homeostasis. These are the lipoxins, resolvins, protectins and maresins, discussed in detail below [79, 86, 213]. These are also derived from ω-6 AA and in addition omega-3 (ω-3) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). During the inflammatory process there is a switch from pro-inflammatory to pro-resolution lipid mediators (figure 1.6). There is therefore a trend to shift from ω-6 to ω-3 derived lipids [214]. AA is derived from the phospholipid cell membrane by the action of phospholipase A2, whereas ω-3 derived lipids are mainly formed from dietary polyunsaturated fatty acids (PUFA) [213, 215]. A number of groups have hypothesised that dietary supplementation with ω-3 may be beneficial in CF. One study showed an increase in essential fatty acid content in neutrophil membranes and associated reduction in pro-inflammatory LTB_4 to B_5 ratio in CF patients receiving ω-3 supplementation, as compared to placebo [216]. However, a Cochrane review concluded that regular ω-3 supplements may have some benefits in CF, but that there was insufficient evidence to recommend routine use [217].

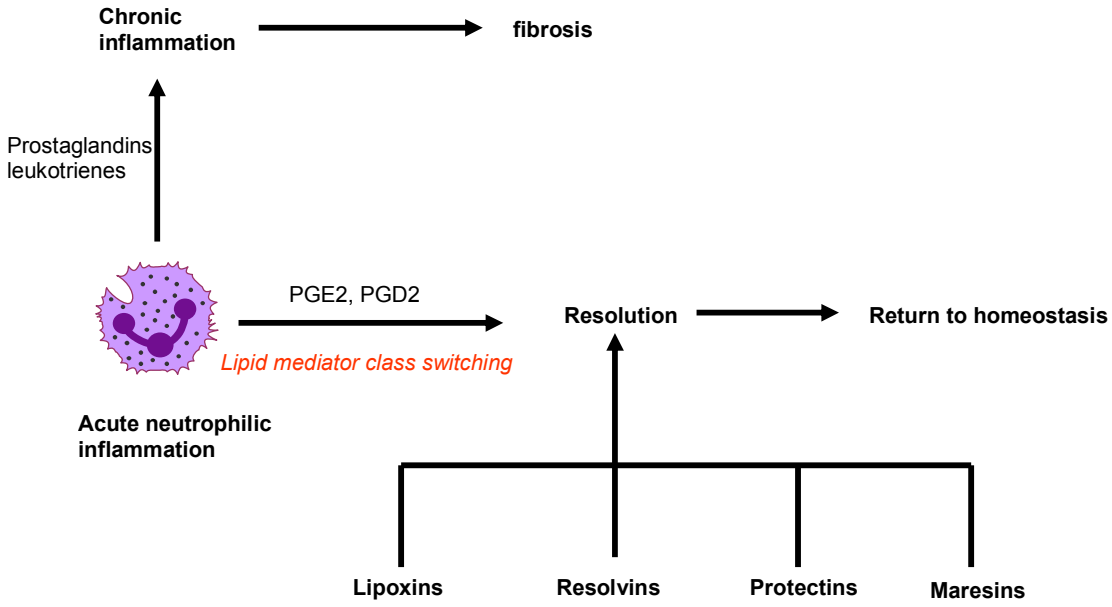


Figure 1.6: The outcome of acute inflammation. Adapted with permission from Macmillan Publishers Ltd; Nat Rev Immunol [80]. Copyright 2008.

1.11.1 AA-derived ω-6 lipid mediators

Once formed by the action of phospholipase A_2, AA is further metabolised by three main enzymatic routes: cycloxygenase (COX), cytochrome P450 (CYP) and lipoxygenase (LOX) pathways. Non-enzymatic pathways of metabolism also exist, mainly involving oxidative stress or reactions with free-radicals, leading to the production of isoprostanes [218]. A schematic representation of the enzymatic pathways is shown in figure 1.7. The next section specifically discusses metabolism of AA by the LOX pathway.

1.11.2 LOX pathway

There are numerous lipoxygenase (LOX) enzymes that convert AA to a variety of hydroperoxyeicosatetraenoic acids (HPETEs) and subsequently HETEs (hydroeicosatetraenoic acids). The most studied of these is 15-HETE, which will be discussed in more detail. The HETEs are subsequently converted to a variety of pro-inflammatory and pro-resolution lipid mediators, and the pathways leading to these are complex. Unlike prostaglandins, which are produced by most cells in the body, the products of LOX metabolism are primarily produced by inflammatory cells such as neutrophils, monocytes, mast cells and DCs.

The synthesis of these different eicosanoids is dependent on production of different precursors and enzymes within the tissue [215, 218].

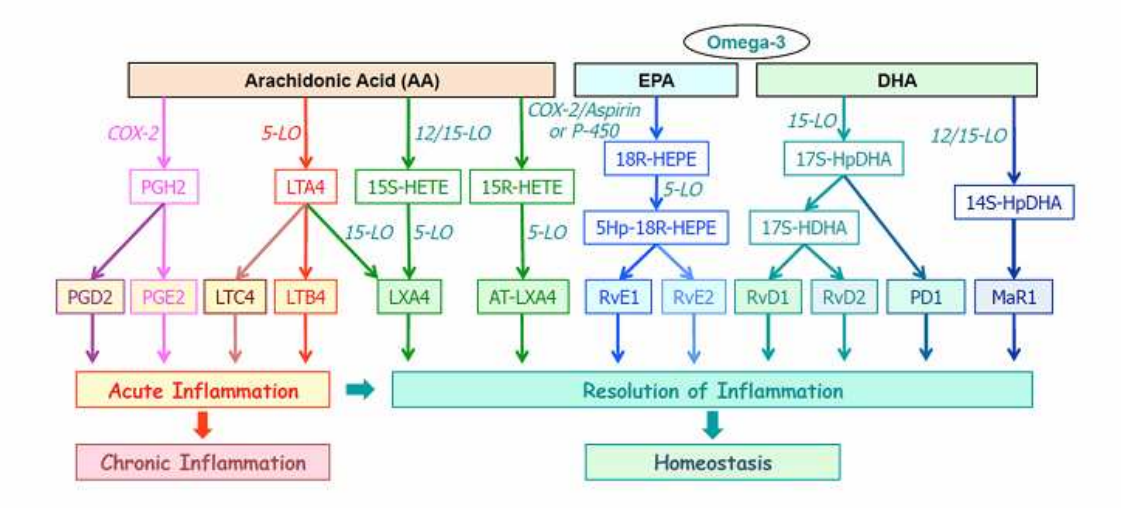


Figure 1.7: Biosynthetic cascades and actions of selected pro-inflammatory and pro-resolution lipid mediators derived from arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). LO=lipoxygenase, COX=cycloxygenase, PG=prostaglandin, LT=leukotriene, LX=lipoxin, RV=resolvin, PD=protectin, Ma=maresin. Reproduced with permission from the American Chemical Society; Chem Rev [79]. Copyright 2011.

1.11.3 15 LOX and 15-HETE

15-HETE is one of the most abundant AA metabolites in the human airway and is mainly synthesised by bronchial epithelial cells, through the enzymatic actions of 15-LOX [219]. It is predominately an intracellular mediator within epithelial cells and monocytes, conjugated with phosphatidylethanolamine (PE). There is no known receptor. The relationship of 15-HETE to inflammation is complex and it appears to have both pro-inflammatory and pro-resolution actions. In a human bronchial epithelial cell line (A549), up-regulation of 15-LOX led to increased levels of the pro-inflammatory cytokines CCL3, CCL5 and CXCL10 and promoted recruitment of immature DCs, mast cells and activated T cells [220]. However, the anti-inflammatory effects of 15-HETE are also well documented, in particular with regard to their role in lipoxin production. Upregulation of 15-LOX favours LXA_4 production at the expense of leukotriene synthesis through the actions of 5-LOX. This occurs through 15-HETE

competing for catalytic sites on 5-LOX, leading to reduced 5-HETE production and by competition for leukotriene A_4 [214].

1.11.4 15-HETE and 15-LOX in neutrophilic lung disease

The responses of neutrophils to the various HETEs are not well documented. The position of the hydroxyl residue determines firstly the naming of the HETEs and secondly their actions on neutrophils. 5- HETE appears to have the most significant effect on promoting neutrophil chemotaxis, followed by 8-HETE. 12-HETE has a less marked effect, followed by 11-HETE. Both 5-HETE and 12 –HETE aggregate neutrophils and this effect is not seen with the other HETE groups. 15-HETE is not known to induce neutrophil chemotaxis [221].

Research into the role of 15-HETE and 15-LOX in neutrophilic lung disease is limited. 15-HETE along with a number of other HETEs has been detected in the sputum from adult CF patients. However, no specific associations were seen with disease severity [222].

Expression of COX and LOX has been examined in nasal polyps of adult and paediatric CF patients using immunohistochemistry [223]. No statistically significant difference was seen in 5-LOX and 15-LOX staining between CF patients and controls.

As 15-HETE is not known to affect neutrophil chemotaxis, has not been associated with percent predicted FEV_1 in CF patients and 15-LOX sinonasal expression does not differ between CF and controls, this lipid pathway could be considered unimportant in neutrophilic lung disease. However there is some evidence to suggest 15-HETE and 15-LOX may be important in the neutrophilic airway. Previous work has involved adult patients, and not children with milder disease, and expression of 15-LOX in nasal mucosa may not be representative of the lower airways.

In a CF model using human epithelial cell lines, 15-LOX mRNA expression was increased in response to *Aspergillus fumigatus* sensitisation. Expression was also increased in response to a number of cytokines, in particular TNFα, IL-1β, IL-2, IL-3,

IL-4, IL-6 and IL-13 [224]. Furthermore, inhibition of 15-LOX has been proposed as a potential therapeutic target for both asthma and CF. In a systemic ova sensitisation model, 12/15-LOX knockout mice (the murine equivalent of human 15-LOX), have impaired airway inflammatory responses with reduced BAL eosinophils, lymphocytes, monocytes and tissue eosinophils. There was also a reduction in airway mucus, airway remodelling and levels of IL-4 and IL-13. However, although there was a trend there was not a statistically significant reduction in BAL neutrophils [225]. It is therefore uncertain whether targeting 15-LOX would necessarily directly affect airway neutrophilic inflammation, although it may have indirect actions through the inhibition of LTB_4, as discussed above.

These mediators have also not been examined in other neutrophilic lung diseases in either adults or children. The conflicting role of 15-HETE and 15-LOX in the inflammatory process makes this area of lipid metabolism an interesting focus for further study. While there appears to be a role in allergic and eosinophilic airway disease, the role of 15-HETE and 15-LOX in CF and neutrophilic lung disease is less clear, particularly in children with milder airway disease, and warrants further research.

1.11.5 Lipoxins

Lipoxins are produced at sites of inflammation during cell-cell interactions, generally between neutrophils and resident cells, and are potent anti-inflammatory lipid mediators. LXA_4 and lipoxin B4 (LXB_4) were the first lipid anti-inflammatory and pro-resolution mediators to be described [226]. There appears to be a phenotypic change in neutrophils to produce different lipid mediator profiles, in response to environmental changes. Neutrophils in resolving exudates switch lipid production from leukotrienes to lipoxins and resolvins [214]. Lipoxins promote inflammatory resolution through a variety of mechanisms. They inhibit the influx of neutrophils into tissue, reduce vascular permeability, prevent the release of azurophilic granules from neutrophils, inhibit the release of reactive oxygen species and promote the uptake of apoptotic neutrophils by macrophages [93, 227, 228]. Lipoxins are also potent chemoattractants for monocytes, which are needed for wound healing and repair [229]. They also reduce the production of pro-inflammatory cytokines including CXCL8 and increase the

production of anti-inflammatory cytokines [80, 228]. Lipoxins are mostly produced by the enzymatic action of 15-LOX, leading to the conversion of 15-HPETE to 15-(S) HETE. 15-(S) HETE can then be converted by 5-LOX in neutrophils and eosinophils to produce lipoxins [226]. LTA_4 derived from 5-LOX can be converted by platelet 12-LOX into LXA_4 [230].

The lipoxins, similar to many lipid mediators, signal via a G-protein coupled receptor [231]. The LXA_4 receptor is ALX, expressed on haematopoietic cells, including neutrophils and monocytes, and also on other tissues such as airway epithelial cells and synovial fibroblasts [232-234]. As discussed previously, ALX is also the receptor for the inflammatory pro-resolution protein AnxA1 – although it binds LXA_4 with greater affinity. Following ALX binding, intracellular signalling cascades vary between different cell types. Within monocytes, signalling via ALX, as with many G-protein coupled receptors, leads to intracellular calcium mobilisation. This promotes cell chemotaxis and adherence. However, this does not lead to proinflammatory responses in these cells [235]. In contrast, within neutrophils, LXA_4 – ALX signalling does not result in calcium mobilisation. This leads to an accumulation of presqualene diphosphate- a potent negative intracellular signal in neutrophils. Presqualene diphosphate inhibits phospholipase D and superoxide anion generation [236]. LXA_4 also reduces the formation of peroxynitrite (thought to be important in CXCL8 production) and therefore reduces neutrophil peroxynitrite signalling [237]. In addition, expression of CXCL8 appears to be reduced by inhibition of mRNA production by LXA_4 [237]. LXA_4 also down-regulates CD11b/CD18, and inhibits neutrophil migration through regulation of β2-integrin [238]. The synthesis of lipoxins can also be triggered by the action of aspirin on COX-2, leading to a lipoxin epimer. Epimeric forms of lipoxin function in the same way as natural lipoxins, but have a longer half-life and appear to be more potent than naturally occurring lipoxins [85]. Aspirin-triggered lipoxins have been shown to inhibit neutrophil migration through nitric oxide dependent mechanisms [239]. They also block components of the p38 MAPK pathway, thus inhibiting neutrophil chemotaxis and pro-inflammatory responses [240].

There is some evidence that LXA_4 – ALX signalling leads to regulation of pro-inflammatory and protective genes, including the transcriptional co-repressor NAB1 (a glucocorticoid-responsive gene) which was found to be upregulated by aspirin-triggered lipoxins in a murine model [241].

Transgenic mice which over-express ALX show an intense anti-inflammatory phenotype with reduced TNFα-induced NFκB signalling. As a result there is significantly reduced neutrophil infiltration following the administration of endogenous LXA_4. These mice also show an enhanced sensitivity to low doses of LXA_4 [242]. Airway inflammation and injury increases the expression of ALX on airway epithelial cells [243]. These findings suggest that defective lipoxin pathways may be associated with persistent inflammation.

1.11.6 Lipoxins in neutrophilic lung disease

Lipoxins have been studied in adults with CF. However, the role of lipoxins has not been looked at in other specific neutrophilic lung diseases, and there is little research involving children. One group examined LXA_4 in BAL from paediatric CF patients, and expressed this as a ratio of BAL LTB_4 and CXCL8 as a method of controlling for the level of inflammation. These levels were then compared to a control group of non-CF patients with varying degrees of airway neutrophilia and pathogen isolation. The control patients in this instance were therefore a poorly defined inflammatory group and were not those with isolated upper airway disease. This group reported no difference in LXA_4 or LTB_4 BAL concentration between CF patients or inflammatory controls. There was also no association between pathogen isolation and LXA_4 levels. However, CF children had lower LXA_4/LTB_4 and LXA_4/CXCL8 ratios as compared to inflammatory control subjects, and this did not vary with infection status. In contrast control subjects with pathogens had lower LXA_4/LTB_4 and LXA_4/CXCL8 ratios than those without pathogens [244]. This work therefore may imply a CFTR-specific effect, although this study compared CF to other non-neutrophilic diseases, and thus needs further exploration.

Sputum levels of LXA_4 increase in CF patients after antibiotic treatment and correlate with a fall in CXCL8 [245]. BAL LXA_4 is significantly reduced in adult CF patients as compared to inflammatory control subjects, including when controlling for the level of neutrophilic inflammation, implying a CF-specific defect in lipoxin function [13]. However another group showed that there was no difference between BAL LXA_4 levels in adult CF patients and inflammatory controls, implying the contrary [246]. An analogue of LXA_4 has also been shown to inhibit *Pseudomonas aeruginosa* induced production of CXCL8 from bronchial epithelial cells [13]. *Pseudomonas aeruginosa* also encodes for a secretory 15-LOX capable of generating 15-HETE from host arachidonic acid, leading to lipoxin production [254]. These bacteria may therefore be able to influence the local inflammatory response to infection, highlighting the fact that inflammation is needed to clear infection and therefore inappropriate lipoxin production may be detrimental. In a mouse model of CF, mice pre-treated with LXA_4 prior to pulmonary infection with *Pseudomonas aeruginosa* had less weight loss, reduced airway neutrophilia and bacterial load and a shift from an acute to a chronic inflammatory picture, as compared to those who were not pre-treated with LXA_4 [13].

Collectively this suggests an important role for lipoxins in CF, and that perhaps the lower levels of LXA_4 seen in adult CF patients may contribute to increased neutrophilic inflammation. What is unclear, however, is whether reduced levels of LXA_4 are due to the primary CFTR defect or whether they are due to severe bacterial infection and neutrophilia, which overwhelms the inflammatory pro-resolution process and thus prevents the return to pulmonary homeostasis.

1.11.7 Resolvins, Protectins and Maresins

These are inflammatory resolution lipid mediators. Resolvins are produced from DHA and EHA; protectins and maresins are produced from DHA.

1.11.8 Resolvins

Resolvins (resolution phase interaction products) are derived from ω-3 polyunsaturated fatty acids and were first described in resolving inflammatory exudates [247]. They are important in the termination of the acute inflammatory process. There are two groups

of resolvins formed through the actions of CYP. These are D resolvins derived from DHA and E resolvins derived from EPA [248]. As well as aspirin triggered lipoxins, resolvins can also be derived from aspirin-acetylated COX-2 [248]. A receptor for resolvin E1 (RvE1) has been identified as ChemR23 [249]. ChemR23 is present on monocytes, macrophages and dendritic cells. Similar to ALX, ChemR23 is a G protein-coupled receptor and can bind protein as well as lipid mediators. The protein mediator it binds is chemerin – a chemotactic agent found in inflammatory exudates. Chemerin leads to chemotaxis of DCs and macrophages and appears to have a role in inflammatory resolution [250]. A further receptor for RvE1 has been identified as BLT_1, to which RvE1 acts as a partial agonist and antagonist [251]. BLT_1 is also the receptor for LTB_4 and is expressed on neutrophils, eosinophils. monocytes and T cells. Therefore through its effects on BLT_1 RvE1 may also mediate the pro-resolution process. Two leukocyte receptors have been identified for resolvin D1 (RvD1), the AnxA1 receptor, ALX and G protein-coupled receptor 32 (GPR32) [252].

Both D and E resolvins have a number of pro-resolution and anti-inflammatory properties, including shortening the resolution process [252]. RvE1 inhibits DC migration and release of pro-inflammatory IL-12 [249, 253]. Within neutrophils, RvE1 inhibits generation of superoxide as well as inhibiting neutrophil migration, and upregulating CCR5. CCR5 expression on apoptotic neutrophils acts as a terminator of cytokine signalling [254, 255]. In an animal model, RvE1 promotes airway inflammatory resolution through suppression of IL-23 and IL-6, and therefore Th17 cells, and upregulation of IFNγ and LXA4 [256]. RvD1 inhibits LPS-release of TNF by macrophages as well as also inhibiting neutrophil recruitment in a concentration-dependent manner [257, 258].

A number of animal models have been developed highlighting the inflammatory resolution effects of the resolvins. RvE1 promoted resolution of inflammation in a mouse model of an acute exacerbation of allergic asthma [259]. Pre-treatment with RvD1, in a mouse model of lipopolysaccharide-induced acute lung injury, revealed RvD1 to have potent anti-inflammatory actions through reduction in the expression of TNFα and IL-1β, and increased expression of IL-10. RvD1 also inhibited aberrant neutrophil recruitment and stimulated apoptosis of neutrophils [260].

Given the fact that beneficial effects of dietary ω-3 fatty acids have been found in a number of human diseases, particularly cardiovascular disease, it is possible that they may have a role in airway inflammatory disease. However, aside from animal models, there is limited research into the role of resolvins in human airway disease. RvE1 has been detected in sputum from adult CF patients and raised levels of this lipid mediator were associated with improved percent predicted FEV_1 [222]. It is possible therefore that low levels of pro-resolving lipid mediators may be important in the CF airway.

1.11.9 Protectins

Protectins are generated from DHA through the actions of 15-LOX [247, 261]. Protectin D1 (PD1) reduces neutrophil migration and infiltration in a mouse model of peritonitis [262]. PD1 levels are lower in exhaled breath condensates from patients undergoing asthma exacerbations, as compared to healthy controls, suggesting protectins may be important in airway inflammatory resolution [263]. In a mouse model of asthma, PD1 administration prior to aeroallergen challenge led to reduced eosinophil and lymphocyte recruitment and reduced Th2 cytokines [263]. The role of protectins has not been examined in neutrophilic lung disease, but given the findings in asthma it is possible that these lipid mediators may be important in inflammatory resolution in other airway diseases. Any differences seen in neutrophilic lung disease as compared to healthy subjects may be representative of changes in 15-LOX, an area that requires further investigation.

1.11.10 Maresins

Maresins (macrophage mediator in resolving inflammation) are derived from DHA through the actions of 14-LOX. Little is currently known about the actions of maresins, but they are thought to be produced by macrophages and enhance phagocytosis of apoptosing neutrophils. They therefore appear to have a role in maintaining tissue homeostasis, wound healing and host defence [86].

1.12 Summary

Although there has been increasing interest and momentum in the field of active resolution of inflammation, and a number of mediators and mechanisms are now

recognised, their role in paediatric neutrophilic lung disease remains uncertain. Neutrophilic inflammation remains an important mechanism for the removal of invading pathogens, and while it must be remembered that modulating this pathway may not always be beneficial, evidence from previous studies in CF has suggested there may be a role for inflammatory resolution therapies in neutrophilic lung disease. Experimental work examining pro-resolution mediators and mechanisms has concentrated on CF rather than other neutrophilic lung diseases, and predominately involves adults. Paediatric patients with neutrophilic lung disease generally have a milder phenotype and therefore pre-existing adult data cannot merely be extrapolated to children. The differences in prognosis between paediatric neutrophilic lung diseases may be related to the capability for resolution of inflammation; if this were the case, this would lend further support to the concept of the therapeutic manipulation of resolution of inflammation. Furthermore, the use of non-CF neutrophilic diseases as controls may help determine if pathways important in CF are specifically related to CFTR dysfunction, or are a non-specific manifestation of inflammation and infection. For these reasons an understanding of mediators and mechanisms involved in active resolution of infection in children may lead to future therapies which may promote the maintenance of pulmonary homeostasis and thus preserve pulmonary function for longer. Thus the hypotheses, aims and objectives of this thesis are as follows.

1.13 Hypotheses

1. Failure to actively terminate the acute inflammatory process is potentially important in the pathophysiology of childhood neutrophilic airway disease.

2. The differences in prognosis between childhood diseases characterised by airway neutrophilia are related to the extent to which neutrophilic inflammation can actively be resolved.

1.14 Aims

1. To recruit children with neutrophilic lung disease (CF, CF NBS, bronchiectasis, PCD and PBB) and those without lower airway disease (controls) undergoing flexible bronchoscopy, and obtain BAL and EBB samples.

2. To utilise BAL and EBB to measure mechanisms important for the resolution of airway inflammation.

1.15 Objectives

Endobronchial biopsies:

1. To establish an immunofluorescent staining protocol for AnxA1 and its receptor ALX.
2. To measure mRNA levels of AnxA1 & ALX.
3. To establish an immunofluorescent staining protocol for LKLF.
4. To establish an immunofluorescent staining protocol for 15-LOX.

Bronchoalveolar lavage:

1. To measure pro and anti-inflammatory cytokines.
2. To establish a flow cytometry protocol for paediatric BAL to investigate numbers of different lymphocyte subsets - in particular CD4+CD25+FoxP3+ T regulatory cells.
3. To establish an immunofluorescent staining protocol for AnxA1 and its receptor ALX using BAL cytospins.
4. To measure mRNA levels of AnxA1 & ALX in BAL cell pellets.
5. To collaborate with Professor V O'Donnell's laboratory in Cardiff in the measurement of lipid mediators by LC/MS/MS.

The next chapter describes the subjects recruited to the study, and methods generally applicable to subsequent chapters. There are detailed methods for specific experiments in individual chapters looking at aspects of resolution of inflammation.

Chapter 2

Subjects and generic methods

2.1 Introduction

The previous chapter described the background to the development and resolution of neutrophilic inflammation and the potential importance in airway diseases, leading to the propounding of the hypotheses to be tested in this thesis. This next chapter describes the patient groups, the acquisition of BAL and EBB samples from these paediatric patients and methods that are common to subsequent chapters. Specific methods will be discussed in later sections.

2.2 Research subjects

Children undergoing clinically indicated flexible bronchoscopy (bronchoscopy performed solely for research is unethical in children), with one of the following confirmed or suspected diagnoses, were considered for inclusion into the research study:

- CF – established disease
- CF – diagnosed by newborn screening (age $\leq$ 6 months)
- PCD
- Non-CF, non-PCD bronchiectasis (referred to from now on as bronchiectasis)
- Persistent bacterial bronchitis
- Chronic respiratory symptoms (CRS – explained in more detail below)
- Children without lower airway disease – control subjects

2.2.1 CF patients (established disease and CF NBS)

Patients with CF diagnosed in accordance with standard guidelines were eligible for inclusion [264]. Diagnostic criteria were:

- The presence of one or more characteristic clinical features (for example chronic cough and sputum production, persistent infection with typical CF respiratory pathogens, persistent chest radiograph abnormalities or poor growth and nutritional status)
- Or a sibling with CF
- Or diagnosis by standard NBS

  AND
- Laboratory evidence of an abnormality in the CFTR gene or protein

- Abnormal sweat chloride concentration difference (performed twice) or nasal potential difference
- Or identification of a CF disease-causing mutation in each copy of the CFTR gene

2.2.1.a CF Pseudomonas aeruginosa infection status

Airway infection with *Pseudomonas aeruginosa* is a marker of increased disease severity in patients with neutrophilic airway disease, and in particular CF [18]. In this thesis Pseudomonas infection was detected in CF and PCD subjects and one CRS patient. CF and PCD patients were therefore divided into those that had ever isolated *Pseudomonas aeruginosa* in cough swabs, sputum or BAL, and those that had not. Lee *et al* have further refined the definition of Pseudomonas infection in CF, by subcategorising infective status into those that recurrently isolate Pseudomonas, and those that have evidence of previous infection [265]. There were insufficient patient numbers in this thesis to perform this type of categorisation and therefore the crude definition of ever versus never isolated Pseudomonas was used.

2.2.1.b Indication for bronchoscopy

The following were clinical indications for bronchoscopy in CF patients:

- To identify occult lower airway pathogens that may be contributing to a decline in respiratory status. These children had responded poorly to empirical antibiotic treatment.
- As a therapeutic manoeuvre to treat lobar collapse that had not responded to physiotherapy, antibiotics and inhaled mucolytics.
- At new diagnosis - this included those with established disease but diagnosed late and also babies identified through NBS. This was to identify occult infection that would otherwise go untreated, and to assess the extent of pulmonary inflammation [23, 24].

Ethical approval (discussed below) was also given for flexible bronchoscopy if the child was undergoing general anaesthesia for another reason. Potential circumstances included insertion or removal of a totally implantable venous access device,

peripherally inserted central catheter line or a multi-lumen neck line. Children having general anaesthesia for a peripherally inserted central catheter line or a multi-lumen neck line were generally those with either or both of difficult intravenous access and needle phobia. The line was usually sited at the start of an admission for intravenous antibiotics to treat a pulmonary exacerbation

2.2.1.c Definition of CF acute respiratory exacerbation

There is no standardised definition of a respiratory exacerbation in CF patients [266]. The indication for bronchoscopy in most patients was a decline in respiratory status (for example decline in lung function or persisting wet cough) with a view to identifying occult airway pathogens. Therefore by definition the majority of patients were not clinically asymptomatic. This made dividing patients into those who had a pulmonary exacerbation and those who were stable challenging as only a small number had a bronchoscopy while genuinely stable (for example opportunistically while under general anaesthesia for insertion or removal of a totally implantable venous access device). Therefore for the purposes of this study a respiratory exacerbation was defined as an episode severe enough to warrant admission for intravenous antibiotics, as determined by a respiratory paediatrician independent of the study.

2.2.2 PCD

Children with PCD were included as they tend to have a somewhat similar but milder respiratory phenotype to CF patients, making them a useful comparison group. Patients with PCD diagnosed by standard criteria were eligible for inclusion [34]. Diagnostic criteria included the presence of the characteristic clinical phenotype (for example chronic productive or wet cough, persistent rhinorrhoea, chronic sinusitis, otitis media with effusion or heterotaxic syndromes) and either specific ultrastructural ciliary defects identified by transmission electron microscopy, evidence of abnormal ciliary function or low nasal nitric oxide. These children underwent flexible bronchoscopy in order to identify occult pathogens in the lower airway leading to a decline in respiratory status after failure of response to empirical antibiotic therapy; or because of persisting lobar collapse.

2.2.3 Bronchiectasis

As with patients with PCD, patients with a diagnosis of bronchiectasis also provide a suitable comparison group due to their similar respiratory manifestations but milder clinical picture. Diagnostic criteria included a persistent wet or productive cough and radiological evidence of bronchiectasis [46]. In addition there was no evidence of CF or PCD in these patients using the diagnostic criteria outlined above. Bronchoscopy was performed in these children in order to identify occult pathogens in the lower airway leading to a decline in respiratory status after failure of response to empirical antibiotic therapy.

2.2.4 Persistent bacterial bronchitis

Children were suspected to have PBB on the basis of a prolonged persistent wet cough of at least 4 weeks duration, no evidence of bronchiectasis on CT scan and no alternative diagnosis (following full respiratory investigation, including two negative sweat tests (or negative CF mutation analysis), no evidence of gastro-oesophageal reflux disease or aspiration, normal immune investigations and normal cilia or nasal nitric oxide). Flexible bronchoscopy was performed in order to identify lower airway pathogens and BAL cellular profile in children with persistent respiratory symptoms who had not responded to empirical therapy. These children were only considered to have a diagnosis of PBB if, in addition to these features and no evidence of an alternative diagnosis, they were found to have a positive bacterial growth and neutrophilia ($\geq 4\%$) on BAL [56, 267, 268].

2.2.5 Chronic respiratory symptoms

A number of children were investigated for a persistent wet cough and suspected clinically to have PBB on the basis of the criteria described above. However, in the absence of positive bacterial growth or neutrophilia in BAL, by the criteria used these children could not be labelled as PBB. In addition some children thought to be control patients had positive microbiological cultures or neutrophilia on BAL, and could therefore not be included in the control group. These two groups of children were therefore termed as CRS, and were used in the flow cytometry experiments as they had undergone flow cytometry analysis prior to microbiology or cytology results being

available. This group did not include children with clear alternative diagnoses such as asthma.

2.2.6 Control subjects

Ideal control subjects would be from a disease group which resolves inflammation normally. However this was not possible and therefore control patients in this study were those subjects undergoing clinically indicated flexible bronchoscopy for upper airway problems (for example stridor) in the absence of lower airway disease (table 2.1). These control paediatric patients are by definition never truly normal or they could not undergo bronchoscopy. Some patients included had reported haemoptysis, but without evidence for this seen on bronchoscopy and without haemosiderin laden macrophages. These children were only included if they had normal cytology, no bacterial growth on BAL and no evidence of viral infection by BAL immunofluoresence [268]. Occasional EBB from children were included with a macroscopically normal bronchoscopy, but no lavage sample taken. These are discussed in relevant sections where they were used due to small patient numbers. Ethical approval was also given to perform research bronchoscopies on children undergoing general anaesthesia for cardiac catheterisation. Children in this group were excluded if there was a history of respiratory disease, pulmonary hypertension, cyanotic heart disease or if they were to be anti-coagulated during catheterisation. Following informed consent, flexible bronchoscopy was only performed on these children if the anaesthetic, cardiology and respiratory teams were in joint agreement, the child was clinically stable and sufficient time could be allowed for the procedure to be performed. During sample collection for this thesis the author did not collect any samples from these patients. However there are stored samples available from these patients which were included in some experiments.

2.3 Measurement of height and weight

The height (cm) (or length of children unable to stand) and weight (kg) of each patient was recorded on admission. Although this is standard clinical practice, the height was not recorded for some children, and so for these children data was incomplete. Children under 1 year of age were weighed without clothes or nappy. Those between 1

and 5 years of age were weighed in underwear only; those over 5 years of age were weighed in light clothing.

Table 2.1: Characteristics of control patients

Patient number	Indication for bronchoscopy	Bronchoscopy findings
1	Dry cough	Normal
2	Dry cough	Normal
3	Dry cough	Normal
4	Haemoptysis	Normal
5	Stridor	Laryngomalacia
6	Stridor	Laryngomalacia
7	Previous stenosis left main bronchus	Normal
8	Stridor	Vocal cord dysfunction
9	Haemoptysis	Normal
10	Dry cough	Normal
11	Haemoptysis	Normal
12	Haemoptysis	Normal
13	Stridor	Vascular ring
14	Recurrent croup	Adenotonsillar hypertrophy
15	Stridor	Normal
16	Recurrent croup	Tonsillar hypertrophy
17	Dry cough	Normal
18	Stridor	Laryngomalacia
19	Dry cough	Normal

2.4 Spirometry

Spirometry was performed where age appropriate (generally children > 5 years of age) and clinically indicated. Lung function performance, calibration and quality control were conducted as per American Thoracic Society / European Respiratory Society

guidelines [269]. Calibration was performed daily with a standard 1 litre syringe. Three reproducible flow volume loops with <10% variability in FEV_1 were performed and the best result documented. The percent predicted FEV_1 and FVC from the most recent spirometry before bronchoscopy was recorded, using Rosenthal reference data and an Alpha Touch spirometer [270]. CF NBS and the majority of PBB patients were too young to be able to perform spirometry.

2.5 Flexible bronchoscopy in children

Flexible bronchoscopy in children is a well-established tool for the investigation of respiratory disease and the acquisition of lower airway samples. We followed established guidelines for performing flexible bronchoscopy and processing of samples [271, 272]. Reference values also exist for normal cellular contents of BAL from healthy children [268, 273]. The safety of paediatric flexible bronchoscopy has been previously described in a large series [274].

The acquisition of endobronchial biopsies has become an established part of bronchoscopy and guidelines have been published on endobronchial biopsy acquisition, processing and analysis [275]. The safety of acquiring endobronchial biopsies in children has been previously established [276-279].

In contrast to adult subjects, it is not ethical to perform bronchoscopies purely for research purposes. However, with ethical approval and the consent of parents/guardians and age-appropriate assent from children, it is acceptable to take additional samples at a clinically indicated bronchoscopy for research purposes.

2.6 Consent

Consent (and age appropriate assent from children) was obtained by the author from parents/guardians for their child to take part in the study. Consent for the use of historical stored samples was obtained by previous colleagues.

2.7 Anaesthesia

All bronchoscopies were performed under general anaesthesia in an anaesthetic room at the Royal Brompton Hospital. Paediatric flexible bronchoscopy performed under general anaesthesia has been previously described [280]. There was no set anaesthetic protocol, and induction of anaesthesia was intravenous or inhalational at the discretion of the anaesthetist. Volatile anaesthetic agents were used for maintenance of anaesthesia. Airway management was the choice of the anaesthetist after discussion with the bronchoscopist. Thus the bronchoscope was passed through a face mask, laryngeal mask airway or endotracheal tube depending on the clinical circumstances. The choice of spontaneous or controlled ventilation was also based on the clinical situation on discussion with the anaesthetist. On occasions, and where there was evidence of vocal cord spasm, the vocal cords were sprayed under direct vision with 1% lignocaine spray. No anaesthetic was used more distally in the airways. Full anaesthetic monitoring was employed throughout.

2.8 Bronchoscopes

A number of different sized bronchoscopes were used: Olympus BF-XP40 (2.8mm external diameter), BF-3C20 or 3C40 (3.6mm), BF-MP60 (4.0mm videobronchoscope) or BF-P20D (4.9mm) bronchoscopes (Keymed, Southend-on-Sea, Essex, UK) were used, depending on the size of the child. The 4.9mm bronchoscope was generally used for children over the age of 7 years, but it was sometimes necessary to use this in younger children, particularly CF patients, as the suction channel of the smaller bronchoscopes made clearance of mucopurulent secretions more difficult.

2.9 Bronchoalveolar lavage

The bronchoscope was wedged into a bronchus thus isolating the distal airway. Bronchoalveolar lavage was obtained using 0.9% sodium chloride (3 aliquots each 1ml/kg – maximum lavage 40ml per aliquot) generally from the right middle lobe (as this tends to give the greatest yield) [271] and lingula, unless imaging or findings at bronchoscopy dictated that the lavage should be taken from an alternative anatomical site. The fluid was instilled by syringe through the suction channel of the bronchoscope,

aspirated back using gentle wall suction of up to -30kPa and collected in sterile polyproylene containers (Bibby Sterilin, Stone, UK). The fluid was mixed by inversion and pooled. There is no consensus as to whether BAL samples should be pooled [271], and it has been shown that the first BAL return tends to have a higher neutrophil and lower lymphocyte count in children with CF, respiratory infection and healthy controls [268, 280-282]. However standard clinical practice was to pool samples and so this practice was continued for research purposes. BAL samples that remained after clinical samples had been taken were aliquoted, placed on wet ice and retained for research purposes.

Clinical BAL samples were sent to the hospital microbiology laboratory (for standard microbiological culture, staining for acid-fast bacilli and culture for mycobacteria) and virology laboratory (for viral immunofluorescence). Culture of samples was performed using CF Trust protocols (www.cysticfibrosis.org.uk/media/82034/cd-laboratory-standards-sept10.pdf). Cytospins for cell differentials and staining for fat laden macrophages were performed by the cytology department at the Royal Brompton Hospital and a clinical report provided. 300 cells were counted and cell differentials (neutrophils, macrophages, lymphocytes and eosinophils) were calculated as a percentage of the total number of cells in the sample. A note was made of the presence of squamous, epithelial and red blood cells, but these were not included in the denominator as per current guidelines [271]. Some BAL samples (particularly those from CF patients) were too degenerate to adequately assess the cell differential; as a result it was not possible to obtain a BAL cell differential for all samples.

The remaining pooled BAL sample was vortexed and, if a sufficient volume was obtained, 1-2 500µl aliquots of unprocessed BAL were placed in cryovials (Nunc, Hunter Scientific, Essex, UK) and stored at -80^{o}C.

2.10 Cell counts

Samples were processed within 4 hours. 50µl of vortexed BAL was taken and added to an equal volume of trypan Blue (Sigma Aldrich, Dorset, UK) for cell counting. 10µl of the mix was placed on each side of a haemocytometer, and cell numbers counted in 4

large squares from each side (8 squares total). A record was made of live cells and the number of squamous and epithelial cells. Red blood cells were not counted. The overall cell count was calculated using the following formula:

$$\frac{\text{Number of cells counted}}{\text{Number of squares counted}} \times \text{dilution} \times 10^4$$

The remaining BAL was centrifuged at 960g for 10 minutes at 4°C (Biofuge 22R Heraeus Sepatech). The supernatant was stored in 500µl cryovial aliquots and stored at -80°C. 1ml of RNAlater (Ambion, Applied Biosystems, Warrington, UK) was added to the remaining cell pellet and was also frozen at -80°C.

2.11 Endobronchial biopsies

Up to six endobronchial biopsies were obtained from the sub-carinae of 3rd or 4th generation bronchi. Samples were predominately taken from the right lower lobe, unless an alternative anatomical site was preferred for clinical reasons. Samples were however only taken from one side (usually right) for safety reasons.

Re-usable forceps (FB-56D, oval cup with rat tooth jaw, KeyMed) were used with the 2.8mm and 3.6mm bronchoscopes. Larger single use forceps (FB-231D, oval cup standard, KeyMed) were used with the 4.0mm and 4.9mm bronchoscopes.

The biopsy sample size was visually assessed, and if adequate this was placed in the appropriate medium as outlined below. Where successful, the smaller forceps usually yielded a biopsy approximately 1 mm in diameter. However, the smaller forceps were more difficult to use and obtaining tissue samples of appropriate quality was challenging. The larger forceps gave biopsies that were up to approximately 2 mm in diameter.

One biopsy was placed in 1ml of RNAlater in an RNAse, DNAse-free cryovial (Elkay, Hampshire, UK), placed on wet ice and then frozen at -80°C.

The remaining biopsies were placed in 10% formal saline and processed by the histopathology department at the Royal Brompton Hospital into paraffin wax blocks, between 4 and 24 hours after formal saline fixation. Paraffin fixed biopsies were cut using a microtome into 5µm thick serial sections. Sections were stained using haematoxylin and eosin (H&E) in order to assess tissue quality and morphology. If the sample did not yield adequate tissue, the block was cut again. If despite this it was not possible to obtain an adequate sample, a further biopsy was selected. All slides were examined by the author. Those with suitable morphology and architecture with identifiable epithelium, reticular basement membrane (RBM) and submucosa, were selected for further experiments (figures 2.1a and 2.1b).

A clinical report was provided by Professor Andrew Nicholson, department of histopathology, Royal Brompton Hospital. All samples were handled in accordance with the requirements of the Human Tissue Act and other relevant legislation.

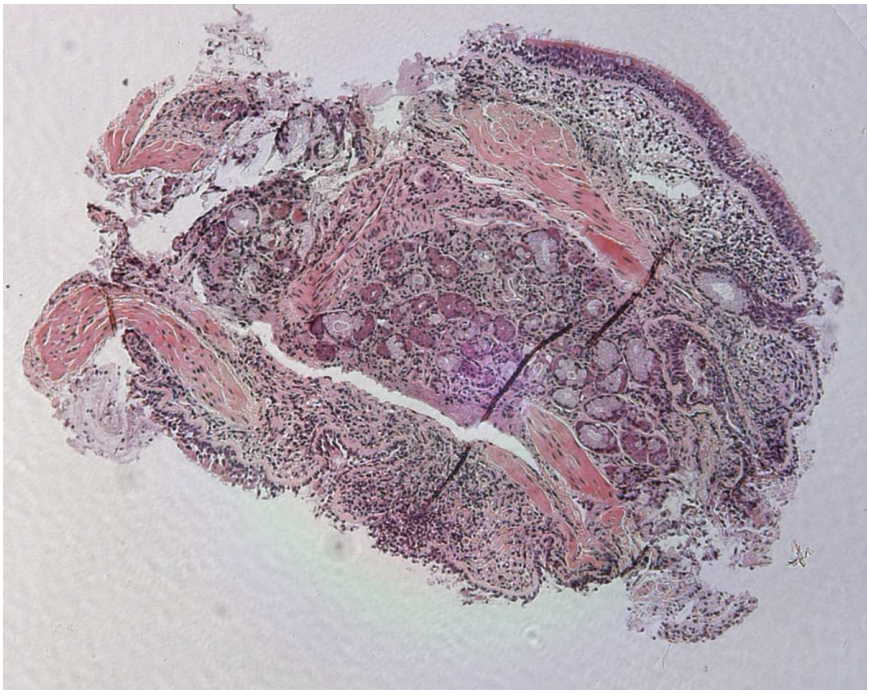


Figure 2.1a: Example of section from endobronchial biopsy. H&E stain. X 5.
Reproduced from T Hilliard MD Thesis: Airway inflammation and remodelling in CF.

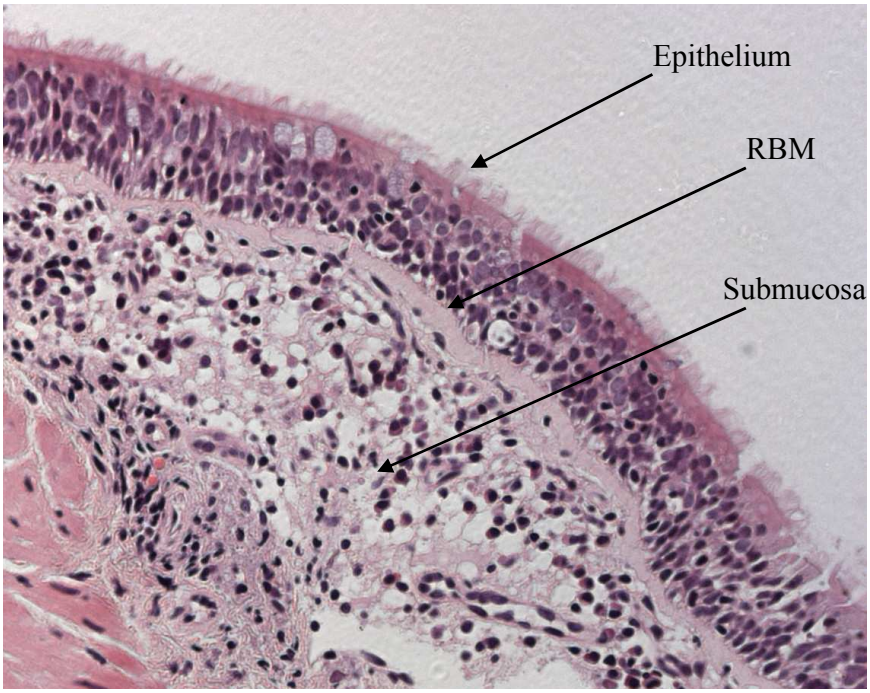


Figure 2.1b: Higher power view of same section, showing epithelium, RBM and submucosa. X 20.
Reproduced from T Hilliard MD Thesis: Airway inflammation and remodelling in CF.

2.12 Previously acquired samples

This work follows on from a number of previous studies, under the same ethical approval looking at airway inflammation in paediatric neutrophilic lung disease. This led to the establishment of a sample bank and clinical database of previous recruits. These samples include BAL stored at -80^{o}C as well as paraffin-fixed endobronchial biopsies. On occasions it was necessary to utilise these samples in order to have access to a sufficient number of, for example, control biopsy samples. All other samples were collected by the author.

2.13 Ethics

Ethical approval was obtained from the research ethics committee of the Royal Brompton and Harefield NHS Foundation Trust and National Heart and Lung Institute (granted on 12th February 2003, reference 02-302). From 27th April 2010 a Biobank tissue bank storage facility (Biomedical Research Unit Advanced Lung Disease Biobank) came into effect, and was used for the storage of samples (reference 10/H0504/9).

2.14 Data collection and statistical analysis

Clinical data and results from the initial processing of BAL were contemporaneously entered into a purpose built Access database (Access 2000, Microsoft, Redmond, WA, USA). The database was stored securely at the Department of Gene Therapy and only patient identifiable data necessary for the study was collected and recorded. All statistical analysis and graph production was performed using Prism version 6, Graphpad software, California, USA.

Non-parametric statistical tests were used throughout due to the small patient numbers in some groups. Comparison between two samples was made using the Mann-Whitney test. Comparison between three samples or more was made using the Kruskal-Wallis test. If a significant difference was found ($p<0.05$) then a Dunn's post-test was applied to look for differences between groups. The Spearman Rank correlation coefficient was applied to see if there was an association between sets of ordinal data. The Chi-Squared test was used to assess categorical data. Statistical significance was defined as $p<0.05$. Variables with a skewed range were $\log_{10}$ transformed before analysis.

A power calculation to calculate the minimum sample size was not possible due to insufficient published data. The aim was to recruit numbers comparable to previous paediatric bronchoscopy studies. On occasions some outlier results were excluded from the analysis. For the purposes of this thesis an outlier was defined as an observation that lay an abnormal distance from other values in the population. If outliers were excluded, this is clearly indicated in the results.

2.15 Specific methods and patient details

The following chapters discuss specific methods, patient details, results and discussion for individual experiments. Table 2.2 shows the overall numbers and details of patients enrolled in the study. In total samples were collected from 189 patients. Since there were limitations in the amount of biological material that could be obtained from each child, not every child contributed to each experiment in this thesis. The details of subjects in the individual studies have been reported in the respective chapters.

	Controls	CF	CF NBS	Bronchiectasis	PCD	PBB	CRS
Number	18	56	29	19	18	27	22
Age yrs (IQR)	**11 (3.2-12.9)**	**7.7 (13.9-12.6)**	**0.25 (0.25-0.33)**	**8.1 (4.8-11.6)**	**10.7 (7.7-12.1)**	**2.9 (1.3-5.1)**	**3.1 (1.5-7.1)**
Sex male (%)	**8 (44)**	**29 (52)**	**11 (38)**	**15 (79)**	**8 (44)**	**10 (37)**	**15 (68)**
FEV_1 % (IQR)	**113 (84-124)**	**69 (59-76)**	_____	**76 (56-95)**	**62 (48-76)**	_____	**88 (85-127)**
BMI kg/m^2 (IQR)	18.8 (17.3-23.5)	16.8 (15.6-18.4)	_____	16.9 (15.5-18.3)	18.5 (15.9-22.2)	17 (16-18)	16.4 (15.1-17.4)

Table 2.2: Total patient characteristics. Patient age was significantly lower in CF NBS as compared to controls, CF, bronchiectasis, PCD, CRS ($p<0.0001$) and PBB ($P<0.001$). The percentage of male patients varied between groups ($p<0.05$). % FEV_1 was significantly higher in controls as compared to CF ($p<0.05$) and PCD ($p<0.01$). IQR = interquartile range.

Each chapter first describes the specific experimental methods. The patient data for each experiment is tabulated, and is expressed as number (percentage) or median (interquartile range). Statistically significant differences between groups are highlighted in bold. Data was also collected for BMI, percent predicted FEV_1, percentage BAL neutrophils and pathogen isolation. There were no differences in BMI, as a marker of nutritional status, in any of the groups. BMI was not recorded for CF NBS subjects as this is not considered to be meaningful in these young patients. In addition data is shown for the number of patients with spirometry and cytology data available, the presence of a pulmonary exacerbation and prescribed medication which may have affected the inflammatory status of the airway. Although data regarding prescribed medication was recorded, overall patient numbers prescribed specific treatments were limited. It was therefore not possible to draw conclusions regarding

the effects of these treatments on inflammatory resolution in any of the experiments. Any notable findings regarding prescribed treatment are however discussed. Overall no association was seen between the mediators analysed and percent predicted FEV_1, unless specifically stated. The number of patients undergoing a pulmonary exacerbation was also limited and therefore it was not possible to assess the effect of an exacerbation on the mediators analysed. In addition, no association was seen specifically with airway infection (defined as a positive microbiological culture on BAL) or *Pseudomonas aeruginosa* status (generally due to small patient numbers) unless specifically specified.

The analysis and results follow a similar pattern in each section. For each mediator, results are displayed as levels in individual patient groups. Results are also expressed as a ratio of BAL neutrophil count, to normalise for airway inflammation. Thus differences may be seen between groups, but if on data normalisation no differences are seen then the initial differences are not disease-specific. Ratios of mediators to CXCL8 were not used throughout as CXCL8 may not be the only or most important neutrophilic chemoattractant. As patient numbers were often limited, a combined group was created of bronchiectasis / PCD and PBB. This combined group was labelled as chronic suppurative lung disease (CSLD). Combining bronchiectasis and PCD patients together for analysis has previously been performed [283]. Results were compared between CSLD, CF (termed established CF to distinguish from CF NBS) and controls, to see if there were differences due to neutrophilic diseases as compared to the control groups, and whether differences were related to disease severity. In addition, all CF (established CF and CF NBS) were compared to CSLD to see if differences were due to the CFTR-defect.

Results were analysed for evidence of association with markers of airway inflammation (BAL neutrophil count, CXCL8 and IL-10). CXCL8 was used as a pro-inflammatory cytokine as this is mostly used by studies examining neutrophilic inflammation given its marked chemoattractant effect on neutrophils, and IL-10 as this is typically thought of as an anti-inflammatory cytokine. However, there are limitations with using these two cytokines alone as analysis is on the assumption that these are the predominant

cytokines involved in the airway inflammatory process. The choice of these markers was largely hypothesis generating. Correlation analyses were performed by collating all patients together (controls, CF, CF NBS, bronchiectasis, PCD and PBB), CSLD and all CF patients.

A discussion follows for each section – with the principle findings, strengths and weaknesses, relation to previous published work and the meaning of the results outlined for that section. The final chapter outlines the overall results in relation to the original hypotheses, with a discussion of strengths and weaknesses pertinent to all chapters. The thesis concludes with unanswered questions and proposals for future research.

Chapter 3

Cellular and Cytokine analysis of BAL

3.1 Analysis of BAL for cytokines and T regulatory cells

This chapter discusses the methods and results from multiplex cytokine and flow cytometry analysis of BAL. Multiplex cytokine analysis of BAL was undertaken in order to relate findings of other mediators analysed in this thesis (AnxA1, Tregs, LKLF and lipid mediators) to the pro- and anti-inflammatory cytokines (CXCL8 and IL-10 respectively). IL-10 was of interest given this cytokine is generally reported to have anti-inflammatory properties, although there is some evidence suggesting it may also have pro-inflammatory properties under certain circumstances. There is strong evidence that Tregs are critical in maintaining pulmonary homeostasis. However, although there is published literature on the role of Tregs in allergic airways disease, there is at present no research into their role in paediatric neutrophilic lung disease. The flow cytometry results in this chapter aim to investigate this further. The first section discusses cytokine analysis of BAL. The hypotheses of this first section are as follows:

- BAL CXCL8 is increased in severe neutrophilic inflammation (established CF) as against more mild disease (PCD, bronchiectasis and PBB), relative to the levels of the putative anti-inflammatory cytokine IL-10
- BAL CXCL8:IL-10 ratio is higher in CF than other CSLDs due to a direct effect of CFTR dysfunction

3.2 Multiplex cytokine analysis of BAL (Luminex)

Cytokine analysis of stored BAL samples was performed by Dr Rebecca Thursfield, National Heart and Lung Institute, Royal Brompton Hospital as part of her MD(Res) thesis. Cytokine measurements were performed on BAL supernatant using a multiplex cytokine assay (Bio-rad Inc., Human cytokine assay) for the following: IL-1β, IL-2, IL-6, CXCL8, IL-10, IL-12 p70, IL-17, IFNγ, TNFα and GM-CSF. The Bio-rad multiplex assay was chosen as it contained pro and anti-inflammatory cytokines pertinent to this thesis. In addition samples were analysed for IL-4 using a specific Luminex plate. IL-4 was chosen due to its known association with 15-HETE and 15-LOX function – given this cytokine is associated with higher levels of 15-LOX, and 15-HETE and 15-LOX increase IL-4 production [284, 285]. The assay was performed as per the manufacturer's

instructions. Data in this section is shown for CXCL8 and IL-10, with IL-4 data discussed in the lipid chapter (chapter 6).

The standard curve for IL-10 is shown in figure 3.1 as an example. The other standard curves for CXCL8 and IL-4 can be found in the appendix. Table 3.1 shows the lower limit of detection for the respective cytokines. None of the samples had cytokine levels above the assay upper limit of detection. Samples with values below the assay detection limit were assigned a value equal to the assay detection limit.

Cytokine	**Concentration (pg/ml)**	
CXCL8	0.35	
IL-10	1.76	
	Plate 1	**Plate 2**
IL-4	3.87	2.02

Table 3.1; Lower limit of detection (pg/ml) for CXCL8, IL-10 and IL-4 tested in the Luminex assay. Two plates were run for IL-4. Where two plates were used with differing lower detection limits, the higher of the two detection limits was used. Samples with values below the lower limit of detection were assigned a value equal to the lower limit of detection.

3.3 Cytokine results

3.3.1 Patient characteristics (table 3.2)

Three CF patients in this cohort underwent bronchoscopy while clinically stable. The bronchoscopy was performed under general anaesthetic during insertion of a venous access device. One control patient (who underwent bronchoscopy for reported haemoptysis) did not have a BAL cytology result. However, the bronchoscopy was macroscopically normal and the patient included due to limited numbers in the control group. BAL samples from 111 patients were available for Luminex analysis. The majority of CF NBS patients had results below the assay detection limit. Patients with values below the LLD were assigned a value equal to the LLD.

The first section in this chapter presents results from BAL CXCL8 analysis. IL-10 analysis follows. CXCL8 results are then expressed as a ratio of IL-10 to examine the relationship between these two cytokines.

Plate: Plate_*2BC1QA5116X*
Assay: IL-10 (Human)
Group: Standard

Sample^	Well	Concentration (pg/ml)	Signal	Mean	CV	Calc. Concentration (pg/ml)	Calc. Conc. Mean (pg/ml)	Calc. Conc. CV
S001	A02	2500	137440	133554	4.12	2626	2554	4.02
	A01		129667			2481		
S002	B02	625	30699	30378	1.5	609	603	1.46
	B01		30056			596		
S003	C01	156	9173	8054	19.6	187	164	19.3
	C02		6935			142		
S004	D01	39.1	1756	1868	8.44	36.1	38.4	8.57
	D02		1979			40.8		
S005	E01	9.77	484	496	3.28	9.07	9.32	3.8
	E02		507			9.57		
S006	F02	2.44	156	184	21.2	1.8	2.42	36.3
	F01		211			3.04		
S007	G02	0.61	77	118	49.1	0	0.934	141
	G01		159			1.87		
S008	H02	0	78	75	6.64	0	0	N/A
	H01		71			0		

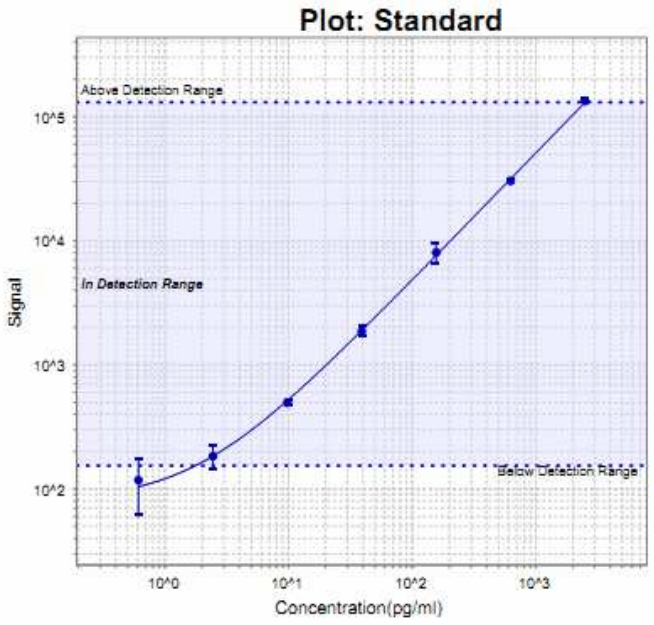


Figure 3.1: Standard curve for IL-10

Table 3.2: Cytokine analysis patient characteristics (total n=111)

	Controls	CF	CF NBS	Bronchiectasis	PCD	PBB
Number	6	47	19	15	6	18
Age yrs (IQR)	**11.8 (1.7-13.5)**	**9 (4.3-12.6)**	**0.3 (0.3-0.3)**	**8.3 (6.4-13.3)**	**9.5 (7.5-12.7)**	**3.9 (2-7.8)**
Sex male (%)	3 (50)	24 (51)	5 (26)	13 (87)	3 (50)	6 (33)
Number with recent spirometry (%)	1 (17)	22 (47)	_____	9 (60)	4 (67)	0
FEV1 % predicted (IQR)	80	69.5 (57.3-76)	_____	89 (69-95)	66 (57-76)	_____
Number with BAL cytology results (%)	5 (83)	21 (45)	11 (58)	11 (73)	5 (83)	18 (100)
BAL % neutrophils (IQR)	**2.7 (0.5-2.7)**	**24.7 (16.7-59.4)**	**10 (4-18)**	**44.3 (1-78)**	**1.7 (0.9-41.9)**	**13.7 (7.9-37.4)**
Pathogens isolated on BAL (%)	**0**	**24 (51)**	**6 (32)**	**9 (60)**	**0**	**18 (100)**
Infected with *P. aeruginosa* (%)	0	31 (66)	2 (11)	1 (7)	1 (17)	0
Respiratory exacerbation	0	7	0	0	2	0
Inhaled steroids	0	12	0	10	2	5
Oral steroids	0	3	0	0	0	0
Azithromycin	0	9	0	3	1	2
Oral antibiotics	0	25	17	1	2	2
Nebulised antibiotics	0	24	1	0	2	0

CF NBS patients were significantly younger than control subjects ($p=0.001$), CF ($p<0.0001$), bronchiectasis ($p<0.0001$), PCD ($p<0.001$) and PBB ($p=0.01$). The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.0001$). The percentage BAL neutrophilia was significantly higher in CF ($p<0.01$) and bronchiectasis ($p<0.05$) patients as compared to controls. IQR=interquartile range.

3.4 BAL CXCL8

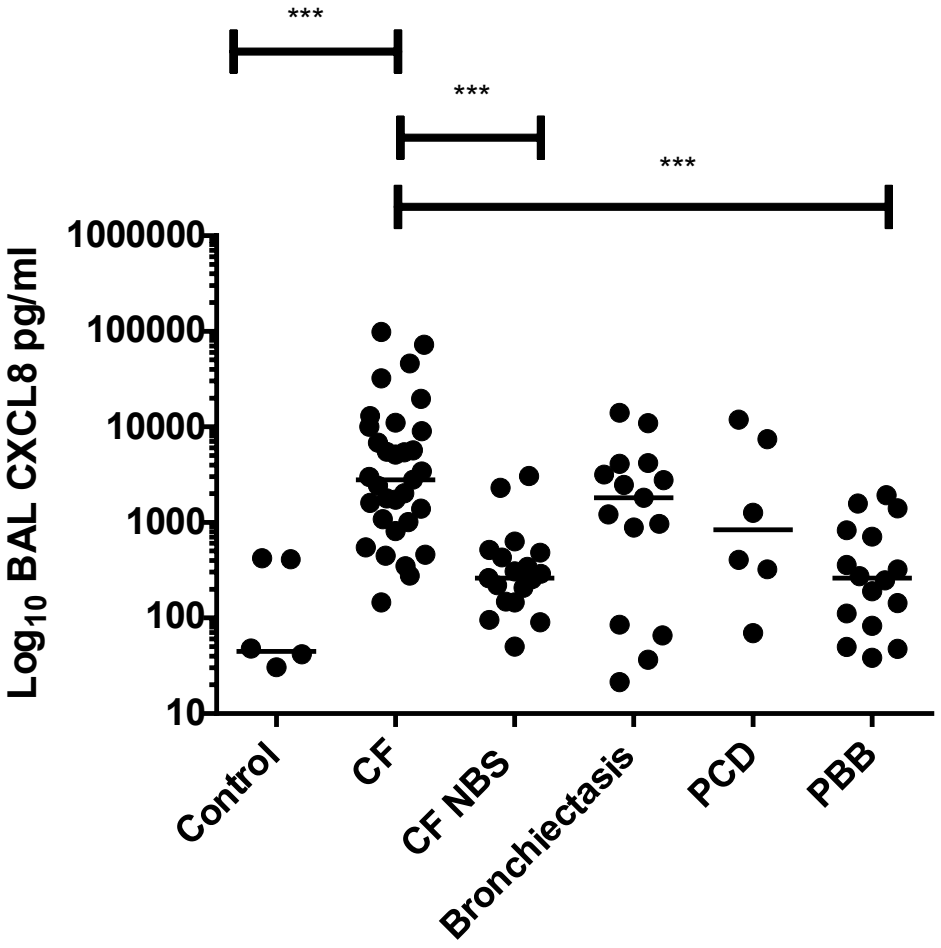


Figure 3.2a: BAL CXCL8 in individual patient groups. CXCL8 levels were higher in CF than controls, CF NBS and PBB (all $p<0.001$). Data has been Log_{10} transformed to expand the axis.

All BAL CXCL8 levels were above the LLD. Levels were significantly higher in CF as compared to controls, CF NBS and PBB (all $p<0.001$) (figure 3.2a). In order to examine whether this was a related to disease severity or the CFTR defect, results were analysed for differences between controls, established CF and CLSD (bronchiectasis / PCD / PBB), and all CF (established and CF NBS) and CSLD (figure 3.2b).

CXCL8 levels were statistically higher in established CF patients than controls or CSLD. However this significance was lost with the inclusion of CF NBS in the CF group. This implies this is not related to the CFTR defect and the differences seen between established CF and CSLD may be related to disease severity in this cohort.

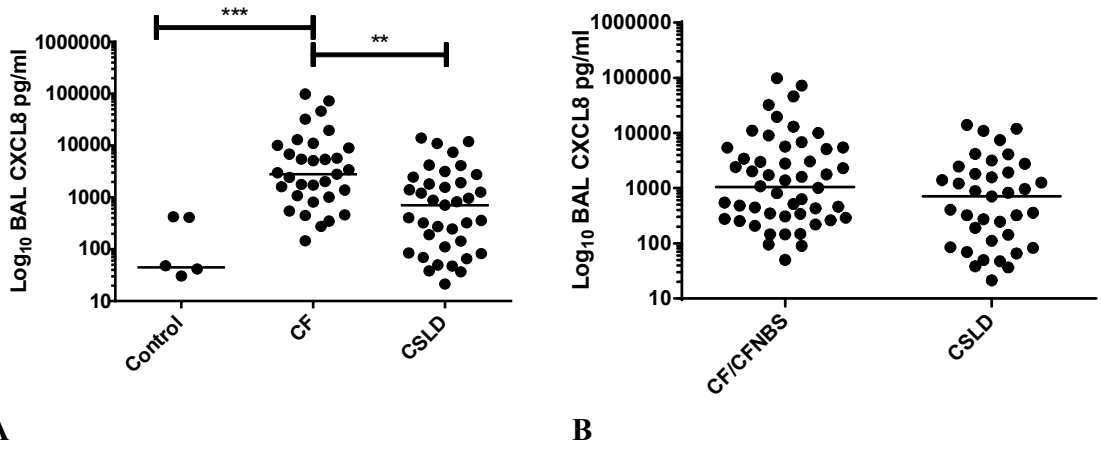
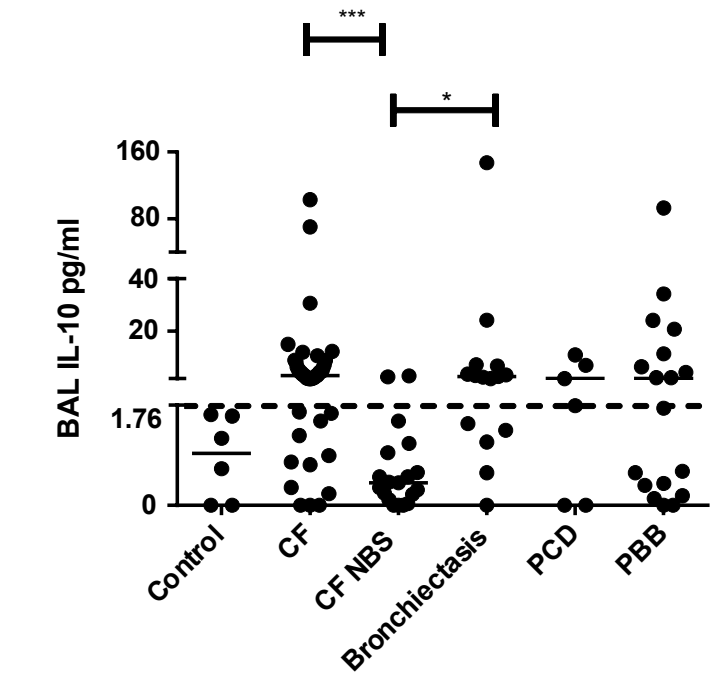


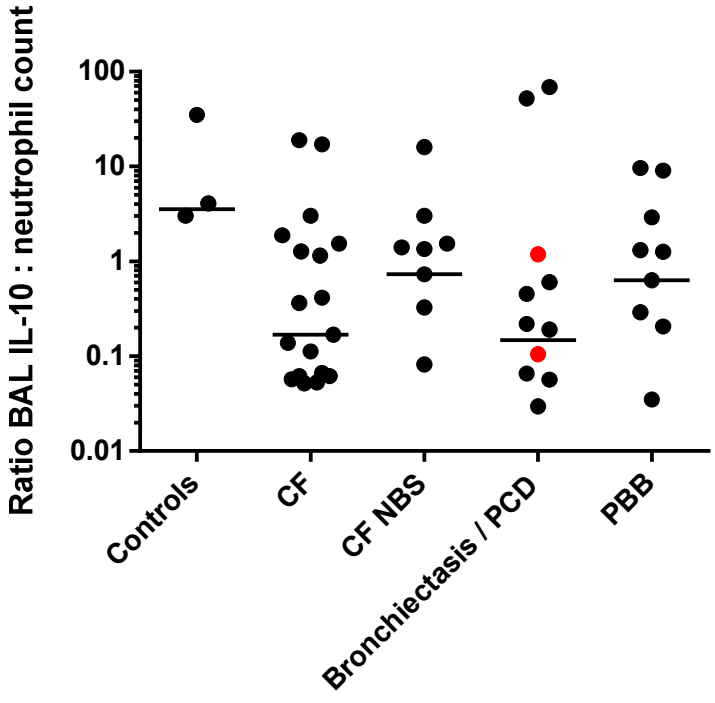
Figure 3.2b: Graphs of BAL CXCL8 levels in controls, established CF and CSLD (A) and all CF and CSLD (B). Graph A: CF vs controls $p<0.0001$, CF vs CSLD $p<0.001$. Graph B shows that this is not a CF-specific effect. Data in both graphs has been Log_{10} transformed to expand the axis.

3.5 BAL IL-10

BAL IL-10 levels were similar in CF NBS and control subjects, and significantly higher in CF as compared to CF NBS (figure 3.3a). Previous published evidence has shown that levels of BAL IL-10 are lower in the CF airway in those with established disease as compared to healthy controls, thus suggesting an impaired inflammatory resolution capability in the CF airway [69, 286]. However, this does not appear to be the situation with this patient cohort and there is increasing evidence in the literature that IL-10 may also have pro-inflammatory properties [160, 162]. In order to investigate this further, additional analysis was undertaken to examine the relationship between BAL IL-10 and markers of increased airway inflammation. The first step in this analysis was to normalise the data for airway inflammation by expressing as a ratio of BAL neutrophil count (figure 3.3a). No significant differences were seen between groups.



A



B

Figure 3.3a: Graph A**:** IL-10 levels in BAL. Levels were statistically higher in CF ($p=0.001$) and bronchiectasis ($p<0.05$) as compared to CF NBS. Dotted line represents the assay lower limit of detection. Graph B: IL-10 levels normalised for neutrophilic inflammation. Data has been Log_{10} transformed to expand the axis. There were no significant differences between groups. Red dots represent PCD patients.

Data was then analysed to see if IL-10 levels were affected by disease severity, by comparing levels between controls, established CF and all other neutrophilic diseases (CSLD) (figure 3.3b). This showed that IL-10 levels were significantly higher in both CF and CSLD than controls, although this was lost on normalising data for neutrophilic inflammation.

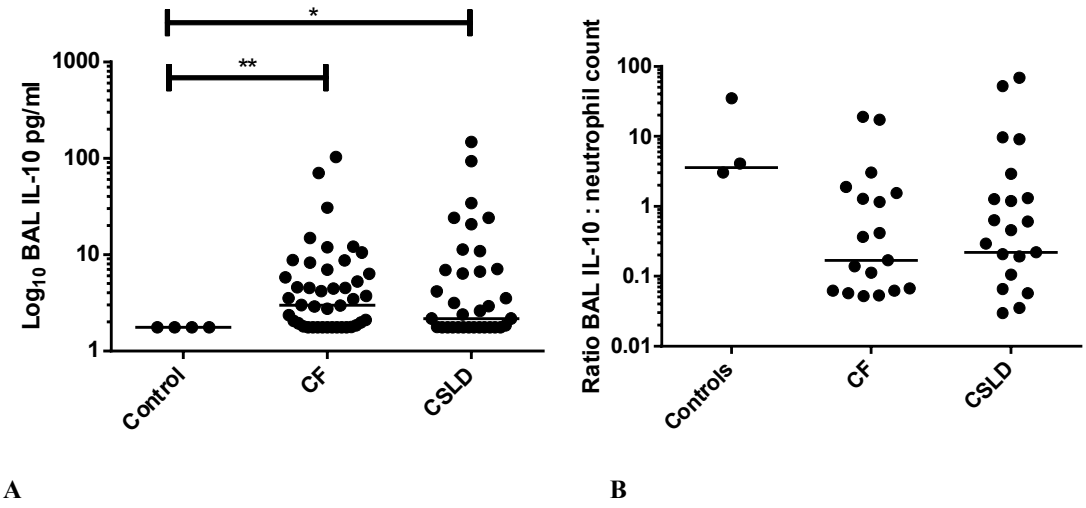


Figure 3.3b: Graph A: Levels of BAL IL-10 in controls, established CF and CSLD. Levels were higher in CF ($p<0.01$) and CSLD ($p<0.05$) than controls. Graph B: Data normalised for neutrophilic inflammation. There was no statistically significant difference between groups. Data in both graphs has been Log_{10} transformed to expand the axes.

Data was also analysed to see if there was a CF-specific effect, by comparing all CF patients (established CF and CF NBS) with CSLD. No significant difference was seen between these groups, including on normalising for neutrophilic inflammation (figure 3.3c).

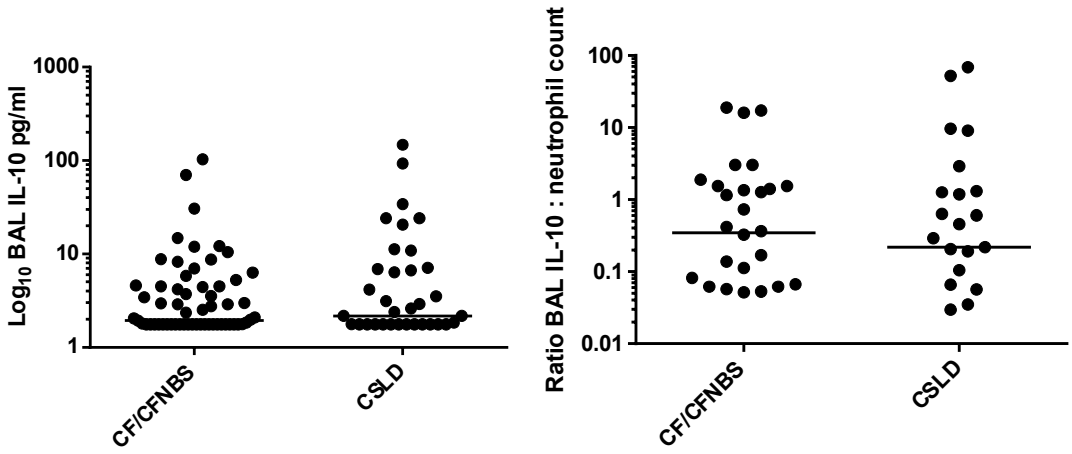


Figure 3.3c: Graph A: Levels of BAL IL-10 in all CF and CSLD. Graph B: Data normalised for neutrophilic inflammation. Data in both graphs has been Log_{10} transformed to expand the axes There was no statistically significant difference between groups.

3.6 Ratio of BAL CXCL8:IL-10

This section examines whether CXCL8 : IL-10 ratios vary between groups, to see if the ability of IL-10 to resolve CXCL8 mediated inflammation is impaired in more severe disease and CF.

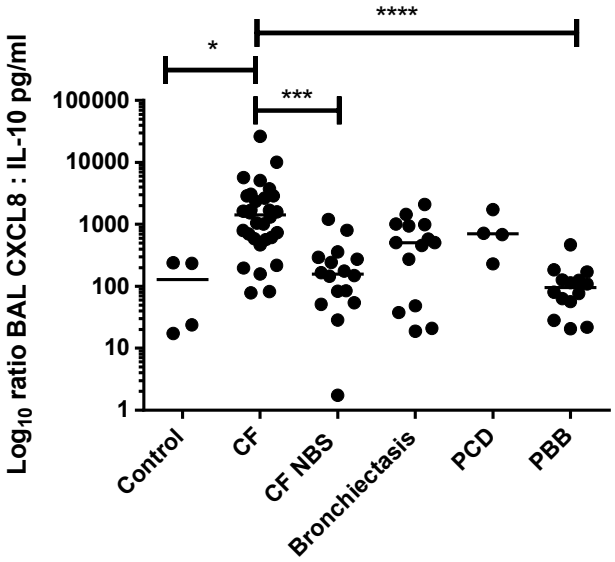


Figure 3.4a: Graph showing ratios of BAL CXCL8:IL-10. Ratios were significantly higher in CF as compared to controls ($p<0.05$), CF NBS ($p<0.001$) and PBB ($p<0.0001$). Data has been Log_{10} transformed to expand the axis.

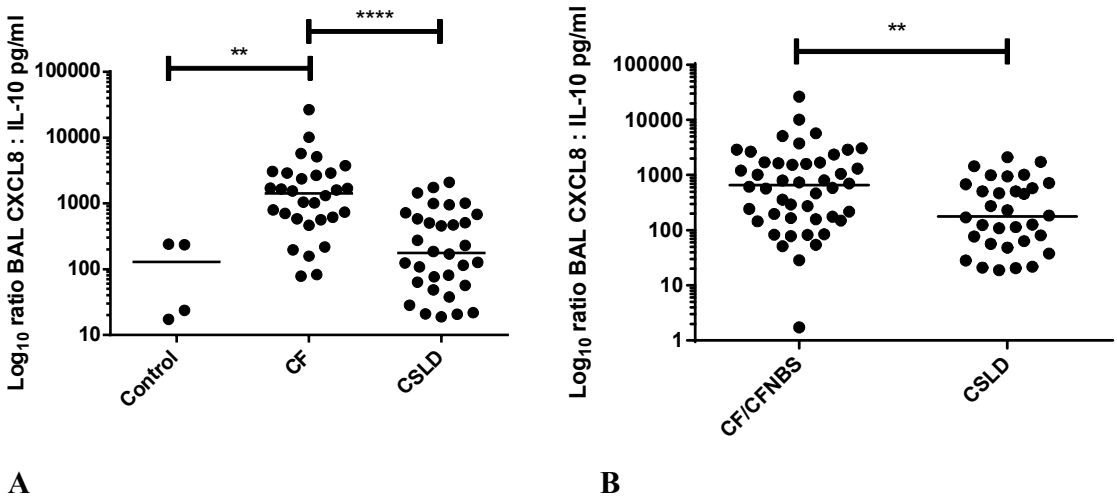


Figure 3.4b Graphs showing ratios BAL CXCL8:IL-10 in (A) controls, established CF and CSLD and (B) all CF and CSLD. Ratios were higher in CF than other disease groups and controls. Graph A: established CF vs controls $p<0.01$ and vs CSLD <0.0001; Graph B: all CF vs CSLD $p<0.01$. Data in both graphs has been Log_{10} transformed to expand the axes.

The ratio of BAL CXCL8:IL-10 was significantly higher in established CF patients as compared to controls, CF NBS and CSLD (figure 3.4a and 3.4b). Ratios remained higher in all CF patients (CF and CF NBS) as compared to CSLD, implying CFTR dysfunction has an effect on inflammatory regulation by IL-10 (figure 3.4b). Thus although IL-10 levels were higher in this cohort of CF patients, the effectiveness at reducing inflammation is impaired as shown by higher ratios of CXCL8:IL-10. Correlations between the ratios of CXCL8:IL-10 and BAL neutrophil count were also examined in all CF and the CSLD group. No correlation was seen in the CSLD group, and a moderately significant positive correlation in the CF group ($r=0.43$, $p<0.05$), again implying a CF-specific phenomenon. This implies that with increasing airway neutrophilic inflammation in CF, the ability of IL-10 to resolve inflammatory CXCL8 is reduced.

3.7 IL-10 and airway infection

Levels of BAL IL-10 were also related to airway infection (positive BAL microbiological culture). This showed that levels were higher in those patients with positive microbiological cultures when grouping all patients together and in the CF group. However no association was seen in the CSLD group. On correcting for

inflammatory status, the ratio BAL IL-10: neutrophils did not vary in all patients together or CF patients with pathogen status. However the ratio BAL CXCL8:IL-10 was higher in CF patients with pathogens (figure 3.5). Thus the ability of CF patients to resolve CXCL8 mediated inflammation by IL-10 is impaired in those with positive BAL cultures as compared to those with negative cultures.

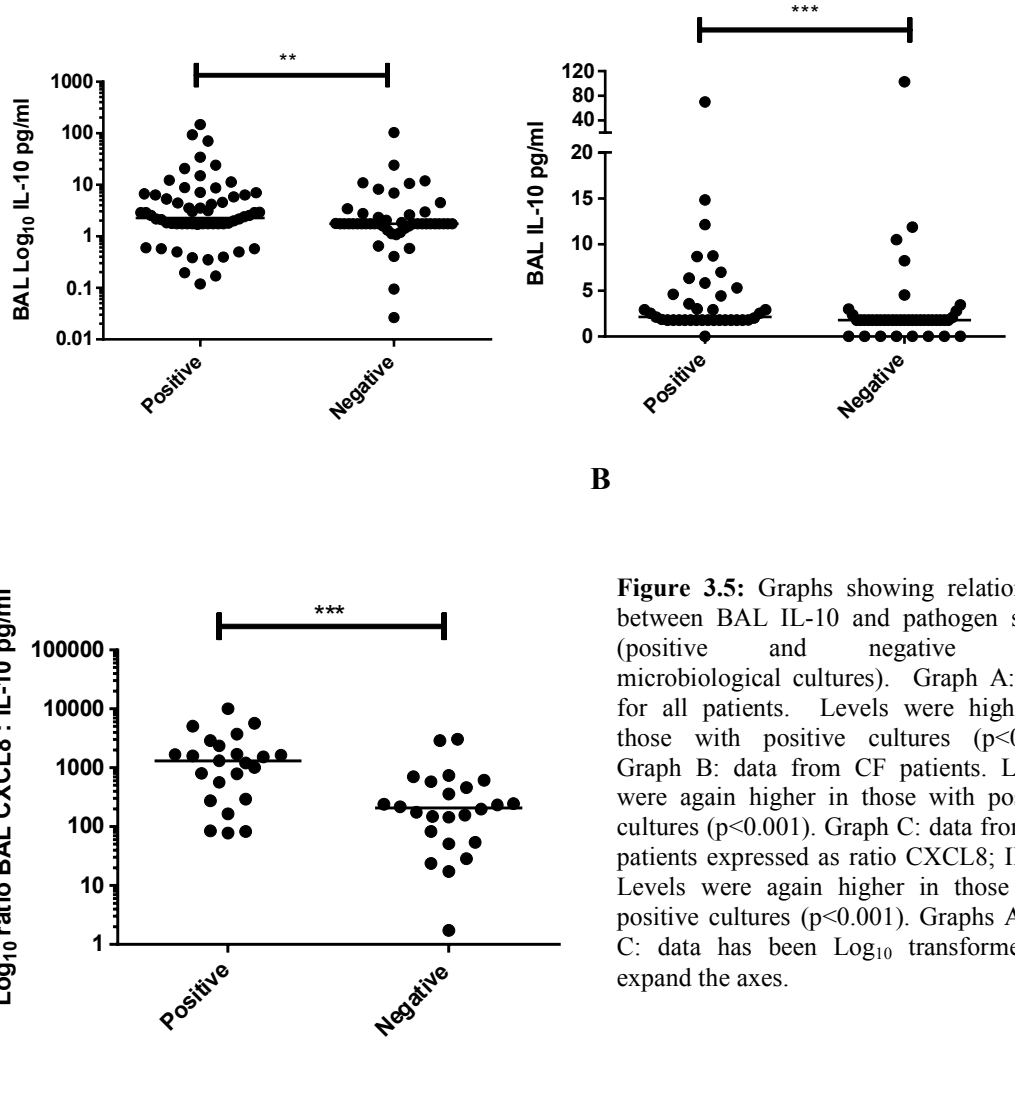


Figure 3.5: Graphs showing relationship between BAL IL-10 and pathogen status (positive and negative BAL microbiological cultures). Graph A: data for all patients. Levels were higher in those with positive cultures ($p<0.01$). Graph B: data from CF patients. Levels were again higher in those with positive cultures ($p<0.001$). Graph C: data from CF patients expressed as ratio CXCL8; IL-10. Levels were again higher in those with positive cultures ($p<0.001$). Graphs A and C: data has been Log_{10} transformed to expand the axes.

The next section examines flow cytometry analysis of BAL, in particular for Tregs.

3.8 Flow cytometry analysis of BAL

There is limited published literature on the lymphocyte content of paediatric BAL, as assessed by flow cytometry, in healthy children and those with lung disease. Professor Hawrylowicz's laboratory at Kings College London has experience of flow cytometric analysis of paediatric BAL, and therefore a protocol was established in collaboration with the King's laboratory. Many paediatric BAL samples are low volume, particularly those obtained from babies, and often have low cellular numbers ($<10^6$/ml). For this reason it was necessary to prioritise lymphocyte subsets for analysis. Given the importance of Tregs in inflammatory resolution, CD4+CD25+FoxP3+ Tregs were prioritised. The King's laboratory had previously been unsuccessful in analysing BAL for IL-10+ Tregs due to the large number of cells needed. For this reason, and given the limited cell numbers available, analysis of Tregs was limited to CD4+CD25+FoxP3+ Tregs. However, given the precious nature of these samples, where cell numbers allowed, BAL was also analysed for other lymphocyte subsets. These were (in order of priority):

- CD3+CD8+ (cytotoxic T lymphocytes)
- CD3+CD4+CD103+ and CD3+CD8+CD103+ (Intraepithelial lymphocytes)
- CD3+CD19+ (B lymphocytes)
- CD3+CD56+ (NK cells)
- CD3+CD69+ (Activated T lymphocytes)

- In addition samples were analysed for dendritic cells:
- HLA-DR+CDllc+ and HLA-DR+BDCA4+ cells

The hypotheses of this section were as follows:

- BAL Treg levels are reduced in severe neutrophilic airway disease (CF) as compared to CSLD, and that control levels lie between these two groups.

- Treg levels are lower in CF patients, as compared to other groups, due to CFTR dysfunction.

Data from analysis of other lymphocyte subsets and DCs is included, as there is very little published research in this area. Given these samples are so precious it was important to extract as much data as possible. However, the results from these additional analyses did not form part of the main hypotheses.

3.8.1 Processing of BAL and surface antibody staining

BAL samples were filtered using a cell strainer to remove mucus plugs. The filtered BAL fluid was collected in a 15ml Falcon tube and centrifuged at 200g for 10 minutes at 4^{o}C. The supernatant was removed and stored in 500µl aliquots at -80^{0}C. The cell pellet was resuspended in 1ml FACSFlow (BD Biosciences, Oxford, UK) containing 1% mouse serum. A note was made if the cell pellet contained erythrocytes. Cells were counted as previously described and the cell concentration adjusted to $1x10^{6}$/ml. Most samples did not need further dilution.

3µl of each cell marker antibody was added to numbered FACS tubes and 18µl of lineage cocktail was added to panel 5 as indicated in table 3.3. 100µl (1×10^{5}) of BAL cells was added to each tube. If the sample contained few cells, then the volume equivalent of 1×10^{5} cells was added to each tube. This meant that for some patients it was only possible to analyse panels 1 and 2 (see table 3.3). The tubes were mixed and incubated on wet ice in the dark for 30 minutes. 500µl of cold FACSFlow was then added to the tubes and centrifuged at 200g for 5 minutes. The liquid was decanted from each tube, the pellet resuspended in FACSflow and spun as before.

Table 3.3: Antibody profile for each lymphocyte analysis panel

Panel Number	FITC	PE	PerCP	APC
1 (for FoxP3)	CD25 [b]	Isotype control [b]	CD3 [b]	CD4 [b]
2 (for FoxP3)	CD25 [b]	FoxP3 [a]	CD3 [b]	CD4 [b]
3	CD8 [b]	CD103 [a]	CD3 [b]	CD4 [b]
4	CD56 [b]	CD69 [b]	CD3 [b]	CD19 [c]
5	**Lineage cocktail * [b]**	CD11c [b]	HLA-DR [b]	BDCA-4 [c]

FITC - Fluorescein isothiocyanate

PE - Phycoerythrin

PerCP - Peridinin chlorophyll protein

APC – Allophycocyanin

[a] eBioscience, Hatfield, UK

[b] BD biosciences, Oxford, UK

[c] Miltenyi Biotec, Hatfield, UK

*** Lineage cocktail**

CD3 FITC
CD14 FITC
CD16 FITC
CD19 FITC
CD20 FITC
CD56 FITC

3.8.2 Cell permeabilisation and Foxp3 staining

As FoxP3 is intracellular, staining required cell permeabilisation. 1ml of FoxP3 fixative (diluted 1:4) was added to panels 1 and 2 and incubated at room temperature in the dark for 45 minutes. 1ml of permeabilisation buffer (diluted 1:10) was added and the tubes vortexed and centrifuged at 200g for 5 minutes. The liquid was decanted, a further 1ml of permeabilisation buffer was added and the tubes spun as before. The liquid was again decanted and 5µl mouse serum was added to each sample. The tubes were incubated at room temperature in the dark for 10 minutes.

5µl Mouse IgG PE (isotype control) was added to panel 1 and 5µl anti-human FoxP3 PE was added to panel 2. The isotype control was used to set quadrants for analysis of FoxP3 positive cells as shown in figure 3.6. The tubes were vortexed and incubated for 45 minutes at room temperature in the dark. 1ml of FoxP3 permeabilisation buffer was added to each tube and the samples vortexed and centrifuged at 200g for 5 minutes. The liquid was decanted and 500µl of FACSFlow was added to each tube. The samples were once again mixed and centrifuged as before. Finally the liquid was decanted once more, the samples were resuspended in 500µl of FACSFlow and run on a flow cytometer (FACSCalibur, BD Biosciences, Oxford, UK).

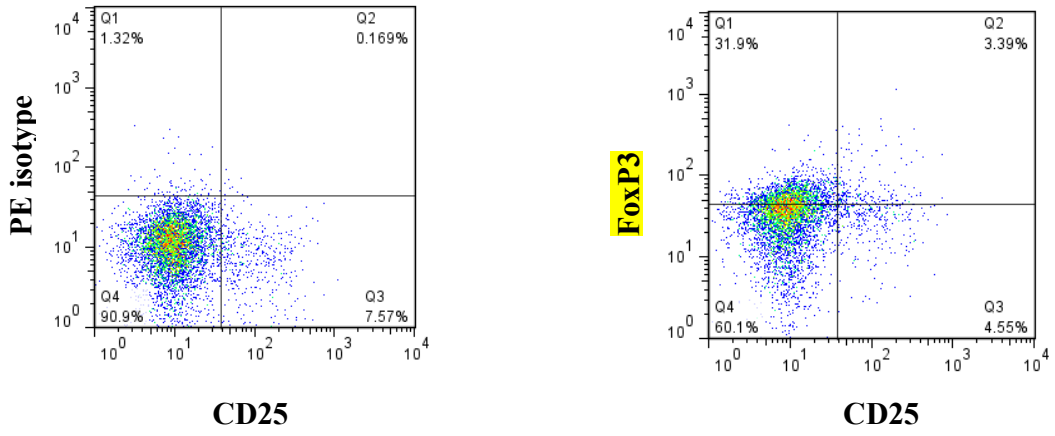


Figure 3.6: Plots illustrating analysis of FoxP3. Left hand plot shows use of the PE isotype control to set positive and negative staining quadrants. Right hand plot shows how these quadrant settings are applied to the FoxP3 stained cells.

3.9 Flow cytometry analysis

Compensations were set using compensation beads (BD Biosciences, Oxford, UK). One drop of each bead was added to a tube along with 1µl of each mouse IgG isotype control antibody conjugated to a fluorochrome (BD Biosciences, Oxford, UK) and incubated for 15 minutes. 1ml of FACSflow was added and the tube centrifuged at 200g for 5 minutes. The supernatant was discarded and the pellet resuspended in a further 1ml of FACSflow and used to set compensations on the flow cytometer.

A live gate was established by excluding dead cells by eye on the scatter plot. Dead cells occupy the lower left corner of the plot as these cells are small and with low granularity. A lymphocyte gate was also established by eye (figure 3.7), using prior knowledge as to the location of these cells within a scatter plot. Analysis of results was performed using FlowJo 7.6 software (Tree Star, Oregon, USA).

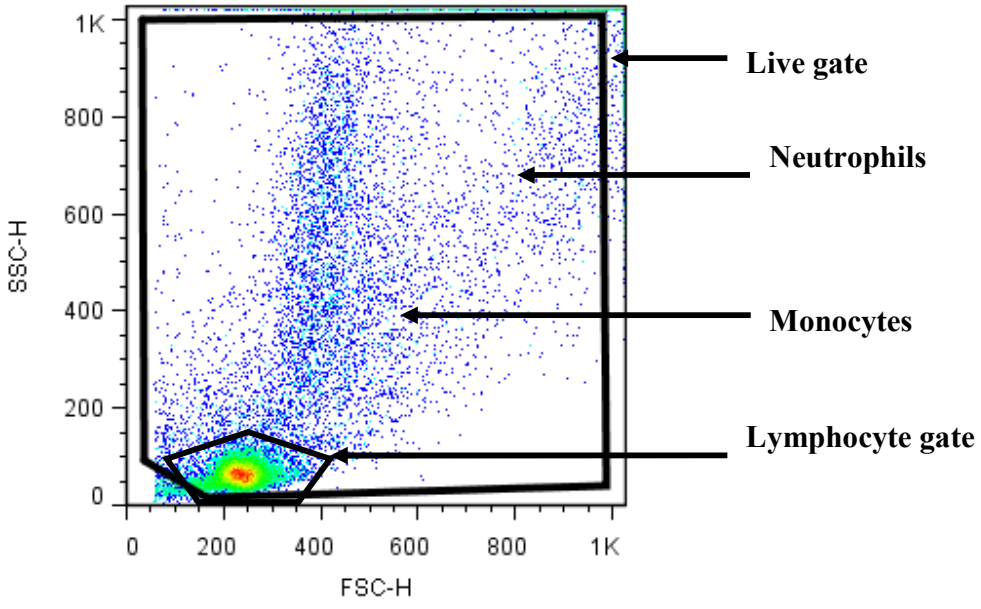


Figure 3.7: Scatter plot from peripheral blood showing position of live gate, lymphocyte gate and cell populations.

Unfortunately and unavoidably, routine paediatric bronchoscopy lists took place in the afternoon and finished at around 5pm. Samples generally reached the laboratory by early evening and therefore a decision was made to see if it was possible to leave the

samples overnight after surface antibody staining by adding 100µl CellFIX (BD Biosciences, Oxford, UK) and performing cell permeabilisation and Foxp3 staining the next morning.

In order to test whether leaving samples overnight was possible without compromising results, adult peripheral blood mononuclear cells (PBMC) from a healthy volunteer were utilised in place of scarce and precious paediatric BAL samples. Blood was collected in a 50ml syringe containing 0.5ml heparin. 10ml of Histopaque (Sigma-Aldrich, Dorset, UK) was added to two 20ml universal containers. Blood was slowly layered over the histopaque using a pipette. The samples were then centrifuged at 1400 rpm with the brake off for 20 minutes. At the end of centrifugation, granulocytes and red blood cells collect at the bottom of the tube. The buffy coat containing PBMC is between this layer and the platelet rich top layer. The buffy coat was removed by gentle pipetting and collected in a fresh tube. This was then spun at 1000rpm at 4^{o}C for 10 minutes. The cell pellet was resuspended in 1ml FACSFlow, a cell count performed as previously described and the sample diluted to a cell count of 1 x 10^{6} / ml. 100µl (1 x 10^{5}) of BAL cells was added to each tube as previously described and samples run with the experimental procedure as outlined in table 3.3.

The results obtained were sufficiently similar between both experiments to allow samples to be left overnight prior to permeabilisation and FoxP3 staining the following day (table 3.4). There was some discrepancy between percentages of HLADR+BDCA4+ and HLADR+CD11c+ cells, although there were more cells in the experiment where samples were left overnight. As there are such small cell numbers then any difference is likely to be magnified. For ease of analysis if panels 1 and 2 were to be left overnight, panels 3, 4 and 5 were also fixed and left for analysis the following day at the same time as panels 1 and 2.

The next section describes the method of identifying BAL Tregs.

Table 3.4: Results from experiment assessing whether BAL samples could be left overnight prior to cell permeabilisation and analysis. A: samples analysed immediately; B: samples left overnight.

	Experiment A		**Experiment B**	
TUBE 1	**Cell count**	**Percentage cells**	**Cell count**	**Percentage cells**
Live gate	20182	96.1	20156	93.6
Lymphocyte gate	15461	73.6	15036	74.6
CD3+CD4+	7987	51.7	8042	53.4
FoxP3 isotype	1	0.013	0	0
TUBE 2				
Live gate	20168	95.1	20385	93.6
Lymphocyte gate	15190	71.6	15070	73.9
CD3+CD4+	7645	50.3	7965	52.8
CD3+CD4+CD25+FoxP3+	5	0.07	7	0.09
TUBE 3				
Live gate	20557	56.4	19944	71.9
Lymphocyte gate	11702	32.1	14268	71.5
CD3+CD4+	6199	53	8943	62.4
CD3+CD4+CD103+	68	1.1	68	0.8
CD3+CD8+	941	8.0	1296	9.0
CD3+CD8+CD103+	0	0	0	0
TUBE 4				
Live gate	20677	55.9	20318	68.3
Lymphocyte gate	11569	56	14660	72.2
CD3+CD19+	30	0.3	47	0.3
CD3+CD69+	110	1.0	156	1.1
CD3+CD56+	230	2.0	275	1.9
TUBE 5				
Live gate	19689	50.7	22116	71
HLADR+ BDCA4+	44	0.5	69	1.4
HLADR+ CD11c+	63	0.8	86	1.8

3.10 Phenotypic analysis of regulatory T lymphocytes in BAL

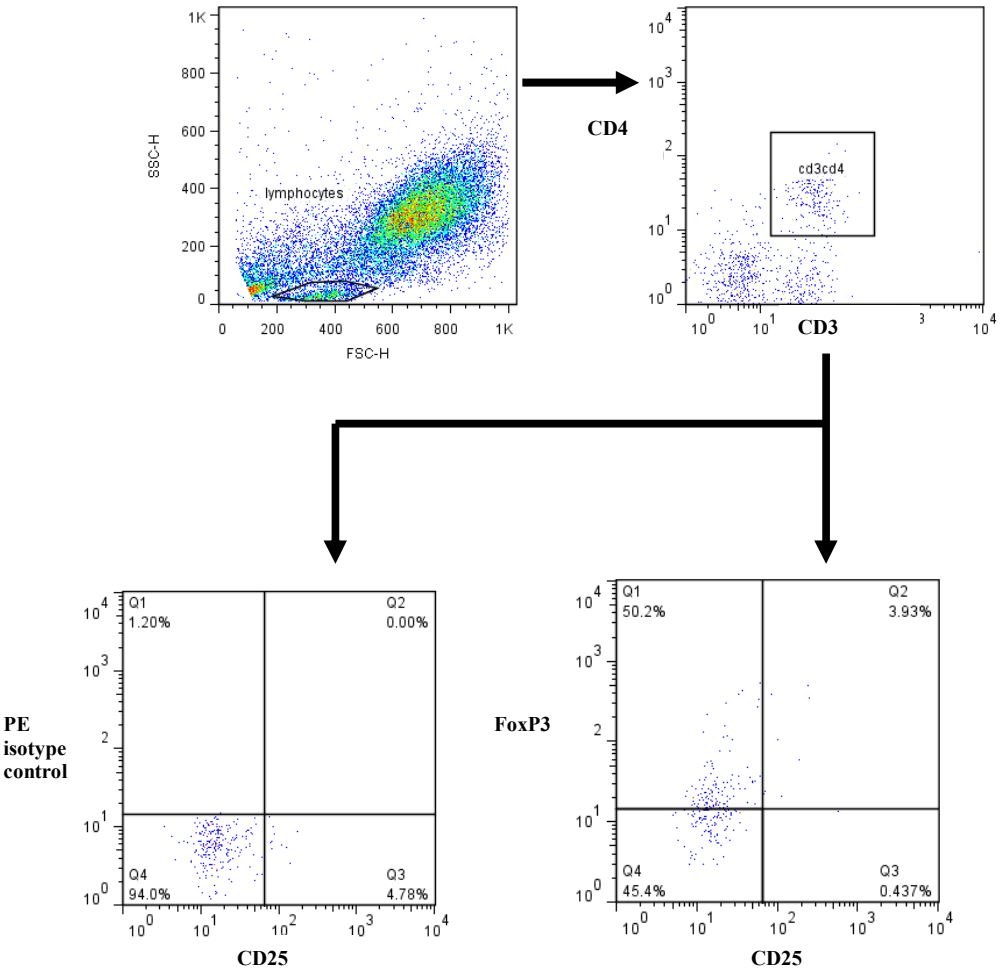


Figure 3.8: Illustration of analysis method for CD3+CD4+CD25+FoxP3+ lymphocytes. For each sample after setting a live gate, a lymphocyte gate was delineated by eye. A CD3CD4 population was then identified. The isotype control tube (panel 1) was used to set the quadrant for analysis of CD25+FoxP3+ lymphocytes.

BAL Tregs were identified by firstly delineating the lymphocyte gate and subsequently those cells that are CD3+CD4+. The FoxP3 isotype control was used to set the quadrants for analysis of CD25+FoxP3+ cells (figure 3.8). One of the main difficulties with this method was identification of the initial lymphocyte gate. The scatter plot from BAL is not as clear as that from peripheral blood samples and thus the lymphocyte population can be difficult to delineate. An example of this is shown in figure 3.9. Under these circumstances the gate was placed where the lymphocyte population tends to be in the scatter plot. On the knowledge that most lymphocytes will

be CD3+, having identified the gate, this was analysed to check that there was a population of CD3+ cells within the gate.

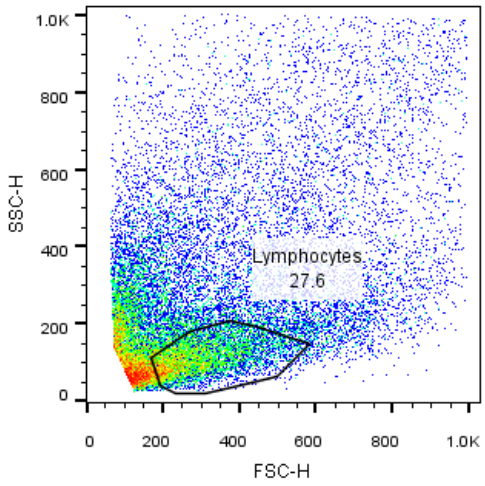


Figure 3.9: Example of BAL sample showing difficulties identifying the lymphocyte gate

3.11 Flow cytometry results

3.11.1 Patient characteristics (table 3.5)

One PCD and two CF patients were clinically stable at the time of bronchoscopy which was performed opportunistically while under general anaesthetic in order to site a venous access device. One CRS patient was prescribed nebulised antibiotics for *Pseudomonas aeruginosa* isolated on a cough swab. This patient had two normal sweat tests and negative CF genetics for common mutations.

Table 3.5: Flow cytometry patient characteristics (total n=65)

	Controls	CF	CF NBS	Bronchiectasis	PCD	PBB	CRS
Number	4	15	9	8	3	4	22
Age yrs (IQR)	**2.4 (1.5-13.0)**	**7.8 (4.3 - 12.8)**	**7.8 (4.3 - 12.8)**	**10.3 (5.6 -13.7)**	**7.8 (6.5 - 8.8**	**3.3 (2.1 - 7.2)**	**3.1 (1.5-8.3)**
Sex male (%)	1 (25)	11 (73)	2 (22)	5 (63)	1 (33)	1 (25)	15 (68)
Recent spirometry (%)	1 (25)	9 (60)	_____	6 (75)	1 (33)	_____	3 (14)
FEV_1 % predicted (IQR)	113	71 (64-80)	_____	83 (56-95)	77	_____	88 (85-127)
Number with BAL cytology results (%)	4 (100)	9 (60)	4 (44)	5 (63)	1 (33)	4 (100)	16 (72)
BAL % neutrophils (IQR)	**2.7 (1.5-4.0)**	**20.3 (15.5-37.9)**	**10.0 (8.9-15.9)**	**24.2 (1-58)**	**65.7**	**21 (7.5-38.9)**	**6.2 (2.0-22.2)**
Pathogens isolated on BAL (%)	**0**	**6 (40)**	**4 (44)**	**5 (63)**	**0**	**4 (100)**	**17 (77)**
Infected with *P. aeruginosa* (%)	0	12 (80)	1 (11)	0	0	0	1 (5)
Respiratory exacerbation	0	3	1	0	1	0	0
Inhaled steroids	0	6	0	4	0	0	5
Oral steroids	0	0	0	0	0	0	0
Azithromycin	0	6	0	1	0	0	0
Oral antibiotics	0	11	8	1	2	1	2
Nebulised antibiotics	0	10	1	0	0	0	1

CF NBS patients were significantly younger than CF subjects ($p<0.0001$), bronchiectasis ($p<0.001$) and those with CRS ($p<0.01$). The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.01$) as did the percentage BAL neutrophilia ($p<0.05$). However, there were no specific intergroup differences. IQR=interquartile range.

3.12 BAL cell counts

BAL samples from 65 patients were available for flow cytometry analysis. In order to analyse all the panels at least 1 x 10^6 cells/ml BAL were needed. The median cell count was 0.48 x 10^6/ml [interquartile range 0.25-0.97 x 10^6/ml]. A large proportion of sample cell counts were < 10^6/ml and therefore it was necessary to prioritise cells for analysis as previously discussed. The BAL cell count varied between patient groups as shown in figure 3.10. In particular BAL cell counts were higher in CF patients (median [interquartile range]) (1[0.5-1.26] x 10^6/ml) as compared to CF NBS (0.13[0.03-0.48] x 10^6/ml).

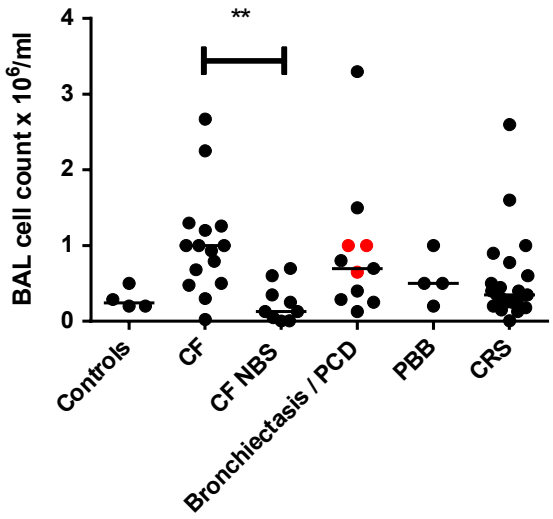


Figure 3.10: Graph showing BAL total cell counts in different patient groups. Horizontal bars represent medians which varied significantly between groups ($p<0.01$). BAL cell counts were higher in CF (1[0.5-1.26] x 10^6/ml) patients as compared to CF NBS (0.13[0.03-0.48] x 10^6/ml) ($p<0.01$). Red dots represent PCD patients.

3.13 Absolute neutrophil count and percentage BAL neutrophils

Neutrophil levels were expressed as total counts and percentage BAL neutrophils (figure 3.11). The BAL neutrophil cell count did not vary significantly between groups, and there was no correlation between total cell count and neutrophil count. The median percentage BAL neutrophils was higher in CF ($p<0.01$), bronchiectasis ($p<0.05$) and PBB ($p<0.05$) as compared to controls. The lack of significant differences in the neutrophil count group may be due to the small patient numbers in the control group.

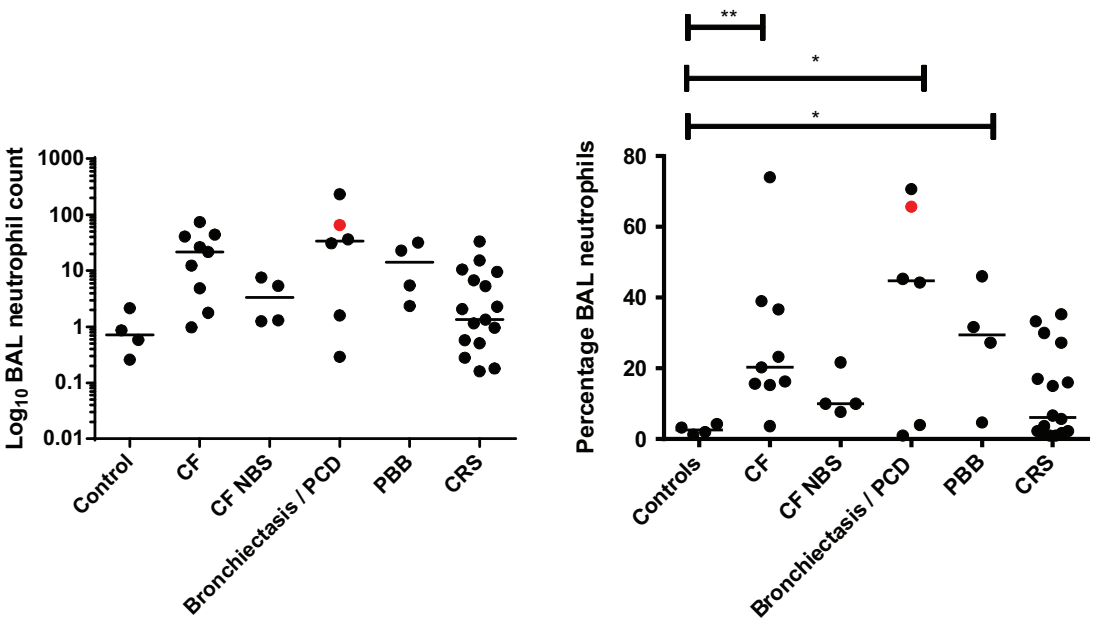


Figure 3.11: Graphs showing total neutrophil cell count (graph A) and the percentage of neutrophils (graph B) in BAL in each patient group. Horizontal bars represent medians. The single PCD patient has been included in the bronchiectasis group and is represented by the red dot. The medians varied significantly ($p<0.05$) in graph A, but there were no significant differences between groups. In graph B the neutrophil percentage was higher in CF ($p<0.01$), bronchiectasis ($p<0.05$) and PBB ($p<0.05$) as compared to controls.

The following section discusses the results from analysis of lymphocyte subsets and dendritic cells.

3.14 CD3+CD4+ Lymphocytes

CD3+CD4+ cells were expressed in the following ways (figures 3.12 and 3.13):

- Cell numbers in BAL
- Percentage of the live cell gate
- Percentage of the lymphocyte gate.

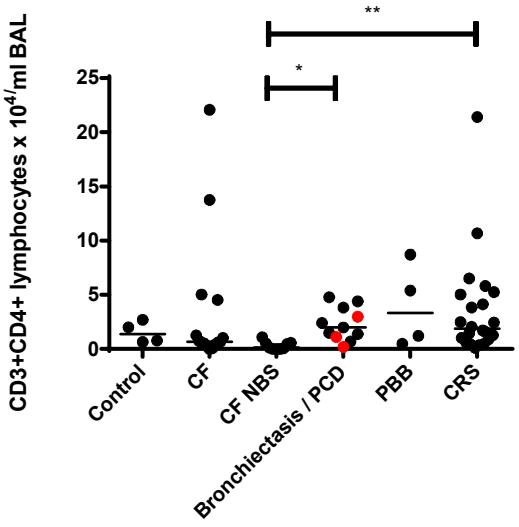
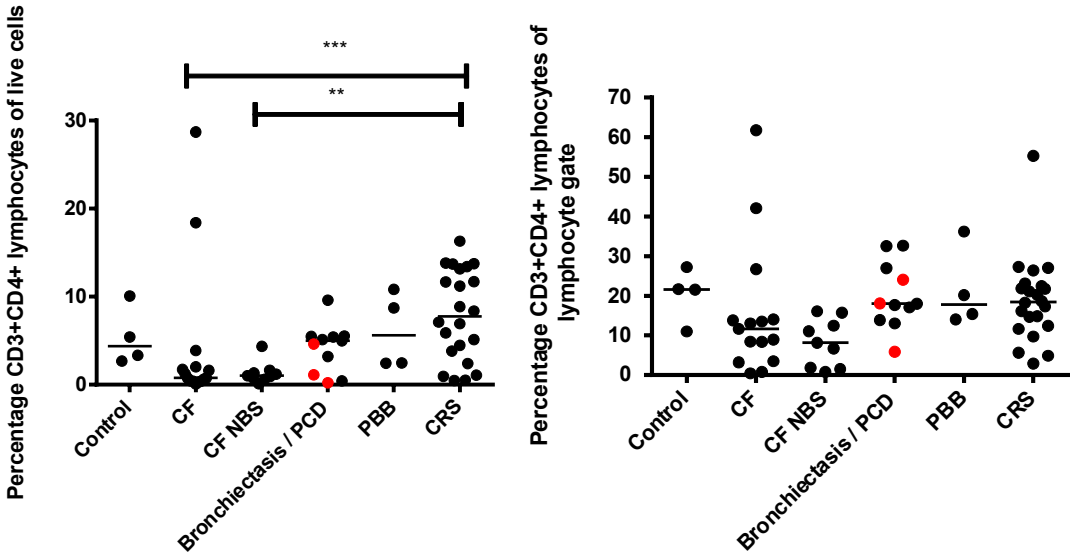


Figure 3.12: Graph showing number of CD3+CD4+ lymphocytes in BAL samples in different patient groups. Horizontal bars represent medians, which varied significantly between the groups ($p<0.01$). CD3+CD4+ cell numbers were higher in bronchiectasis / PCD (1.99[1.11-3.83] x 10^4/ml) ($p<0.05$) and CRS (1.88[0.82-5.08] x 10^4/ml) ($p<0.01$) as compared to CF NBS patients (1.13[0.009-0.54] x 10^4/ml). Red dots represent PCD patients.



A **B**

Figure 3.13: Graphs showing percentage CD3+CD4+ lymphocytes of the live gate (A) and percentage CD3+CD4+ lymphocytes of the lymphocyte gate (B). In graph A, CD3+CD4+ cells were significantly higher in CRS (7.7[3.5-13.2]) as compared to CF (0.8[0.4-2.0]) ($p=0.0001$) and CF NBS (1.0[0.7-1.5]) ($p<0.01$) patients. While the medians varied significantly ($p=0.01$), there were no significant differences between individual groups in graph B.

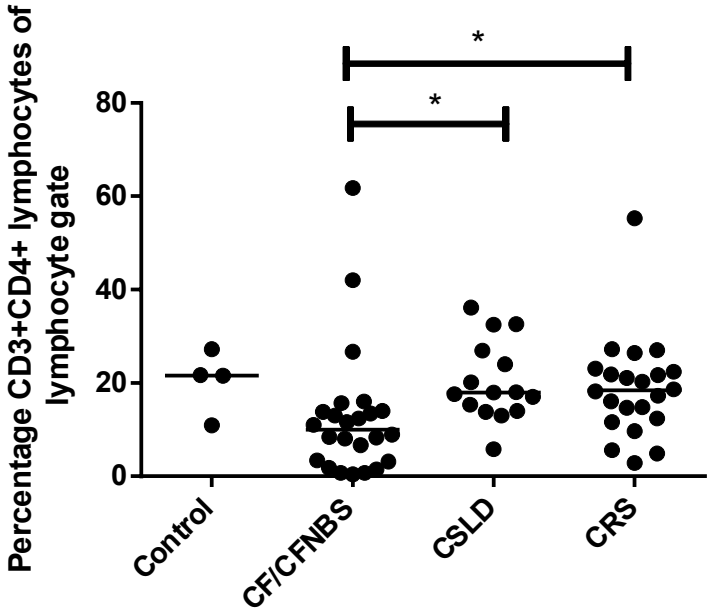


Figure 3.14: Graph showing percentage CD3+CD4+ cells of the lymphocyte gate in controls, all CF patients, CSLD and CRS. The percentage was significantly higher in CSLD and CRS as compared to all CF ($p<0.05$ for both).

Although there is variability in the results dependent on the method of analysis, these data show that levels of BAL CD3+CD4+ lymphocytes were lower in all CF patients than the other disease groups. This is most clearly seen in figure 3.14 above, where percentages were compared between all CF patients, CSLD and CRS. Having identified the CD3+CD4 lymphocyte population, it was then possible to identify Tregs.

3.15 CD3+CD4+CD25+FoxP3+ (referred to as CD25+FoxP3+) Lymphocytes

CD25+FoxP3+ cells were expressed as a percentage of CD3+CD4+ lymphocytes, as this is the traditional way these cells are expressed in the literature given there are such small numbers. They were then expressed as a ratio of BAL neutrophil count, to normalise for airway inflammation (figure 3.15). While it could be argued that cell counts rather than percentages would be a more accurate method of presenting data (if there are a large number of inflammatory cells, the cell percentage could fall while absolute numbers rise), this was not the case. The only finding seen was that there was no significant difference between the ratio of Tregs: neutrophils in controls and CSLD when expressed using cell numbers, whereas the ratio was higher in controls when results were presented as percentage Tregs.

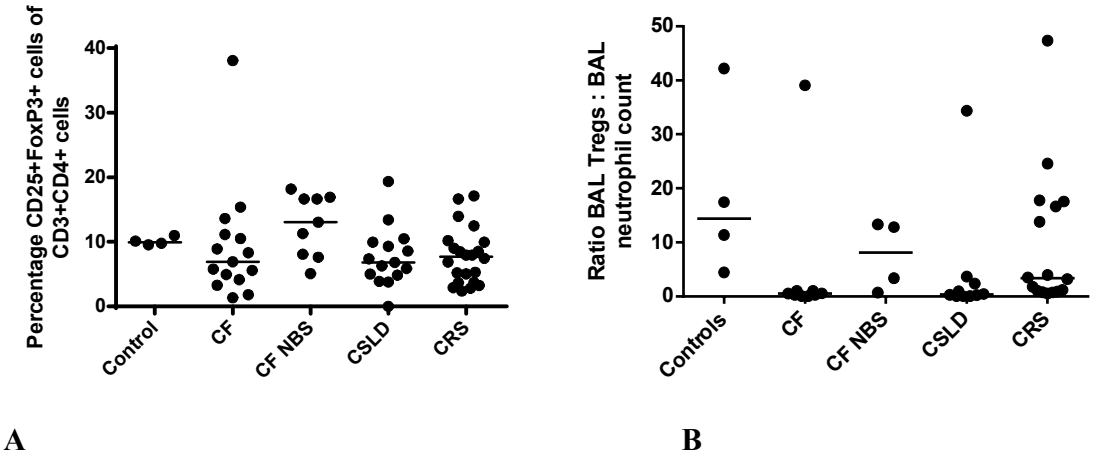


Figure 3.15: Graph A: Percentage CD25+FoxP3+ lymphocytes of CD3+CD4+ cells. Graph B: data normalised for neutrophilic inflammation. There were no significant differences between groups.

With results expressed as percentage Tregs, no significant differences were seen between patient groups, including when data was normalised for neutrophilic inflammation. However, although differences were not statistically significant (possibly due to small patient numbers) - normalised results were lower in CF and CSLD as compared to controls and CF NBS. Data were then analysed to see if there were specific differences between established CF, CSLD and controls (figure 3.16).

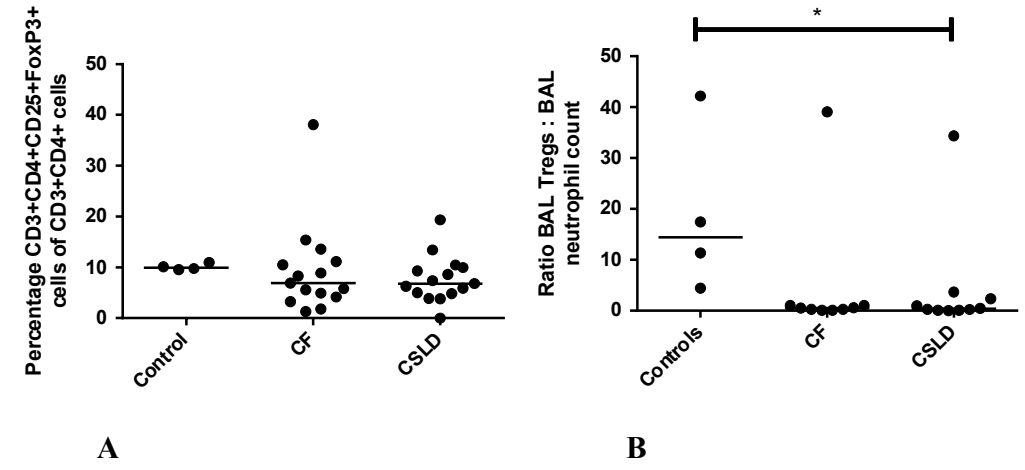


Figure 3.16: Graph A: percentage CD25+FoxP3+ Tregs in controls, established CF and CSLD. There were no differences between groups. Graph B: data normalised for neutrophilic inflammation. Ratios were statistically higher in controls as compared to CSLD ($p<0.05$).

Levels of CD25+FoxP3+ Tregs did not differ between controls, established CF and CSLD. However, when the data was normalised for inflammation by expressing as a ratio of BAL neutrophil count, ratios were higher in controls as compared to CF and CSLD (only statistically higher as compared to CSLD $p<0.05$) (figure 3.16). Finally data was analysed to examine whether there was a CFTR specific effect on Treg expression. No significant difference was seen between all CF and CSLD (figure 3.17).

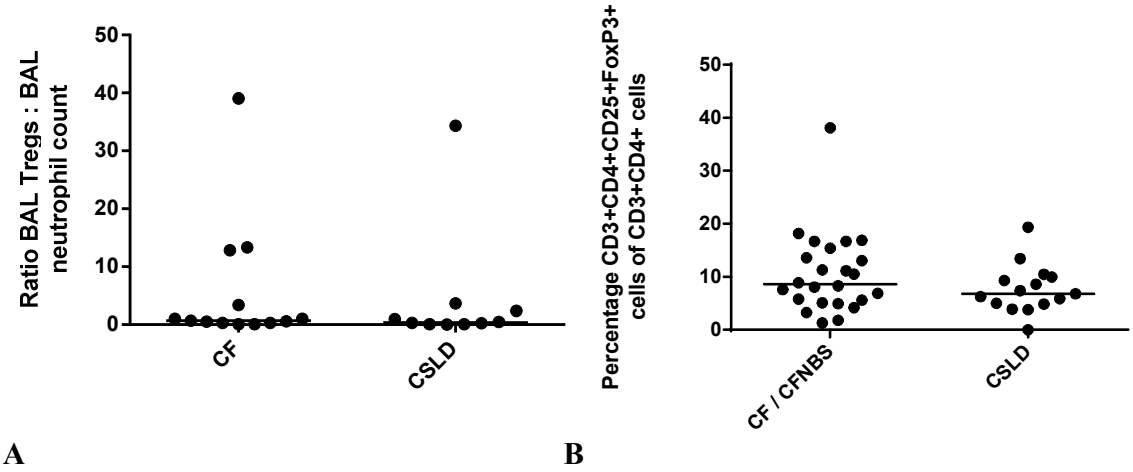


Figure 3.17: Graph A: percentage CD25+FoxP3+ Tregs in all CF and CSLD; Graph B: data normalised for airway inflammation. There were no differences between groups

The next section examines the effect of makers of inflammatory severity on BAL Treg expression. These include BAL neutrophils, CXCL8 and IL-10.

3.16 Correlation between Tregs and BAL neutrophil count

A positive correlation was seen between percentage Tregs and BAL neutrophil count in all CF patients ($r=0.6$, $p<0.05$) (and a weakly positive correlation when grouping all patients together $r=0.3$, $p<0.05$) (figure 3.18). There was no correlation seen in the CSLD group and therefore it was not possible to compare slopes and intercepts of best fit lines. No correlation was also seen on presenting Treg data as cell counts.

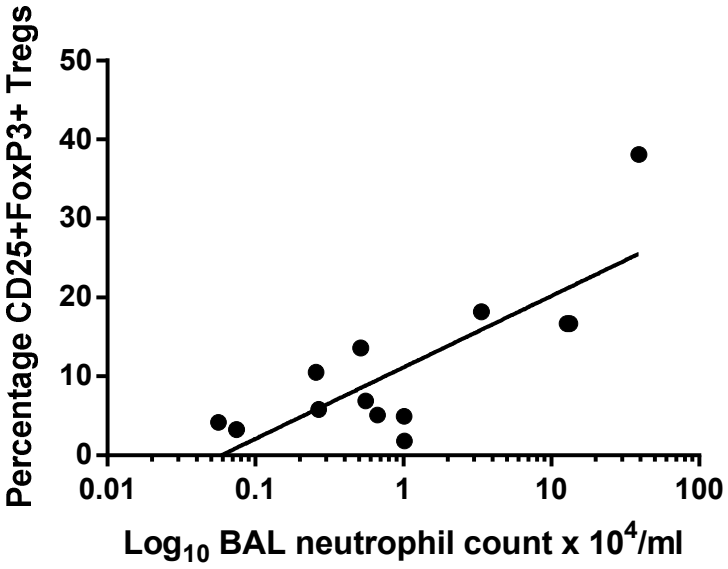


Figure 3.18: Graph showing positive correlation between BAL Tregs and neutrophil count in all CF patients ($r=0.6$, $p<0.05$; Y intercept 11.13, slope 9.03). Neutrophil counts have been Log_{10} transformed to expand the axis.

3.17 Correlation between Tregs and BAL CXCL8 and IL-10

There were a total of 33 patients with both Treg and cytokine results. No association was seen with BAL CXCL8 levels. Negative correlations were seen with IL-10 in all patients together and all CF patients (figure 3.19). However, again no correlation was seen in the CSLD group and thus again it was not possible to compare slopes and intercepts of CF and CSLD groups. No correlation was seen on expressing data as the total Treg cell count.

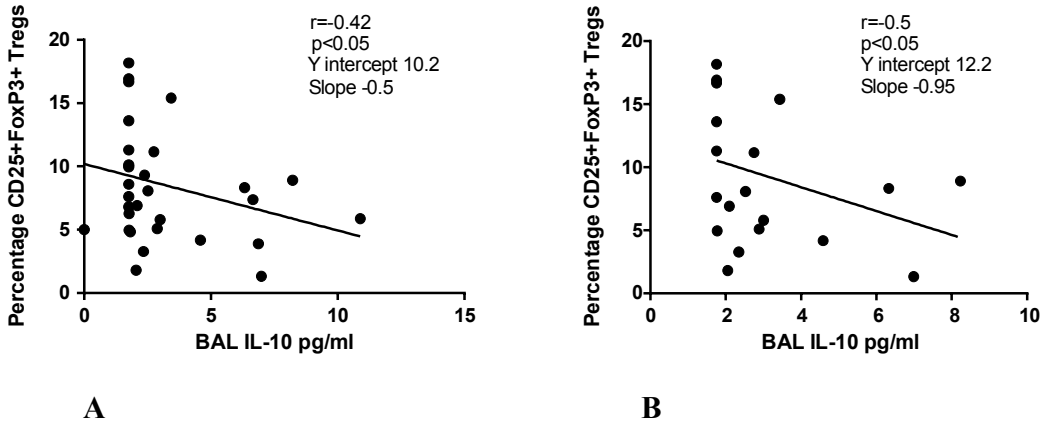


Figure 3.19: Graphs showing correlation between BAL Tregs and IL-10 in all patients (A) and all CF patients (B)

3.18 Treg Summary

Levels of BAL CD3+CD4+ cells were lower in CF as compared to other neutrophilic lung diseases. The percentage of BAL Tregs did not vary between patient groups – including no difference between CSLD and controls and no difference between CF and CSLD. The only exception was on data normalisation for inflammation, levels were statistically higher in controls as compared to CSLD. No correlation was seen with CXCL8 (data not shown). A positive correlation was seen with BAL neutrophil count and a negative correlation with BAL IL-10 in the CF but not the CSLD group. The potential implications of these findings are explored in the discussion section of this chapter.

3.19 Phenotypic analysis of other lymphocyte subsets and dendritic cells

There were insufficient cells in the control and CF NBS samples to perform further analysis of BAL lymphocytes. Therefore analysis of the other panels used only CF, bronchiectasis / PCD, PBB and CRS subjects. Bronchiectasis, PCD and PBB patients were grouped together as CSLD. For each lymphocyte subset, results were analysed for cell numbers, percentage cells of the live cell gate and percentage cells of the lymphocyte gate. Dendritic cells were expressed as numbers and percentage of the live gate.

3.19.1 Analysis of CD3+CD8+ lymphocytes, CD3+CD4+CD103+ lymphocytes and CD3+CD8+CD103+ lymphocytes

A total of 31 patients were included in the analysis: CF (12), Bronchiectasis (6), PBB (3) and CRS (10). No significant differences in levels of these lymphocyte subsets were seen between the patient groups (CF *vs* CSLD *vs* CRS). The method of identifying CD3+CD8+CD103+ lymphocytes is shown in figure 3.20. A similar method was used to identify CD3+CD4+CD103+ cells.

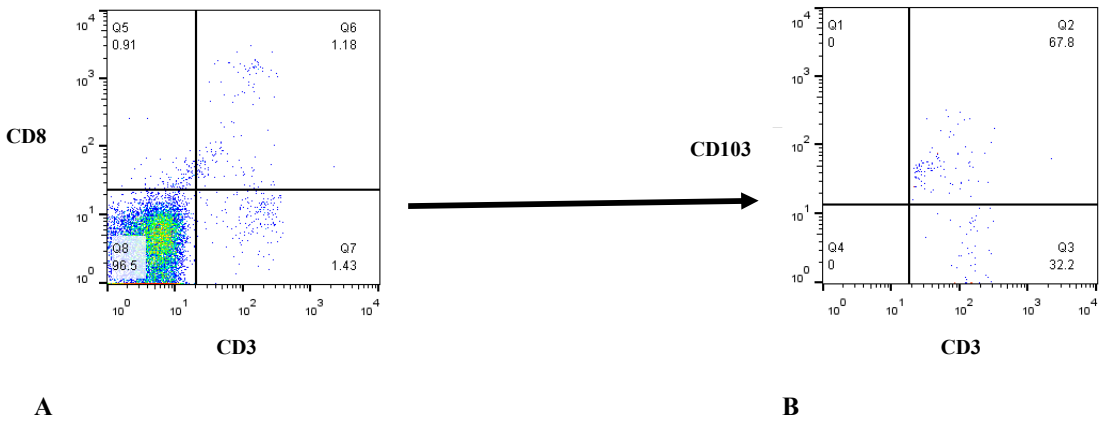


Figure 3.20: Identification of CD3+CD8+CD103+ lymphocytes. Having gated on the lymphocyte population, CD3+CD8+ cells were selected (A). The quadrant of CD3+CD8+ cells was then selected and those cells that were also CD103+ identified (B).

3.19.2 Analysis of CD3+CD56+ lymphocytes, CD3+CD69+ lymphocytes and CD3+CD19+ lymphocytes.

A total of 31 patients were included in the analysis: CF (13), Bronchiectasis (6), PBB (3), and CRS (9). No significant differences in levels of CD3+CD56+ or CD3+CD19+ were seen between groups. CD3+CD69+ cells were higher in CRS as compared to CF ($p<0.05$ – data not shown), but the significance of this is uncertain given the diverse range of patients in the CRS group. Illustrative examples of the identification of these three lymphocyte subsets is shown in figure 3.21.

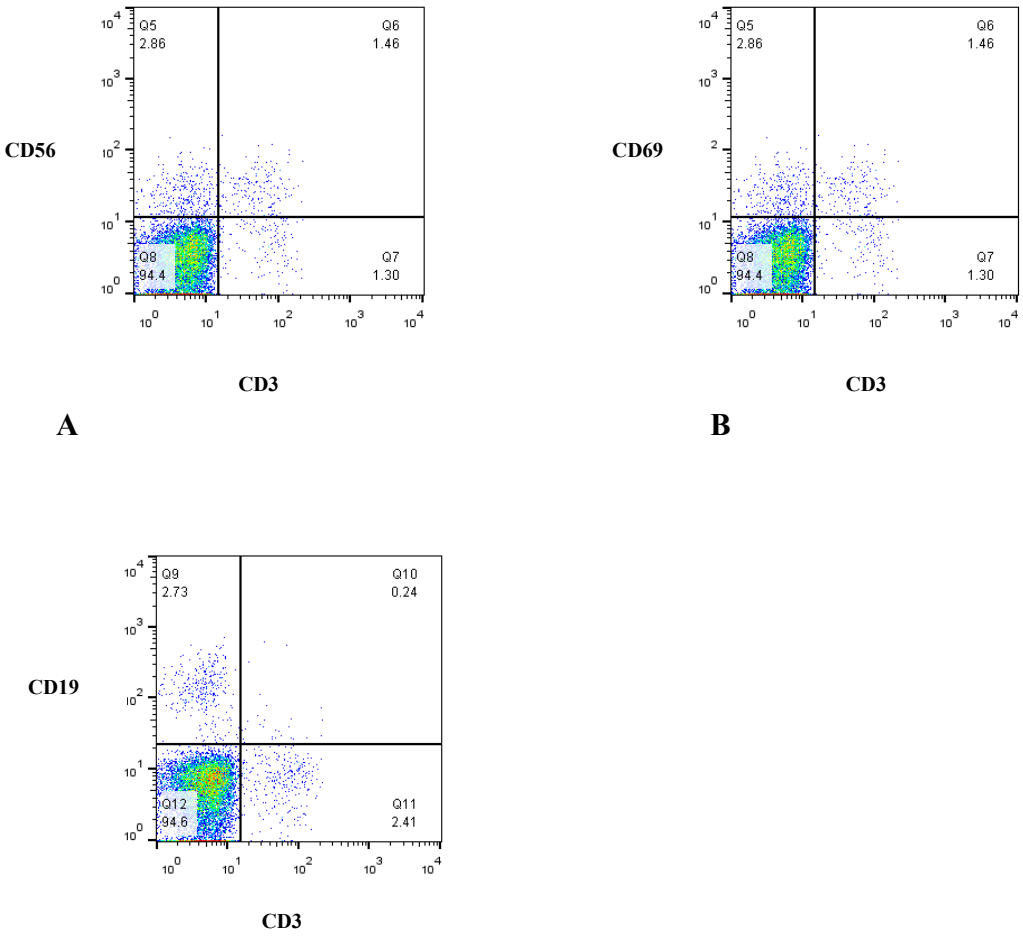


Figure 3.21: Examples of identification of a) CD3+CD56+, b) CD3+CD69+ and c) CD3+CD19+ lymphocytes

3.19.3 Analysis of HLA-DR+CD11c+ and HLA-DR+BDCA4+ cells

A total of 29 patients were included in the analysis: CF (15), Bronchiectasis (5), PBB (3) and CRS (6). BDCA4 levels were lower in CF and CSLD as compared to CRS (both $p<0.05$ – data not shown). CD11c levels were lower in CSLD as compared to CRS ($p<0.05$ – data not shown). Examples of the method of analysis of dendritic cells are shown in figure 3.22. Analysis firstly involved identification of the FITC negative population, and then analysing for HLADR+CD11c+ and HLADR+BDCA4+ cells.

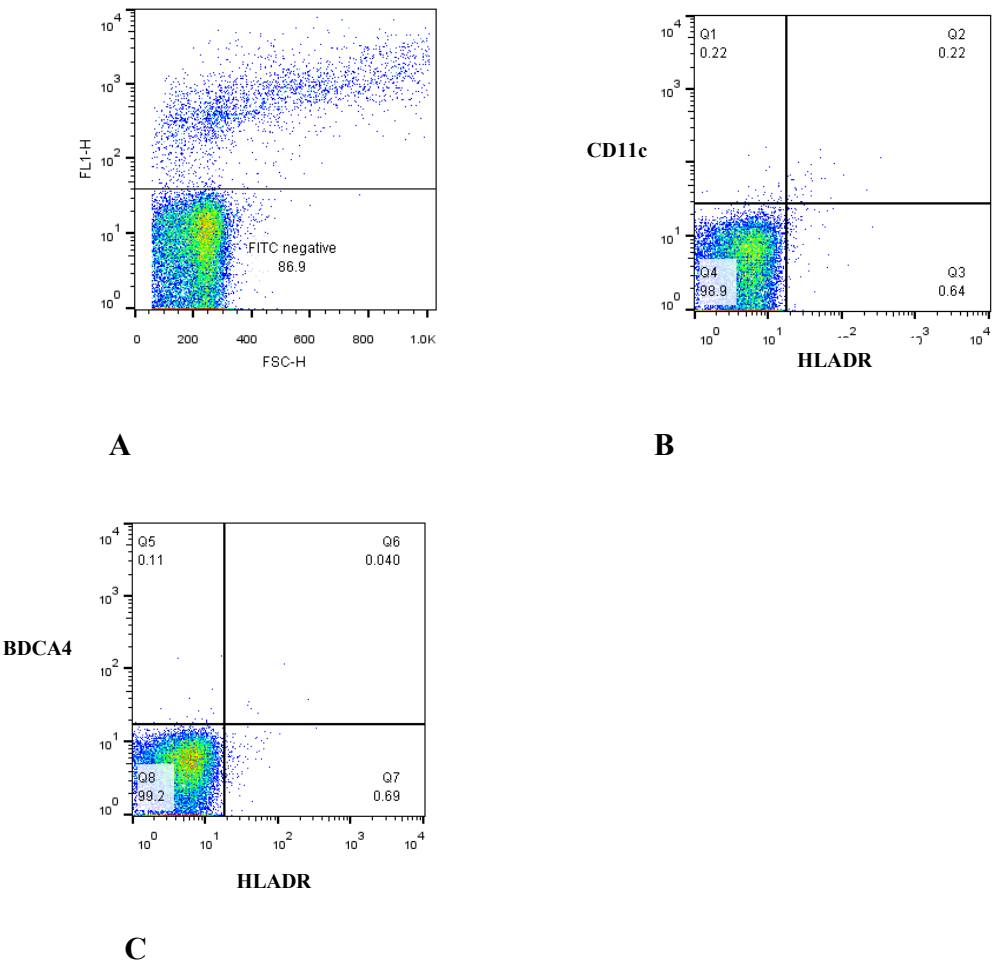


Figure 3.22: Examples of analysis of dendritic cells. A FITC-negative population was first identified (A) prior to setting quadrants for analysis of specific dendritic subsets (B) and (C).

3.19.4 Other lymphocyte subsets and dendritic cells summary

Levels of other measured BAL lymphocytes generally did not vary between groups. The only exceptions were CD3+CD69+ and dendritic cells where CRS levels were higher than CF (CD3+CD69+ and BDCA4) and CSLD (BDCA4 and CD11c). However the robustness of this finding is uncertain given the small patient numbers involved.

3.20 Discussion

Levels of CXCL8 and IL-10 were analysed from BAL in order to assess levels of these traditional pro- and anti-inflammatory cytokines in this cohort, and relate these to the

mediators analysed in this thesis. BAL samples were obtained from clinically indicated paediatric bronchoscopies and levels of cytokines measured by Luminex analysis. Samples were also analysed by flow cytometry in particular for Tregs. The findings from this research and the methodology will be discussed in the following sections, alongside limitations of the study.

3.20.1 Principle findings

Cytokine analysis

All BAL CXCL8 levels were above the LLD, and were higher in CF patients as compared to other groups, which has not been consistently reported by others. However, in this thesis higher levels were only seen in established CF patients, and no difference was seen between all CF (CF NBS and established CF) and CSLD.

Most BAL IL-10 levels were below the assay detection limit. Allowing for this, BAL IL-10 levels were higher in CF and bronchiectasis patients as compared to CF NBS, and CF and CSLD as compared to controls, which was an unexpected finding given that IL-10 is generally reported to be an anti-inflammatory cytokine. However, this difference was lost on normalising data for neutrophilic inflammation. In addition, on combining all CF patients together, no difference was seen between CF and CSLD. The ratio CXCL8:IL-10 was higher in all CF patients as compared to CSLD implying an impaired pro-resolution signal for the degree of inflammation in these patients. This finding was also seen in the context of positive BAL microbiological cultures.

Flow cytometry

A large proportion of BAL cell counts were $< 10^6$/ml, which therefore limited the number of different lymphocyte subsets that could be analysed in some patient groups. BAL cell counts were significantly higher in established CF patients as compared to CF NBS subjects. The percentage of CD3+CD4+ cells was lower in CF as compared to other neutrophilic lung diseases.

The percentage of Tregs did not vary between groups. On normalisation of data for neutrophilic inflammation, the ratio Tregs:BAL neutrophil count was statistically

higher in controls as compared to CSLD. No differences were seen between all CF and CSLD. A positive correlation was seen between the percentage Tregs and BAL neutrophil count in CF patients but not CSLD. Similarly a negative correlation was seen with BAL IL-10, again only in CF patients. No correlation was seen with BAL CXCL8.

Levels of other measured BAL lymphocytes generally did not vary between groups. The exceptions were CD3+CD69+ and dendritic cells where CRS levels were higher than CF (CD3+CD69+ and BDCA4) and CSLD (BDCA4 and CD11c). However, this may have been a chance finding due to the small numbers involved.

3.20.2 Strengths and weaknesses of the study

Cytokine analysis

The main weakness of BAL cytokine analysis was that a significant number of patients had BAL levels below the assay detection limit. This is often the case in paediatric samples where levels of mediators are inherently low, plus the dilution effect of lavage. By definition, the lower limit of detection (LLD) is the level at which a measurement has a 95% probability of being different than zero [287]. There remains ongoing debate as to the most appropriate way of including data below the LLD. Various methods are described in the literature. The most common and easiest method involves replacing the value with another numerical value – either the actual LLD value, LLD + 0.1 or a fraction of the LLD (LLD x 0.5 or LLD $1/\sqrt{2}$). However no overall consensus has been reached and therefore the LLD value was used in this thesis. Protease inhibitors were not used in the experimental work. The effects of neutrophil elastase may reduce cytokine levels and thus bring down any differences between established CF and other groups.

Flow cytometry

Isolation of live cells in the flow cytometry plot was performed manually by gating on live cells by eye. A more accurate method of identifying live cells would be to use markers such as annexin V conjugated to a fluorochrome, for instance FITC, which identifies phosphatidylserine in apoptosed cells; and propidium iodide which stains

necrotic cells with red fluorescence. After exposure to both probes, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. Using these markers would require increased BAL cell numbers, different lymphocyte surface marker fluorochromes and a flow cytometer capable of analysing a number of different fluorochromes. Therefore this was not considered to be practical for the samples in this thesis.

Another potential issue with the method is the identification of the lymphocyte population, again by eye. The identification of the gate was not repeated and so there are no data on reproducibility, although realistically this is rarely used. A variety of methods to establish lymphocyte gates have been used in the literature. These include:

- Analysis by light scatter only (the most commonly used, and used in this thesis)
- The combination of CD14 and CD45 along with light scatter to estimate the percentage of non-lymphocyte contamination of the gated area
- Light scatter with CD3 positive selection
- The combination of side scatter and CD45
- BAL lymphocyte gates defined by the light scatter characteristics of peripheral blood lymphocytes
- The use of commercial software programs

The light scatter pattern from BAL often shows overlapping clusters of cells and debris. This makes the lymphocyte population difficult to delineate, and can lead to the exclusion of cells of interest as well as the inclusion of unwanted cells. In addition cellular autofluorescence and non-specific binding can obscure or mimic specific staining of low-expression markers. Contamination of BAL with erythrocytes can also be problematic as erythrocytes show similar light scatter characteristics as lymphocytes. Some studies have used erythrocyte lysing agents, but lysing can lead to the release of cellular debris which can also interfere with accurate lymphocyte gating [288].

An alternative method of identifying the lymphocyte population by eye is the use of cell markers. CD45 is expressed on macrophages and granulocytes, but at lower levels than

lymphocytes [289]. CD14 is used to identify alveolar macrophages, but is limited by the fact that only 70 to 90% of these cells express this marker [290].

The combination of CD45 and side scatter has been used to gate on BAL lymphocyte populations [291]. The advantage of using CD45 and side scatter to gate on lymphocytes is that large lymphocytes are not excluded from analysis, since forward scatter is not used to select the cells. However selecting lymphocytes by CD45 expression and side scatter can underestimate high side scatter, large granular lymphocytes expressing CD16. By expanding the side scatter gate CD45-positive macrophages may be included in the analysis. Another potential problem is the presence of CD45-positive degranulated neutrophils in the BAL fluid. These cells, which may have arisen as a result of activation upon passage into the alveolar spaces, have side scatter characteristics which allow them to fall within the lymphocyte gate.

The markers used to identify Tregs are only able to identify naturally occurring Tregs, which express FoxP3 (although as previously discussed, FoxP3 alone is not a unique Treg marker). However, it was not possible to identify peripheral Tregs, such as those that are IL-10+, due to the large BAL cell numbers needed, which could not be obtained in this study. In addition the markers used to identify naturally occurring Tregs are not necessarily specific to this cell population. Indeed currently no single marker can provide an unequivocal identification of all human Tregs. The use of FoxP3 as a marker for Tregs came from murine data. In humans FoxP3 can be expressed by activated cells and is also expressed on some non-regulatory lymphocyte groups [137, 138]. In addition some groups have reported that staining of FoxP3 is accompanied by a reduction in sensitivity of other parameters. This includes a change in forward and side-scatter making identification of the live gate by eye using forward and side-scatter inaccurate. However, this problem could be overcome by including a fixable dead cell stain as discussed above. Another issue reported with FoxP3 staining is that it reduces fluorescence in other channels which may affect the results obtained with other fluorochromes [292].

The expression of other surface markers also differentiates Tregs including the downregulated expression of CD127 [139]. CD127 expression is often used if the identified cells are needed for further analysis, as permeabilisation for FoxP3 analysis renders the cells unsuitable for further examination. Use of the combination of CD127 and FoxP3 in addition to CD4 and CD25 may provide greater accuracy in identifying Tregs. There has been recent interest in other Treg markers: special AT-rich binding protein 1 (SATB1), latency associated peptide (LAP) and glycoprotein A repetitions predominant (GARP). SATB1 is crucial for T cell development through regulation of chromatin structure and gene expression. FoxP3 suppresses SATB1 and in experiments where FoxP3 is silenced with small interfering RNA, SATB1 expression increases. This increase in SATB1 leads to loss of suppressive function and subsequent effector cell development [293].

LAP and GARP are important in the role of TGFβ and Treg function. The latent form of TGFβ forms a complex with LAP and the proteolytic removal of LAP releases the active form of TGFβ [294]. The LAP/ TGFβ complex is expressed on activated Tregs and is anchored to the cell membrane by a protein termed GARP, although the role of GARP in Treg function is unknown [295].

In summary, although there are some weaknesses with the chosen method, within the constraints of small sample volumes, the method chosen was the most practical.

3.20.3 Strengths and weaknesses in relation to other studies

There are no previous studies examining BAL lymphocyte subsets in paediatric neutrophilic airway disease. Therefore the work in this thesis is novel in looking at a variety of children of differing ages with a number of different neutrophilic airway diseases of varying severity. One of the main initial issues in performing flow cytometry analysis of paediatric BAL was the low cell counts. In this thesis the median cell count of all patients was 48 x 10^4/ml (IQR 25-97 x 10^4/ml), and the median cell count of control subjects was 25 x 10^4/ml (IQR 20-45 x 10^4/ml). A number of previous groups have reported data on BAL cell counts and differentials from children without

lower airway disease. Table 3.6 shows the cell counts obtained by previous groups from these control paediatric patients. The results are quite varied; however the cell counts in this thesis fall within the previously described ranges. However, in some of the studies the upper limit of the normal range of BAL neutrophils was as high as 17% which therefore suggests that the lower airway was not necessarily normal in these control subjects. This highlights the difficulty of obtaining true healthy controls in paediatric research.

Two previous published studies in "normal" children have examined lymphocyte markers; one using immunohistochemistry techniques [268] and one using flow cytometry [273]. The median percentage CD3+CD4+ lymphocytes of total lymphocytes in these two studies were 34.5% (IQR 10-57) and 27% (IQR 22-32) respectively. The median percentage of CD3+CD4+ lymphocytes in this thesis was 21.6% (IQR 13.6-25.8). The results reported here are comparable to these published papers, although with only four control patients in the flow cytometry work in this thesis, plus different methodology to the published data, only limited conclusions can be drawn. The previous published papers also examined other lymphocyte markers. However in the work of this thesis cell counts were too small to be able to perform further lymphocyte subset analysis on control subjects.

Given there are no previous studies examining the expression of Tregs in BAL in neutrophilic lung disease, it is uncertain what the expected Treg cell percentage should be in these diseases. In order to answer this ideally a model of appropriate resolution of inflammatory airway disease is needed. However given this is not possible, the alternative is to measure Tregs in different paediatric neutrophilic diseases and relate these findings to markers of airway inflammation.

There is one previous published study examining BAL Tregs in paediatric asthmatic patients, and this paper included control subjects [168]. Thus there are some data available for the "normal" paediatric airway. The difficulty in analysing these results to look for concordance with BAL Tregs in this thesis is that there are differing methodologies and cell markers used. This paper quantified CD4+CD25^{hi} T regulatory cells and FoxP3

mRNA in BAL from children with asthma, chronic cough and control subjects without lower airway disease. This group showed that BAL FoxP3 mRNA levels increased in asthmatic subjects after being prescribed ICS for four weeks. The median percentage of these cells in BAL from the control subjects was 7%. The median percentage in asthmatics in which inhaled corticosteroids were not prescribed was 2%, and this had almost normalised following the prescription of ICS. The median percentage of Tregs in this thesis in control subjects was 10%. Howver, given there are only four control patients in this thesis and the different cell markers used, it is difficult to be certain that the results are truly comparable.

3.20.4 Interpretation of the results

CXCL8

BAL CXCL8 was higher in established CF as compared to other disease groups. This is in contrast to previous work and may be a reflection of differing samples [8]. The published work used sputum whereas this work used BAL. Although similar results between these two samples could be expected, this may not be the case as samples taken may reflect differing inflammation within the proximal and distal airway. Differences in patient cohorts may also be important as well as dilution effects of lavage. However given in this thesis levels were similar in all CF patients (CF and CF NBS) and CSLD, overall the differences seen were not CF-specific.

Il-10

BAL IL-10 levels were higher in established CF and CSLD than controls, which was an unexpected finding given that IL-10 is generally reported to be an anti-inflammatory cytokine. However, given this difference was lost on normalising data for neutrophilic inflammation, this suggests that the higher levels seen are a reflection of a higher inflammatory state in these patients. In addition, on combining all CF patients together (established CF and CF NBS), no difference was seen between CF and CSLD – implying differences seen were not related to the CFTR-defect. The ratio CXCL8:IL-10 was higher in all CF patients as compared to CSLD, in particular those with positive microbiological cultures. This suggests that there is an inappropriately low pro-resolution stimulus for the degree of inflammation in these patients.

	Clement *et al* [296]	**Ratjen *et al*** [268]	**Riedler *et al*** [273]	**Midulla *et al*** [297]	**Tessier *et al*** [298]
Number	11	48	18	16	11
Age range (years)	1-15	3-5	0.08-10	0.16-3	4-16
Sedation	LA	GA	GA	LA	LA
Number of aliquots	6	3	3	2	6
Saline volume	10% FRC	3ml/kg	3ml/kg	20ml	10% FRC
BAL x 10^4/ml					
Median	24	7.3	15.5	51	30.5
Range	7-50	0.5-57.1	7.5-25.8 *	20-130	9-68
AM %					
Median	89	84	91	87	92.5
Range	85-97	34.6-94	84.2-94 *	71-98	77-98
Lymph %					
Median	10	12.5	7.5	7	8
Range	1-17	2-61	4.7-12.8 *	2-22	2-22
Neut %					
Median	1	0.9	1.7	3.5	1
Range	0-3	0-17	0.6-3.5 *	0-17	0-3
Eos %					
Median	ND	0.2	0.2	0	0
Range	ND	0-3.6	0-0.3 *	0-1	0

Table 3.6: BAL differential cell counts from different studies of "normal" children. LA= local anaesthetic; GA=general anaesthetic; FRC=functional residual capacity; IQR=interquartile range; AM= alveolar macrophages; Lymph= lymphocytes; Neut= neutrophils; Eos=eosinophils; ND=not done. Adapted from De Blic J., ERJ 2000 [271]

Nevertheless the finding that BAL IL-10 levels alone were higher in established CF patients is interesting and in contrast to previous published work [90, 91]. IL-10 levels may be elevated during acute inflammatory states [186, 187]. Almost all the CF patients in this thesis had a pulmonary exacerbation at the time of bronchoscopy. This is in

contrast to adult studies where it is ethically possible to perform bronchoscopies on stable patients for research purposes. This may therefore explain why IL-10 levels were higher in the children in this thesis. Alternatively adults as a group tend to have more severe disease than children – and levels of IL-10 may be reflective of the underlying disease state.

The observation of a negative correlation between Tregs and BAL IL-10 in CF patients alone may be a chance finding. It was not possible to say whether in this situation IL-10 is pro-inflammatory or anti-inflammatory, or whether another factor drives up neutrophilia and drives down IL-10. Longitudinal or interventional studies would be needed to explore this further.

Tregs

CD3+CD4+ cells were lower in the CF airway, and this may be a reflection of the relative neutrophilia seen in these patients. In addition previous work has shown the compartmentalisation of pathology in the CF airway, whereby neutrophilic inflammation is seen within the airway lumen and lymphocytosis within the submucosa [77].

The finding of no significant difference in the percentage of BAL Tregs between groups is perhaps unsurprising given in these neutrophilic airway diseases, neutrophils are the predominant cell in the airway lumen. Although research regarding the compartmentalisation of pathology in all neutrophilic lung diseases is limited, as discussed above lymphocytosis is a predominant feature of the airway submucosa and not the lumen in CF. Therefore significant differences in lymphocyte subsets may not be seen in BAL, but may be seen in the airway wall – an area for future research. However, normalisation of data for neutrophilic inflammation showed higher Treg: neutrophil ratios in controls as compared to CF and CSLD (only significant for CSLD) – and therefore the ability of Tregs to increase in response to neutrophilic inflammation may be impaired in these disease groups. There was however no evidence of a CF-specific effect. Correlations with neutrophils and cytokines were inconsistent. The positive correlation with neutrophils suggests Tregs increase in-line with neutrophilia in

CF patients, but no association was seen with CXCL8. This may however mean that chemokines other than CXCL8 are important in this situation. The meaning of the negative correlation with IL-10 is uncertain, as discussed above.

Therefore, as regards the original hypotheses, the ratio of BAL CXCL8:IL-10 was higher in neutrophilic lung diseases as compared to controls, and levels were higher in those with more severe disease (established CF) as compared to phenotypically milder disease (CSLD), which proves the first part of the cytokine hypothesis – that differences seen between groups are related to disease severity. This ratio was also higher in combined established CF and CF NBS, as compared to CSLD, suggesting a direct effect of CFTR dysfunction, thus also proving the second cytokine hypothesis. In addition the ratio BAL CXCL8:IL-10 was higher in CF patients with positive microbiological cultures as compared to those with negative cultures, thus suggesting an impaired ability of CF patients to resolve CXCL8 mediated inflammation by IL-10 in the presence of acute infection.

As regards the Treg hypotheses, on normalisation for neutrophilic inflammation, BAL Tregs were lower in CSLD as compared to controls, but there was not clear evidence of lower levels in more severe lung disease or a CFTR effect. The positive correlation between BAL neutrophils and Tregs in CF is interesting, although the precise meaning of this is uncertain. The negative correlation seen with IL-10 also warrants further investigation. Thus there was no evidence to conclusively prove the hypotheses that Tregs are lower in neutrophilic diseases as compared to controls, that there are lower levels with increasing disease severity or that there is a CFTR-related effect.

Chapter 4

AnxA1 & ALXR in the airway wall and lumen

4.1 Analysis of AnxA1 in the paediatric neutrophilic airway

Although AnxA1 has been investigated in adults with CF, the role of AnxA1 has not been established in other neutrophilic lung diseases or in children. AnxA1 immunofluorescence is reduced in human adult CF nasal epithelial cells, as compared to controls, and differential expression of AnxA1 may be related to disease severity [96]. Levels of BAL AnxA1 are reduced in adult CF patients and those with interstitial lung disease, as compared to controls [97].

The hypotheses of this chapter are that

- AnxA1 and ALX (mRNA and protein) levels are reduced in severe neutrophilic inflammation (established CF) as against more mild disease (PCD, bronchiectasis, PBB, CRS), and that control levels lie between these two groups.

- AnxA1 and ALX (mRNA and protein) levels are reduced in CF NBS due to a direct effect of CFTR dysfunction.

In summary, in order to address these hypotheses, AnxA1 and ALX mRNA was measured in BAL cell pellets and EBB. Subsequently a novel immunofluorescent staining protocol was established for AnxA1 in EBB. Attempts were also made to stain EBB for ALX and also cytospins for AnxA1 and ALX. The next section describes the methods and results of these analyses.

4.2 AnxA1 and ALX mRNA analysis of BAL and endobronchial biopsies

4.2.1 Annexin RNA extraction and real-time polymerase chain reaction (RT-PCR)

RNA was extracted from endobronchial biopsies that had been frozen in RNAlater at -80^{o}C. Samples were defrosted and the biopsy and supernatant transferred to another tube to avoid the persisting presence of RNAlater crystals affecting the RNA extraction process. Following tissue homogenisation, RNA was extracted using an RNAeasy minikit (Qiagen, Crawley, UK) as per the manufacturer's instructions.

Cell pellets stored in RNAlater were also defrosted, although, for reasons that are unclear, large RNAlater crystals were not visible. Samples were re-centrifuged (Sorvall Legend RT) at 300g to keep the cell pellet at the bottom of the tube and mucus debris separate at the top of the supernatant. This mucus was discarded and the sample processed as above with RNA extraction using an RNAeasy minikit (Qiagen, Crawley, UK) as per the manufacturer's instructions.

4.2.2 Reverse transcription

The RNA solution was then transferred to a new RNase-free tube. RNA was then ready for reverse transcription into cDNA using an Abgene thermal cycler. For each sample, 2 pmol of sequence specific primers for the following genes: 18s ribosomal subunit (18s), human β-Actin (β-Act), Annexin A1 (AnxA1) and formyl peptide receptor 2/Lipoxin A4 receptor (FPR2/Alx) (Qiagen, Crawley, UK), 2µl 10mM dNTP Mix, and 19.2µl of RNA were first incubated at 65°C for 5min (to denature RNA secondary structure) and placed on ice for 2min (to allow primers to anneal). Tubes were then centrifuged at 1250*g* for 30s and the other components for reverse transcription were added, including 8µl 5X first-strand buffer, 2µl 0.1M DTT, 2µl RNaseOUT (a recombinant RNase inhibitor), and 2µl of Superscript™ III Reverse Transcriptase (200U/µl) (Invitrogen, Applied Biosystems, Warrington, UK). Samples were gently mixed and incubated at 55°C for 1h (for cDNA extension) and then heated at 70°C for 15min to inactivate the enzyme and hence halt the reaction.

4.2.3 cDNA Quantification

cDNA was then quantified using a Nanodrop ND-1000 Spectrophotometer. With the sampling arm open, 1µl of sample was pipetted onto the lower measurement pedestal, the sampling arm was closed and spectral measurement made using the operating software on the PC; for cDNA quantification, the nucleic acid option was selected. When the measurement was complete, the sampling arm was opened and wiped gently from both the upper and lower pedestals using a soft tissue. The next sample was then measured and procedure repeated. The 260/280 ratio of the sample absorbance was used to assess the purity of the sample, a ratio of approximately 1.8 is generally accepted as 'pure' for DNA and a ratio of 2 generally accepted as pure for RNA (data

shown in appendix). If the ratio is lower, this indicates that protein/phenol or other contaminants are present that absorb near 280nm. The concentration of cDNA was measured in ng/µl from the absorbance at 260nm. cDNA was diluted to 40ng/µl in new tubes using molecular biology H_2O, ready for loading into 384-well plates for Real-Time PCR.

4.2.4 Realtime PCR

The relative gene expression in the patient and control samples was performed using ABI Prism® 7900 Real Time PCR equipment. A mastermix was made for each gene specific primer, containing 1µl primer, 5µl 2X POWER SYBR Green (Applied Biosystems, California, USA) and 2µl H_2O; a final volume of 8µl was dispensed in each well and 2µl of diluted cDNA (60ng/µl) was added. Each sample was tested in duplicate for each of the four genes (above). The thermal profile consisted of 95°C for 15min, then 40 cycles of 94°C for 15s, 55°C for 30s and 72°C for 30s. Following this a dissociation step was added, in order to determine if the PCR reaction was specific to the genes under investigation, with the following thermal profile: 95°C for 15s, 60°C for 15s and 95°C for 15s. SYBR Green binds to newly synthesized double-stranded DNA, emitting fluorescence in direct proportion to the number of amplicons generated. A threshold line of fluorescence detected above background was set in the exponential phase of amplification, and the cycle number at which the samples reached this level was referred to as the cycle threshold value (Ct), thus reflecting the relative abundance of the specific mRNA transcripts. Comparison quantification between samples was performed using the ΔΔCt method. With this method the Ct for the gene of interest (in this case AnxA1 or ALX) in both the patient samples and control samples are adjusted in relation to a normaliser gene or genes (in this case the geometric mean of 18s and β-Act, chosen as these have previously been used successfully by Professor Perretti's laboratory). The resulting ΔΔCt value is incorporated to determine the fold difference in expression as outlined below:

Fold difference = $2^{-\Delta\Delta Ct}$

$\Delta Ct_{patient} - \Delta Ct_{control} = \Delta\Delta Ct$

$Ct_{GOI\ patient} - Ct_{normaliser\ patient} = \Delta Ct_{patient}$

$Ct_{GOI\ control} - Ct_{normaliser\ control} = \Delta Ct_{control}$

GOI = gene of interest

Each patient and control sample was compared to the Ct mean of the control samples.

4.3 Development of an immunofluorescent staining protocol for AnxA1 in EBB

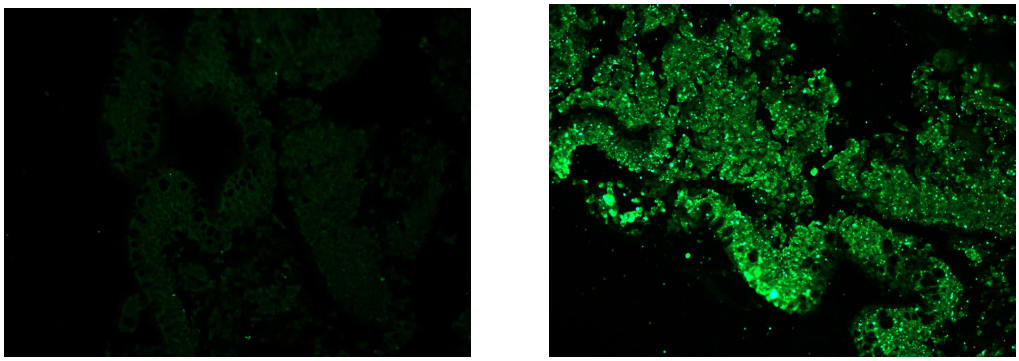
No published protocols exist for immunofluorescent staining of paraffin-fixed endobronchial biopsies for AnxA1 in either adults or children. A protocol was therefore established by the author. Generic immunofluorescent staining protocols from the Lloyd laboratory were used as a starting point for developing this technique and adapted as needed to provide the best staining. Slides from control paediatric subjects (those with isolated upper airway disease) were used to provide positive controls, based on prior adult data that healthy controls (albeit nasal epithelial cells) should express AnxA1 in tissue, whereas disease groups may not [96].

As per standard procedure for preparing paraffin-fixed biopsies, slides were dewaxed in two changes of Histoclear (National Diagnostics, Georgia, USA) each for 5 minutes. They were then rehydrated through 100%, 90% and 70% ethanol (each stage 30 seconds) and finally placed in phosphate buffered saline (PBS). The slides were washed in PBS for 5 minutes.

4.3.1 Antigen retrieval

Tissue samples fixed in formalin and paraffin require antigen retrieval prior to immunohistochemical staining, to counteract the effect of the formation of methylene bridges during the fixation process, leading to cross-linking of proteins and masking of antigenic sites. Antigen retrieval can be performed by heat-mediated or enzymatic methods. Heat mediated antigen retrieval was utilised on the basis of prior laboratory experience and as enzymatic retrieval can lead to damage to tissue morphology.

Heat-mediated retrieval was initially attempted with 10mM sodium citrate (pH 6) using 3 slides. However, staining was unsuccessful (Figure 4.1A). Because the pH of the solution may affect antigen retrieval this was reattempted using a further 3 slides with trishydroxymethylaminomethane ethylenediaminetetraacetic acid (Tris EDTA) (Tris 1.21g, EDTA 0.37g in 1000ml distilled water; pH 9) – with resultant improved staining (figure 4.1B).



A **B**

A) AnxA1 antigen retrieval using 10mM sodium citrate (pH 6). Magnification X 20

B) AnxA1 antigen retrieval using Tris EDTA (pH 9). Magnification X 20

Figure 4.1 Comparison of antigen retrieval techniques. Staining was unsuccessful using 10mM sodium citrate (A), but successful using Tris EDTA (B)

Slides were therefore microwaved in 3 changes of Tris EDTA, each for 3 minutes. The slides were left to cool and then washed in PBS for 5 minutes.

4.3.2 Avidin-Biotin amplification

Avidin-biotin amplification was utilised based on prior laboratory experience to obtain a stronger staining signal. This technique was first established in the 1980s [299]. Avidin is a protein found in chicken egg white, which binds with high affinity to biotin, an enzyme co-factor in carboxylation reactions. Following the addition of the primary antibody of interest, a secondary antibody conjugated to biotin is added, followed by a tertiary avidin antibody conjugated to a fluorochrome. Avidin has four binding sites for biotin and binding is irreversible. To prevent the effects of endogenous avidin and biotin, which are present in a number of tissues and can bind the avidin-biotin complex

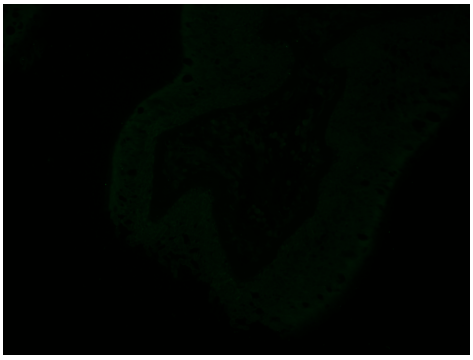
leading to background staining, avidin-biotin blocking solutions (Vector Laboratories, Peterborough, UK) were used.

Slides were therefore flooded with avidin blocking solution for 15 minutes at room temperature. They were then washed for 5 minutes with PBS and 0.3% Triton-X 100 (used to permeabilise the tissue and discussed below) (Sigma Aldrich, Dorset, UK), before the addition of biotin blocking solution for a further 15 minutes at room temperature. Slides were then washed again for 5 minutes with PBS and 0.3% Triton-X 100.

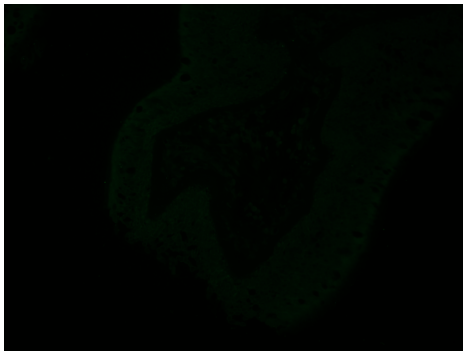
4.3.3 Cell permeabilisation

As AnxA1 is mostly intracellular, cell permeabilisation was utilised. 3 slides were used for each method of cell permeabilisation. Initially 0.05% Saponin (Sigma Aldrich, Dorset,UK) was used to permeabilise cells as it was felt this might not be as harsh as alternatives such as Triton-X 100. However, staining was poor with this technique and so Triton-X 100, initially at 0.025% was tried. This also demonstrated poor staining and so the concentration was increased to 0.3% with good results (figure 4.2).

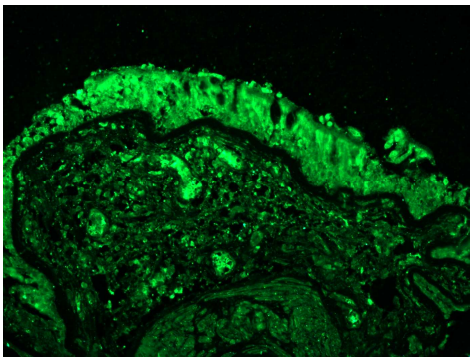
Following avidin-biotin blocking, cells were flooded with protein block (DAKO, Cambridgeshire, UK) and 0.3% Triton-X 100 for 30 minutes at room temperature. This was then tipped off and slides incubated overnight with either AnxA1 antibody, or an isotype control.



A



B



C

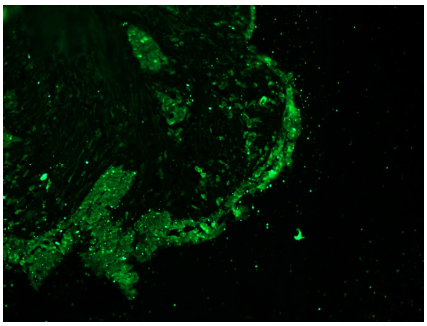
Figure 4.2: Cell permeabilisation methods. Permeabilisation was unsuccessful using 0.05% Saponin (A) and 0.025% Triton-X 100 (B), Permeabilisation was successful using 0.3% Triton-X 100 (C). Magnification X 20.

4.3.4 AnxA1 Staining

AnxA1 antibody (IgG1 raised in mouse, initial concentration 1mg/ml) was generously donated by Prof M Perretti, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, UK. A control slide was prepared for each patient sample by incubating one slide from each patient with a mouse IgG1 isotype control (Becton Dickinson, Oxford, UK). The AnxA1 antibody was used at a dilution of 1:30. A series of experiments were repeated, each with 3 slides, with alternative (1:50, 1:100) diluctions, but staining was unsuccessful (figure 4.3). All antibodies were diluted with PBS, 0.3% Triton-X 100 and 1% human serum.

A

B



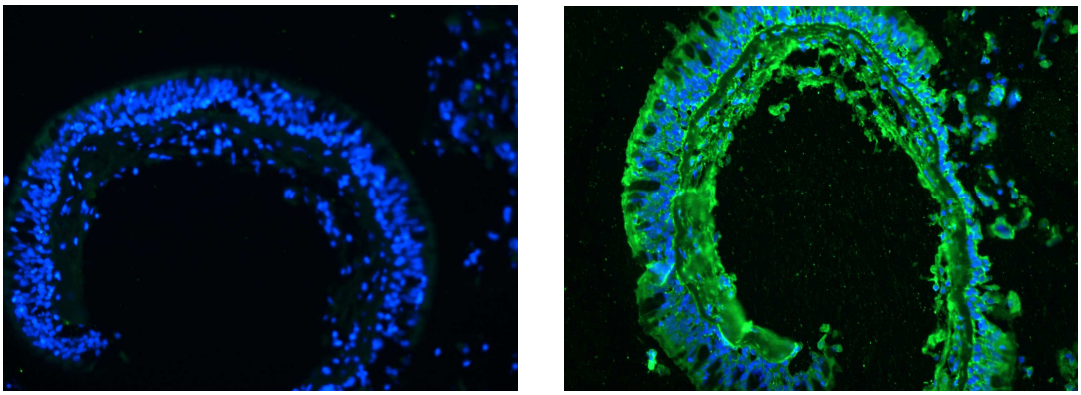
C

Figure 4.3: Examples of AnxA1 staining of EBB using different concentrations of AnxA1 antibody.. Staining was unsuccessful with AnxA1 concentrations of 1:100 (A) and 1:50 (B), but successful at 1:30 (C). Magnification X 20.

Following overnight incubation, slides were washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes. They were then incubated in a secondary rabbit anti-mouse biotin-conjugated antibody (Jackson Immunoresearch, Stratech Scientific, Suffolk, UK) at a dilution of 1:250 at room temperature for one hour.

4.3.5 Streptavidin Amplification

All slides were again washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes. They were then incubated with streptavidin antibody conjugated to Alexa Fluor 488 (Invitrogen, Applied Biosystems, Warrington, UK) at a dilution of 1:250 at room temperature for one hour in the dark. Slides were finally washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes in the dark. They were then mounted with DAPI (Invitrogen, Applied Biosystems, Warrington, UK) and stored at 4^{o}C in the dark until ready for analysis. Once the staining method had been developed it was repeated on two occasions with two slides each from control, CF, bronchiectasis and PBB patients, to ensure staining was reproducible. An example of successful staining in a control patient is shown in figure 4.4.



A **B**

Figure 4.4: Example of stained healthy control EBB: a) control slide stained with mouse IgG isotype (green) and b) staining with AnxA1 antibody at 1:30 (green). Both slides were counterstained with DAPI (blue). Magnification X 20.

4.3.6 Analysis of immunofluorescent-stained paraffin-fixed endobronchial biopsy slides

Slide images were acquired using a Leica microscope (Milton Keynes, UK) in the dark. Analysis of the amount of AnxA1 (green) immunofluorescent staining was performed using Definiens Image Analysis Software, München, Germany. As immunofluorescent staining for AnxA1 was seen predominately in the epithelium, the epithelium on each slide was identified manually (see figure 4.5) using the edge of the RBM to delineate the inferior border. The computer software was then able to measure the area of epithelium identified and the staining intensity of green and blue, as analysed by pixel number. Manually defining the epithelium allowed the area for analysis to be identified. However it was not accurate enough to be able to express AnxA1 staining as a percentage of the epithelial area identified. It was therefore necessary to devise a method so that the amount of AnxA1 staining could be quantified. DAPI stains nuclear material and is therefore a proxy measurement of cell numbers in the epithelium, and thus the epithelial area. It can clearly never be a true measurement of the epithelial area, but the amount of DAPI staining per cell can be assumed to be reasonably consistent. Results were therefore expressed as the percentage of AnxA1 (green) staining of DAPI (blue) staining.

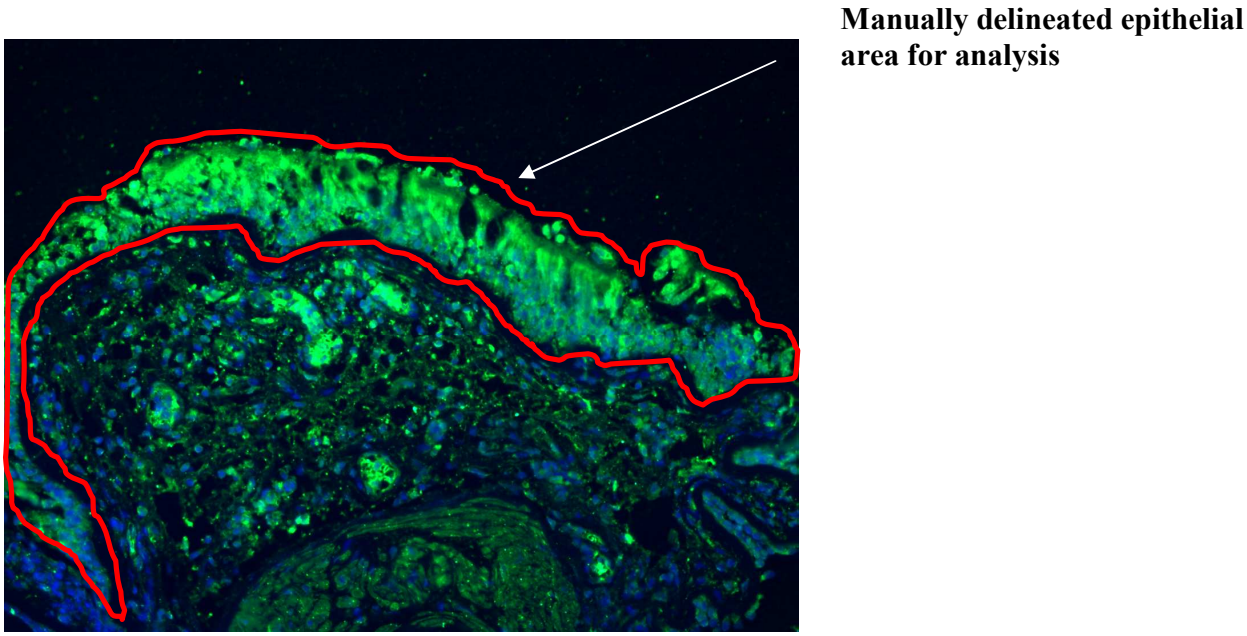


Figure 4.5: Endobronchial biopsy showing method of manual delineation of the epithelial area. There is positive staining of AnxA1 (green), which is predominately in the epithelium, and DAPI staining of nuclear material (blue). Magnification X 20

The next section describes attempts to establish an immunofluorescent staining protocol for ALX.

4.4 Attempts to establish an immunofluorescent staining protocol for ALX using paraffin-fixed paediatric endobronchial biopsies

There are no established immunofluorescent staining protocols for ALX in paraffin-fixed endobronchial biopsies. Attempts to establish a staining method were performed by the author. Slides from healthy children were used to provide positive controls. Again, established staining protocols were used as a basis for method development and the principle method was similar to that used to stain for AnxA1. Slides were dewaxed and heat mediated antigen retrieval using Tris EDTA was utilised as described above. As ALX is expressed on the cell surface cell permeabilisation should not be needed. However, attempts were made to stain with and without cell permeabilisation by 0.3% Triton X-100 (3 slides each). Avidin-biotin amplification was also used with appropriate blocking steps as previously outlined. The primary antibody (rabbit IgG polyclonal, Abcam, Cambridge, UK) was used at dilutions of 1:30, 1:50, 1:100 and 1:500 (using 3 slides for each concentration). A rabbit IgG isotype control was also

used at the same dilutions with a control slide for each patient. Following overnight incubation and washing steps as previously described, biotinylated goat anti-rabbit secondary antibody (Jackson Immunoresearch, Stratech Scientific, Suffolk, UK) was added at a dilution of 1:250. Tertiary streptavidin Alexa Fluor 488 labelled antibody was used as in the AnxA1 staining protocol as outlined above, again with washing steps, at a dilution of 1:250 and with slides kept in the dark. Slides were then mounted with DAPI. Unfortunately despite the method modifications described it was not possible to establish positive biopsy staining.

As AnxA1 and ALX mRNA were detected in BAL, the next section discusses staining of BAL cytospins for AnxA1 and ALX. In an attempt to determine which cells stained positively for AnxA1 and ALX, attempts were also made to double stain for neutrophils and macrophages.

4.5 Attempts to establish an immunofluorescent staining protocol for ALX and AnxA1 using bronchoalveolar lavage cytospins

4.5.1 Preparation of cytospins

BAL samples were centrifuged at 200g for 5 minutes. The supernatant was removed and stored in 500µl aliquots at -80^0C. The cell pellet was resuspended in 500µl RPMI (Sigma-Aldrich, Dorset, UK) and a cell count was performed as before. The cell count was adjusted to 5×10^4 per 100µl. Glass slides were labelled with the study number, loaded into a cytospin centrifuge and spun at 18g for 4 minutes. The slides were then air-dried for at least 5 minutes, and then fixed in methanol for 5 minutes. They were then washed in PBS for 1 minute and finally demineralised water for 1 minute. Slides were then left to dry, wrapped and stored at -20^0C.

4.5.2 Development of an immunofluorescent staining protocol

Immunofluorescent protocols do not exist for cytospin staining of BAL for AnxA1 and ALX. A method was therefore devised by the author. Attempts were made to double-stain cytospin preparations for either AnxA1 or ALX, and neutrophils or macrophages, using CD16b (neutrophil) and CD68 (macrophage) cell markers. Cytospins from

healthy control subjects were used as positive controls, knowing that AnxA1 is expressed in BAL cells in this population. Antigen retrieval and cell permeabilisation steps were not performed as the cytospin fixation process leads to cell permeabilisation.

As per standard protocol, slides were fixed in ice-cold acetone and then washed in PBS at room temperature. Avidin and biotin blocking solutions were added – each for 15 minutes with a 5 minute PBS washing step in between. Following a further 5 minute wash in PBS, protein block was added to the slides for 30 minutes. This was then tipped off and the primary antibody of interest added (AnxA1 or ALXR as described above, at dilutions of 1:100, 1:50 or 1:30 (5 slides each), or an appropriate isotype control as previously described). An isotype control slide was made for each patient. Slides were incubated overnight and subsequently washed three times in PBS – each wash 5 minutes.

For AnxA1 slides, biotinylated rabbit anti-mouse antibody was added. For ALX slides, biotinylated goat anti-rabbit antibody was added. All slides were incubated for 1 hour at room temperature with the biotinylated secondary antibody at a concentration of 1:250. Slides were then washed 3 times in PBS, each for 5 minutes. Streptavidin-labelled Alexa Fluor 488 at 1:250 was added to the slides for 1 hour at room temperature in the dark. Slides were then washed three times in PBS and mounted with DAPI.

Staining for AnxA1 was optimal at a primary antibody dilution of 1:30, and repeatable on two further experiments using two slides each from control and CF patients (figure 4.6). Staining was less successful with ALX. Although staining was again seen at a primary antibody dilution of 1:30, it was not consistent and was not repeatable on subsequent experiments.

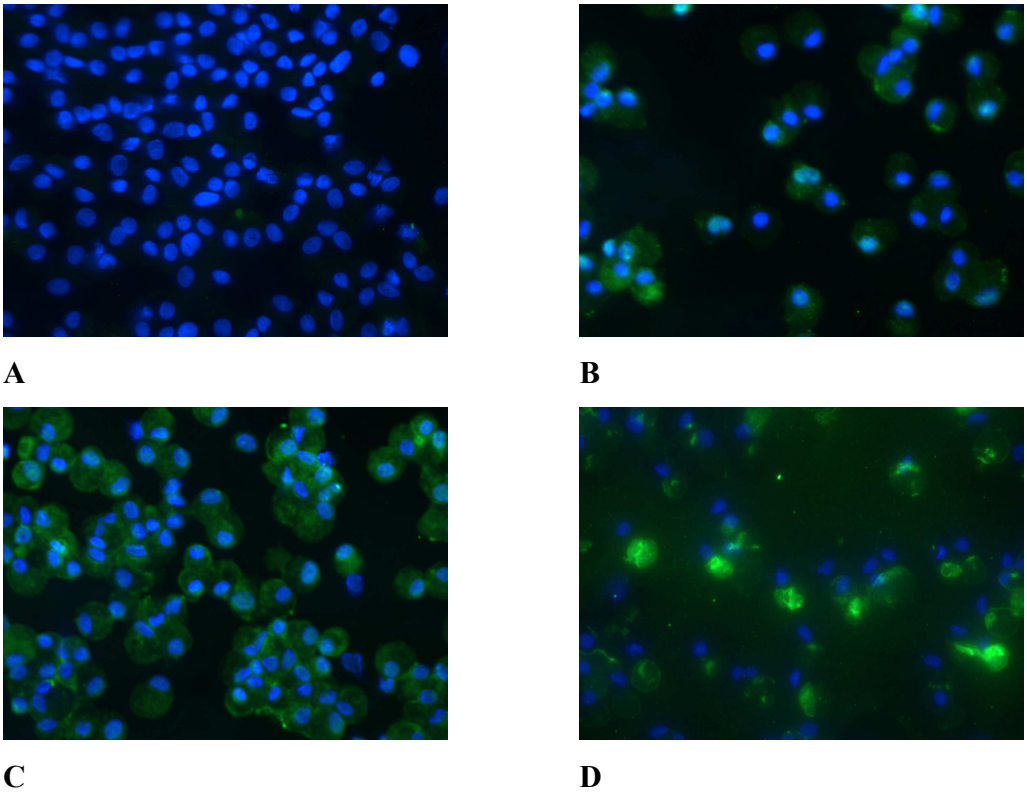


Figure 4.6: Staining BAL cytospins in control patients for AnxA1 at varying primary antibody dilutions and isotype control (A). Staining was attempted with AnxA1 dilutions of 1:100 (B) and 1:50 (C) but was most successful at a dilution of 1:30 (D) Magnification X 20.

Further staining with ALX was not pursued because of time constraints, in order to focus efforts on AnxA1 staining. Experiments were also devised to try to establish which BAL cell types stained with AnxA1.

4.5.3 Cytospin staining with CD16b and CD68 antibodies

To establish whether these antibodies would stain human BAL cytospin samples, staining was first attempted using human peripheral blood cytospins from a healthy adult volunteer. Anti-neutrophil (mouse anti-human CD16b, BD Biosciences, Oxford, UK) and anti-macrophage (mouse anti-human CD68, Biolegend, London, UK) antibodies were sourced, both conjugated to phycoerythrin (PE).

To prepare the cytospins, 40ml of red cell lysis buffer was added to 2ml of blood and left for 15 minutes. The sample was then centrifuged at 200g for 10 minutes. The supernatant was decanted and the resultant pellet resuspended in a further 40ml of red cell lysis buffer. The sample was left for a further 15 minutes and then centrifuged as

above and the supernatant discarded. The pellet was resuspended in 500µl RPMI and a cell count and cytospins performed as described above.

Slides were fixed in ice-cold acetone and then washed in PBS. Protein block was applied for 30 minutes and then the antibodies (either CD16b or CD68) were added at dilutions of 1:50, 1:100, 1:250, 1:500 and 1:1000, using 6 slides for each. Isotype control slides were used at the same concentrations. Slides were incubated in the dark for 1 hour. They were then washed in PBS, 3 washes each 5 minutes, and finally mounted with DAPI. Unfortunately despite trying the antibodies at different concentrations staining was unsuccessful. The experiment was repeated with a further 6 slides leaving the primary antibody to incubate overnight to see if staining was successful with a longer incubation period. However no improvement was seen. Staining was not pursued in BAL cytospins due to time constraints and thus it was not possible to establish whether staining for AnxA1 in BAL cytospins was predominately in neutrophils or macrophages. However alternative markers could be utilised in the future and this is discussed further in Chapter 7.

4.6 AnxA1 / ALX results

4.6.1 Patient characteristics

AnxA1 and ALX mRNA analysis of BAL (table 4.1)

There were only two children with PCD in this cohort and therefore these were included in the bronchiectasis group. These two children with PCD did not show any obvious differences as compared to the other children in the bronchiectasis group. One CF patient underwent bronchoscopy while stable during insertion of a venous access device.

AnxA1 and ALX mRNA analysis of EBB (table 4.2)

Three CF patients were clinically stable when the bronchoscopy was performed during venous access insertion. A cytology result was not available for one control patient (with dry cough and a macroscopically normal bronchoscopy) as they did not have BAL performed.

AnxA1 staining of EBB (table 4.3)

Only four patients in the control group underwent BAL meaning that two patients did not have a BAL white cell differential or microbiology result. They were however included as patient numbers in this group are limited. The indication for bronchoscopy in these two patients was reported haemoptysis and a dry cough and the airways were macroscopically normal. Data from all but one PCD patient had been collected by previous researchers and the cell count was not available. Bronchoscopy was performed in one CF patient while clinically stable during venous line insertion.

Table 4.1: AnxA1 and ALX BAL analysis patient characteristics (total n=66)

	Controls	**CF**	**CF NBS**	**Bronchiectasis / PCD**	**PBB**
Number	5	22	15	11 (2 PCD)	13
Age yrs (IQR)	**12 (2.5-14.5)**	**5.9 (3.5-12.1)**	**0.3 (0.2-0.3)**	**7.8 (5.3-10.3)**	**2.9 (1.3-4.3)**
Sex male (%)	2 (40)	12 (55)	7 (47)	10 (91)	7 (54)
Number with recent spirometry (%)	2 (40)	9 (41)	_____	7 (64)	_____
FEV1 % predicted (IQR)	96	63 (58-77)	_____	71 (56-89)	_____
Number with BAL cytology results (%)	5 (100)	9 (41)	9 (60)	10 (91)	13 (100)
BAL % neutrophils (IQR)	**2.7 (1.9-3.8)**	**42 (15-88)**	**4 (2-13)**	**47 (8-84)**	**15 (7-38)**
Pathogens isolated on BAL (%)	**0**	**16 (73)**	**5 (33)**	**7 (64)**	**13 (100)**
Infected with *P. aeruginosa* (%)	0	15 (68)	0	0	0
Respiratory exacerbation	0	5	0	1	0
Inhaled steroids	0	4	0	7	6
Oral steroids	0	2	0	0	1
Azithromycin	0	2	1	3	1
Oral antibiotics	0	11	13	0	1
Nebulised antibiotics	0	11	0	1	0

CF NBS patients were significantly younger than controls (<0.001), CF and bronchiectasis ($p<0.0001$) and PBB patients (<0.05). The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.001$) although there were no individual differences between groups. The percentage BAL neutrophils was significantly higher in CF as compared to CF NBS patients ($p<0.05$) as well as CF and bronchiectasis patients as compared to controls ($p<0.05$). IQR = interquartile range.

Table 4.2: AnxA1 and ALX mRNA EBB analysis patient characteristics (total n=53)

	Controls	CF	CF NBS	Bronchiectasis / PCD	PBB
Number	6	27	5	7 (1 PCD)	8
Age yrs (IQR)	**9.4 (2.3-14.5)**	**11.3 (5.1-12.7)**	**0.3 (0.2-0.3)**	**7.8 (4.4-10.6)**	**3.5 (2.9-4.3)**
Sex male (%)	**2 (33)**	**15 (56)**	**3 (60)**	**5 (71)**	**4 (50)**
Number with recent spirometry (%)	2 (33)	16 (59)	_____	4 (57)	_____
FEV1 % predicted (IQR)	96	73 (62-77)	_____	72 (48-75)	_____
Number with BAL cytology results (%)	5 (83)	14 (52)	4 (80)	7 (100)	8 (100)
BAL % neutrophils (IQR)	**2.7 (1.5-3.8)**	**23 (16-83)**	**11 (5-18)**	**31 (11-90)**	**12 (7-29)**
Pathogens isolated on BAL (%)	**0**	**19 (70)**	**2 (40)**	**5 (71)**	**8 (100)**
Infected with *P. aeruginosa* (%)	0	21 (78)	0	1 (14)	0
Respiratory exacerbation	0	2	0	1	0
Inhaled steroids	0	8	0	6	2
Oral steroids	0	1	0	0	0
Azithromycin	0	4	0	0	1
Oral antibiotics	0	17	5	0	0
Nebulised antibiotics	0	17	0	1	0

CF NBS patients were significantly younger than CF ($p<0.001$) and bronchiectasis ($p<0.05$) patients. The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.01$) although there were no individual differences between groups. The percentage BAL neutrophils was significantly higher in CF as compared to control patients ($p<0.01$) as well as bronchiectasis patients as compared to controls ($p<0.05$). IQR= interquartile range.

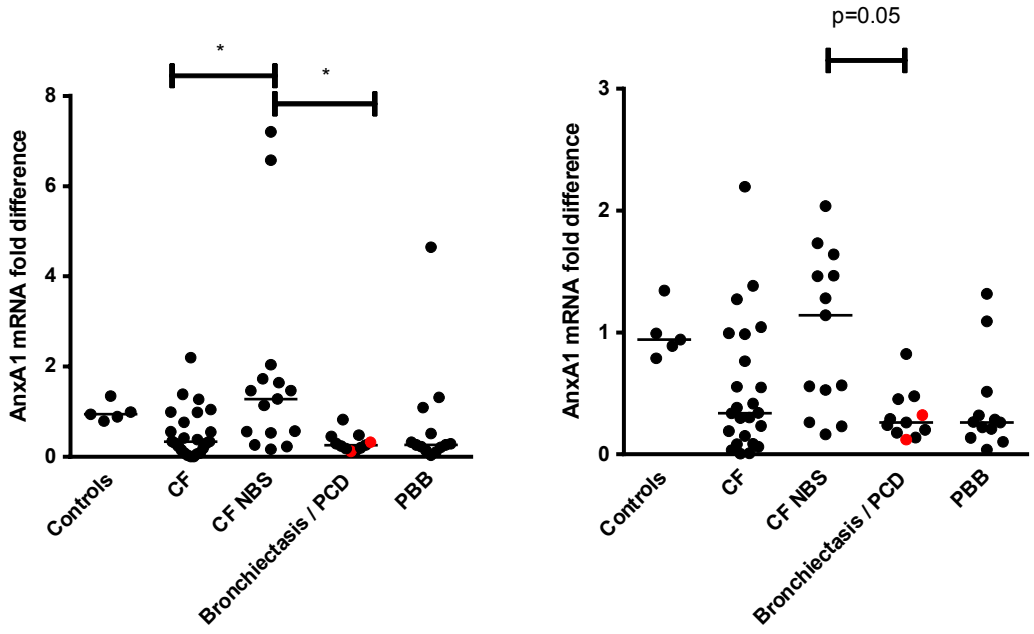
Table 4.3: AnxA1 immunofluorescent staining patient characteristics (total n=39)

	Controls	CF	Bronchiectasis	PCD	PBB
Number	6	12	8	6	7
Age yrs (IQR)	**11.3 (6-14.2)**	**12.3 (6.5-13.1)**	**7.2 (4.4-11.8)**	**11.4 (9.4-13.8)**	**3.4 (2.8-4.3)**
Sex male (%)	2 (33)	8 (67)	7 (88)	1 (17)	4 (57)
Number with recent spirometry (%)	_____	9 (75)	4 (50)	6 (100)	_____
FEV1 % predicted (IQR)	_____	73 (59.5-81)	68.5 (44.5-81)	57.5 (40.5-75)	_____
Number with BAL cytology results (%)	4 (66)	6 (50)	7 (88)	6 (100)	7 (100)
BAL % neutrophils (IQR)	**1.7 (2.0-2.7)**	**76.5 (8.2-90.5)**	**38.7 (11-89.7)**	**31.5 (6.9-84.2)**	**19.3 (9.3-38.7)**
Pathogens isolated on BAL (%)	**0**	**10 (83)**	**5 (63)**	**3 (50)**	**8 (100)**
Infected with *P. aeruginosa* (%)	0	8 (66)	0	3 (50)	0
Respiratory exacerbation	0	1	0	3	0
Inhaled steroids	0	6	6	5	3
Oral steroids	0	0	0	0	0
Azithromycin	0	1	1	1	1
Oral antibiotics	0	6	0	1	0
Nebulised antibiotics	0	6	0	2	0

PBB patients were significantly younger than those with CF ($p=0.01$), and PCD ($p<0.05$). The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p=0.001$). The percentage BAL neutrophils was significantly higher in CF as compared to controls ($p<0.05$). IQR=interquartile range.

4.7 Measurement of AnxA1 and ALX in BAL cell pellets and endobronchial biopsies

4.7.1 BAL cell pellet AnxA1 mRNA



A **B**

Figure 4.7: Graph A: BAL cell pellet mRNA levels of AnxA1. Graph B: outliers removed so the scale can be expanded. Horizontal bars represent medians, which varied significantly between groups ($p<0.01$ in both graphs). With the inclusion of outliers, AnxA1 mRNA levels were statistically higher in CF NBS patients as compared to CF patients and bronchiectasis patients ($p<0.05$) – but not with outliers excluded. Red dots represent PCD patients.

Median levels of AnxA1 mRNA were statistically different between the patient groups (figure 4.7). Levels were similar in CF NBS and control patients, and both these groups were higher than CF, bronchiectasis and PBB samples. With the two CF NBS outliers included, levels of AnxA1 mRNA were significantly higher in CF NBS patients as compared to those with established CF or bronchiectasis ($p<0.05$). Once these outliers were removed, there was no statistically significant difference between the groups (CF NBS as compared to bronchiectasis $p=0.05$). However the study is underpowered in part due to the need for multiple comparisons.

In order to examine whether differences in AnxA1 mRNA expression are related to disease severity, results were normalised for inflammation by expressing as a ratio of BAL neutrophil count (figure 4.8). Although there were no significant intergroup

differences, ratios were similar in controls and CF NBS, and lower in established CF, bronchiectasis/PCD and PBB.

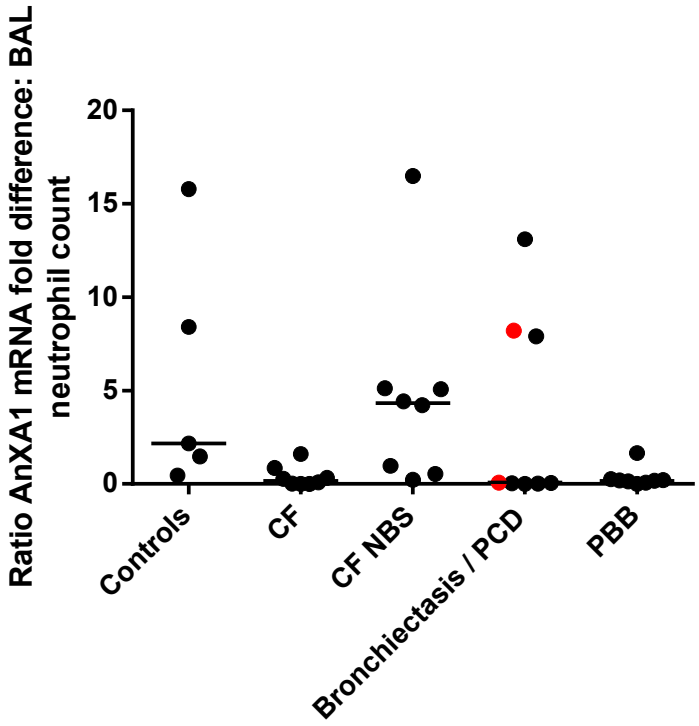


Figure 4.8: Graph showing ratios of BAL cell pellet AnxA1 mRNA:BAL neutrophil count. Horizontal bars represent medians which varied significantly between groups ($p<0.05$). No significant intergroup differences were seen. Red dots represent PCD patients.

To investigate further whether differences in AnxA1 mRNA levels are related to disease severity, and whether levels are overwhelmed in CF, median levels were compared between controls, established CF and all other neutrophilic lung diseases grouped together (bronchiectasis / PCD and PBB - labelled as CSLD). This showed that the differences seen between groups were similarly lower in both CF and CSLD, implying AnxA1 mRNA expression is overwhelmed in both these groups as compared to controls Levels were statistically higher in controls as compared to CSLD ($p<0.01$) (figure 4.9). A similar pattern was seen when the data was expressed as a ratio of BAL neutrophil count (figure 4.9).

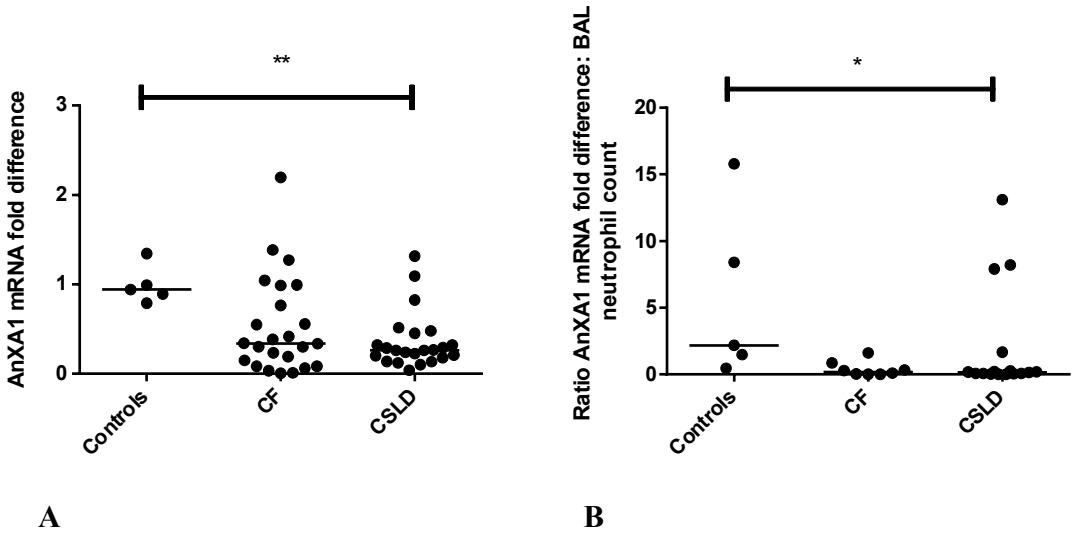


Figure 4.9: Graph A: BAL cell pellet AnxA1 mRNA in controls, established CF and CSLD BAL AnxA1 mRNA levels were significantly higher in controls as compared to CSLD $p<0.01$). Graph B: Ratios of BAL cell pellet AnxA1 mRNA:BAL neutrophil count in controls, established CF and CSLD. Levels were statistically higher in controls as compared to CSLD ($p<0.05$).

Finally, in order to examine whether any differences seen are related to the CFTR defect, AnxA1 mRNA levels were compared between all CF patients (established CF and CF NBS) and CSLD. No significant differences were seen (figure 4.10), and no difference seen when normalising for neutrophilic inflammation.

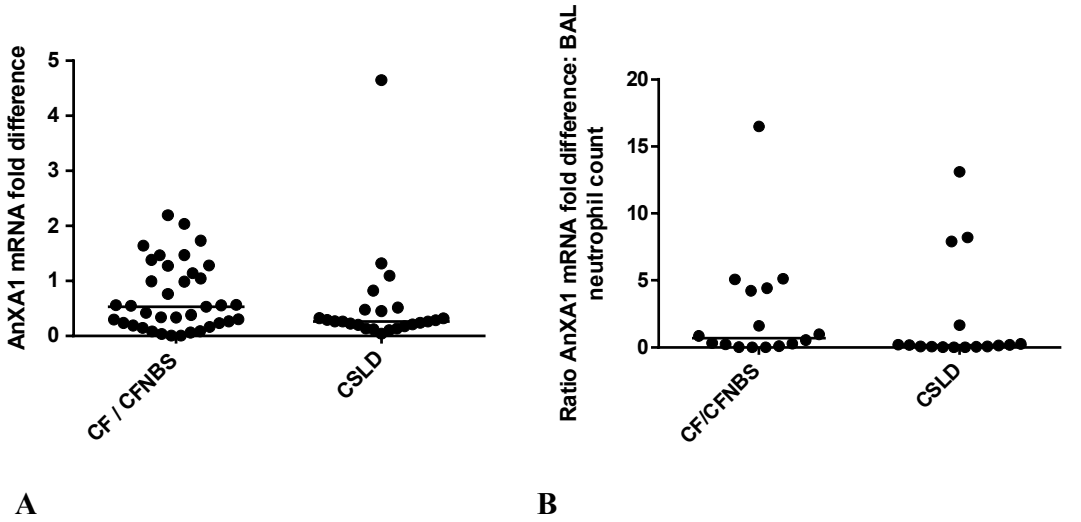


Figure 4.10: Graph A: BAL cell pellet AnxA1 mRNA in all CF and CSLD. Graph B: Ratios of BAL cell pellet AnxA1 mRNA:BAL neutrophil count in all CF and CSLD. There were no significant differences between groups.

No association was seen between BAL AnxA1 and pathogen status. The only positive finding was that levels were higher in CF patients with *Pseudomonas aeruginosa* infection ($p<0.01$), but this finding did not persist on normalisation for inflammation by expressing as a ratio of BAL neutrophil count.

In order to further investigate the relationship between AnxA1 and pulmonary inflammation, expression of ALX mRNA was measured in cells from the airway lumen by RT-PCR.

4.7.2 BAL cell pellet ALX mRNA

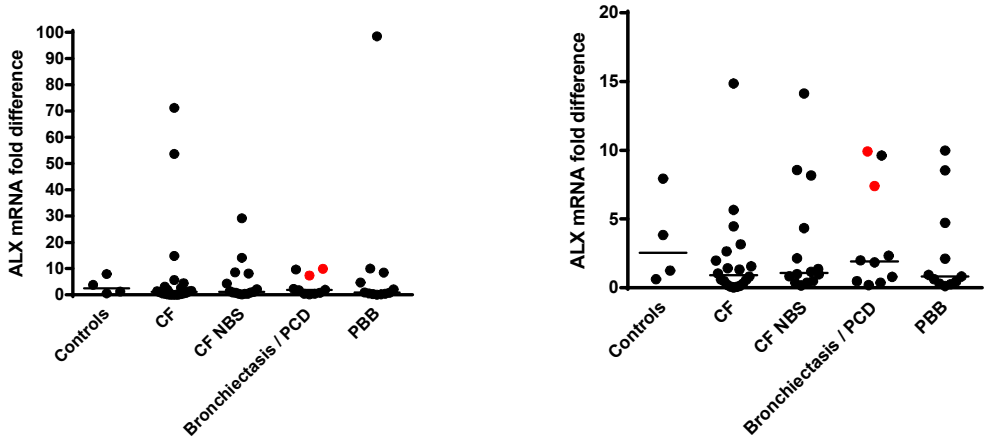


Figure 4.11: Graphs showing BAL cell pellet mRNA levels of ALX. Graph B: two CF outliers and one PBB outlier removed in order to expand the axis. Horizontal bars represent medians which did not vary significantly between groups. Red dots represent PCD patients. 4 patients were not included as results from these subjects were significantly different from those seen in other patients (see text below).

There were no significant differences in the level of ALX mRNA between patient groups, whether or not outliers were removed (figure 4.11). Four patients were not included in the analysis as the ALX mRNA fold difference was significantly different to those seen in other patients. The excluded patients and the ALX mRNA fold difference measured were as follows: Control (0.00005), CF (375), bronchiectasis (441) and PBB (5067). To normalise for inflammation, results were expressed as a ratio of BAL neutrophil counts. However, no statistically significant difference was seen between groups (figure 4.12).

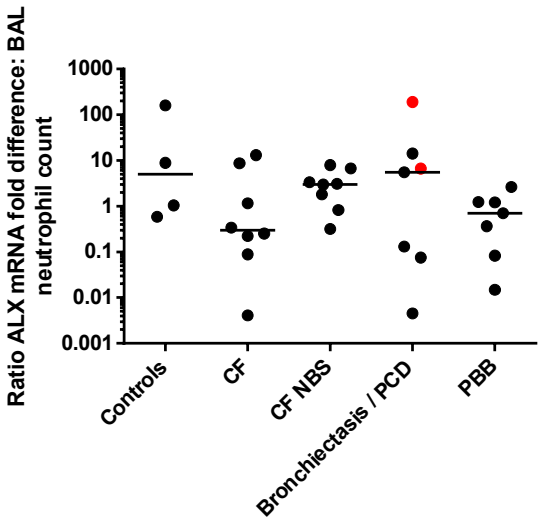


Figure 4.12: Graph showing ratios of BAL cell pellet ALX mRNA:BAL neutrophil count. No significant intergroup differences were seen. Red dots represent PCD patients. Data has been Log_{10} transformed to expand the axis.

BAL ALX mRNA levels were compared between controls, established CF and CSLD patients, to establish whether there was a relationship between levels and disease severity. Again there was no statistically significant difference between groups (figure 4.13), and no difference when normalising for neutrophilic inflammation.

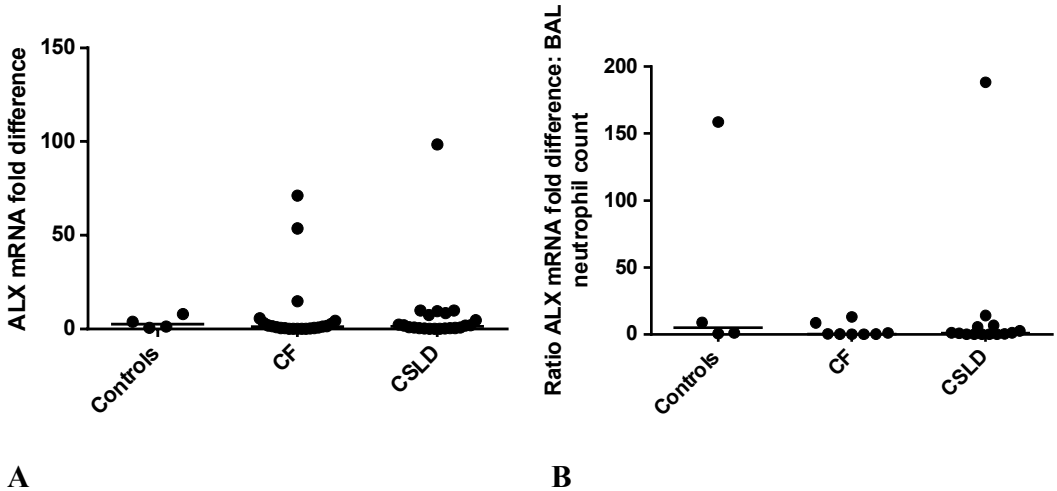


Figure 4.13 : Graph A: BAL cell pellet ALX mRNA in controls, established CF and CSLD Graph B: ratios of BAL cell pellet ALX mRNA:BAL neutrophil count in controls, established CF and CSLD. There were no statistically significant differences between groups.

Finally, to examine whether any differences seen are related to the CFTR defect, ALX mRNA levels were compared between all CF patients (established CF and CF NBS) and CSLD. No significant differences were seen (figure 4.14), and no differences seen when normalising for inflammation.

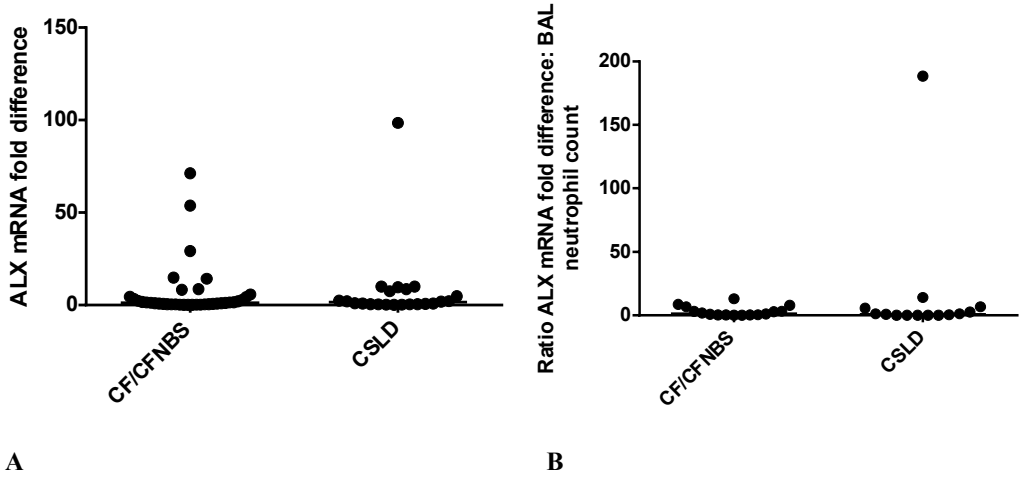


Figure 4.14: Graph A: BAL cell pellet ALX mRNA levels in all CF and CSLD. Graph B; ratios of BAL cell pellet ALX mRNA:BAL neutrophil count in all CF and CSLD. There were no significant differences between groups

The next section investigates levels of AnxA1 mRNA in EBB in these patient groups and the association with airway inflammatory status.

4.7.3 EBB AnxA1 mRNA

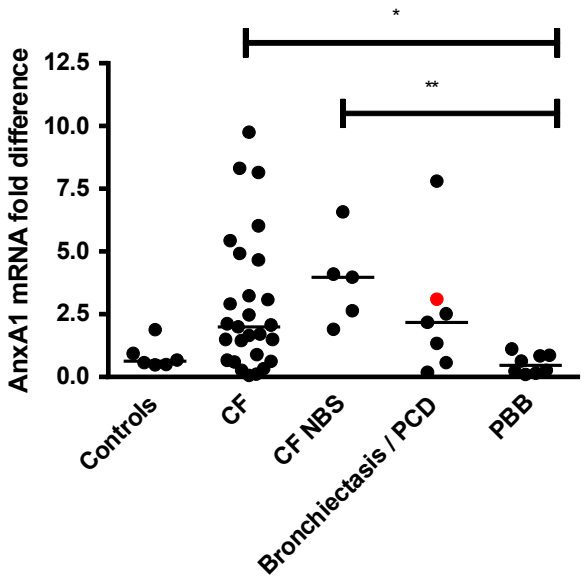


Figure 4.15: EBB AnxA1 mRNA levels in different patient groups. Horizontal bars represent medians,. AnxA1 mRNA levels were higher in CF ($p<0.05$) and CF NBS ($p=0.01$) patients as compared to those with PBB. Red dot represents a PCD patient.

Median levels of AnxA1 mRNA were statistically different between the patient groups (figure 4.15). Median levels were significantly higher in CF ($p<0.05$) and CF NBS ($p=0.01$) patients as compared to those with PBB. Overall PBB and control levels were similarly low, whereas those from other neutrophilic lung diseases were higher.

Again, to normalise for inflammation, results were expressed as a ratio of BAL neutrophil counts. No statistically significant difference was seen between groups (figure 4.16), although ratios were comparable in controls and CF NBS, and similarly low in established CF and other neutrophilic lung diseases.

EBB AnxA1 mRNA levels were compared between controls, established CF and CSLD patients, to establish whether there was a relationship between levels and disease severity. There was no statistically significant difference between groups (figure 4.17) and no difference when normalising for neutrophilic inflammation.

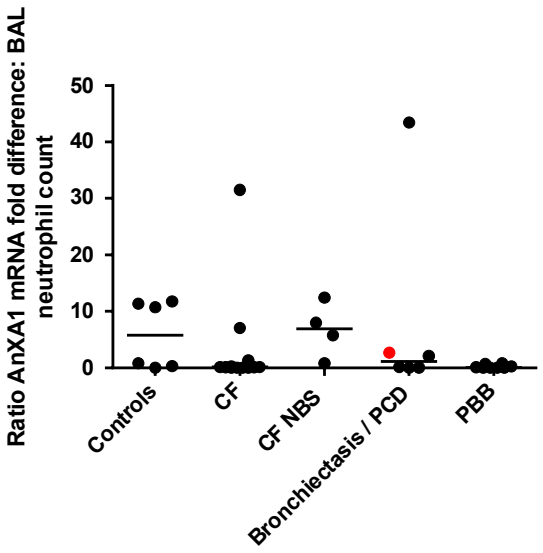
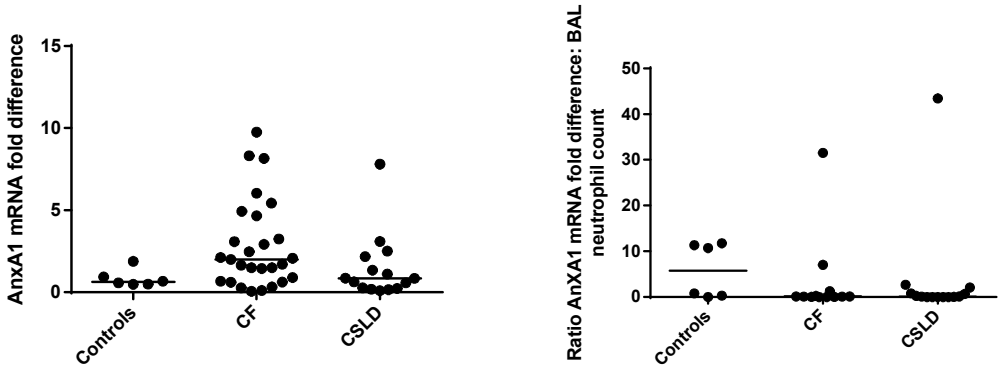


Figure 4.16: Graph showing ratios of EBB AnxA1 mRNA:BAL neutrophil count. No significant intergroup differences were seen. Red dot represents PCD patient.



A **B**

Figure 4.17: Graph A: EBB AnxA1mRNA levels in controls, established CF and CSLD. Graph B: ratios of EBB ALX mRNA:BAL neutrophil count in controls, established CF and CSLD. There were no statistically significant differences between groups.

In order to examine whether any differences seen are related to the CFTR defect, AnxA1 mRNA levels were compared between all CF patients and CSLD. Levels were statistically higher in all CF patients as compared to CSLD (figure 4.18). However, no difference was seen when the data was normalised for severity of neutrophilic inflammation.

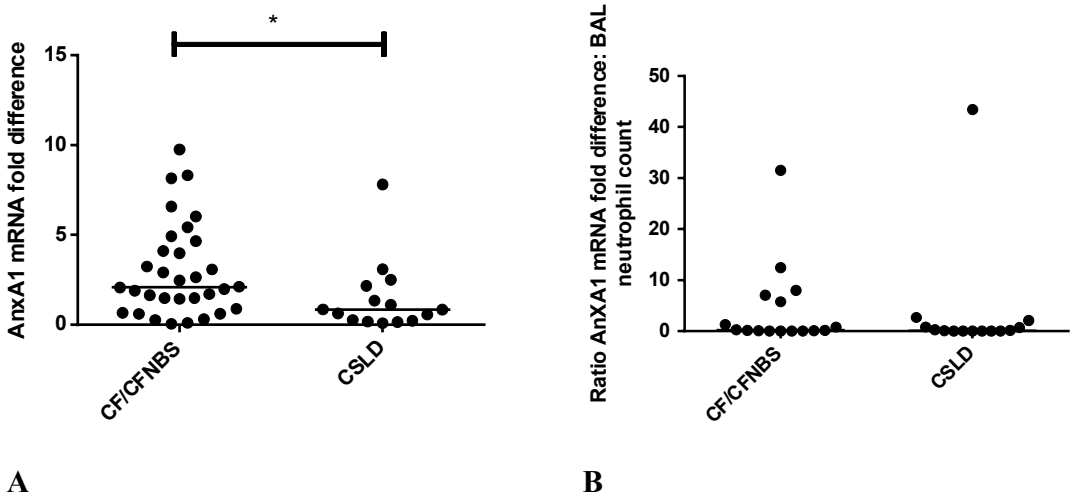


Figure 4.18: Graph A: EBB AnxA1 mRNA in all CF and CSLD. Levels were statistically higher in all CF as compared to CSLD ($p<0.05$). Graph B: ratios of EBB AnxA1 mRNA:BAL neutrophil count in all CF and CSLD. There was no significant difference between groups.

In order to further assess the role of AnxA1 in the paediatric airway, EBB samples were stained for AnxA1, and the results discussed in the following section.

4.7.4 AnxA1 expression in EBB measured by immunofluorescent staining

AnxA1 protein expression in EBB was analysed by immunofluorescent staining in order to ascertain whether there were differences in airway expression between groups, whether there was an association with inflammatory and disease severity and whether protein expression related to EBB AnxA1 mRNA levels. A total of 39 endobronchial biopsies were of suitable quality and made into paraffin-fixed slides. Only 1 CF NBS patient had an endobronchial biopsy that was suitable to be made into a paraffin-fixed slide. There were therefore insufficient CF NBS patients to analyse.

The median percentage staining of AnxA1 did not vary significantly between groups (figure 4.18). The spread of results is quite wide, including in the control group. CF and PBB patients appear to have lower expression as compared to other patient groups. When the data was normalised for BAL neutrophil counts, again there was no difference between groups. (figure 4.18). There were insufficient patients in the control group that could be normalised to BAL neutrophil count – either due to no cytology

result or an absence of neutrophils. Given this issue, ratios were also expressed between AnxA1 staining:BAL CXCL8. This increased the control numbers to 4, but no significant differences were seen. No differences were seen on comparing established CF with CSLD patients (figure 4.19).

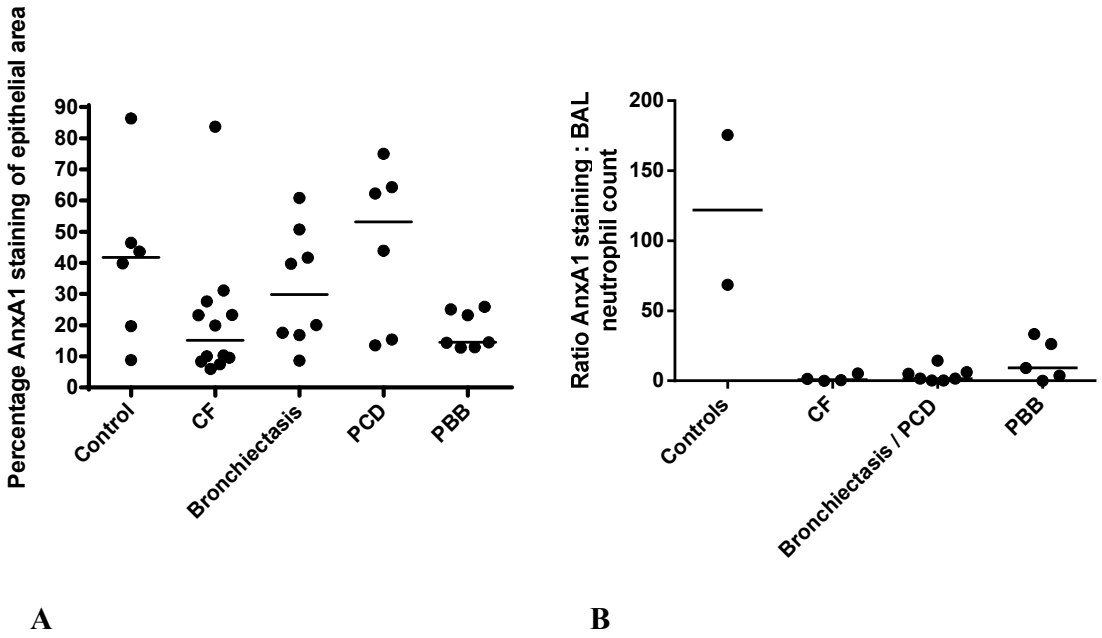
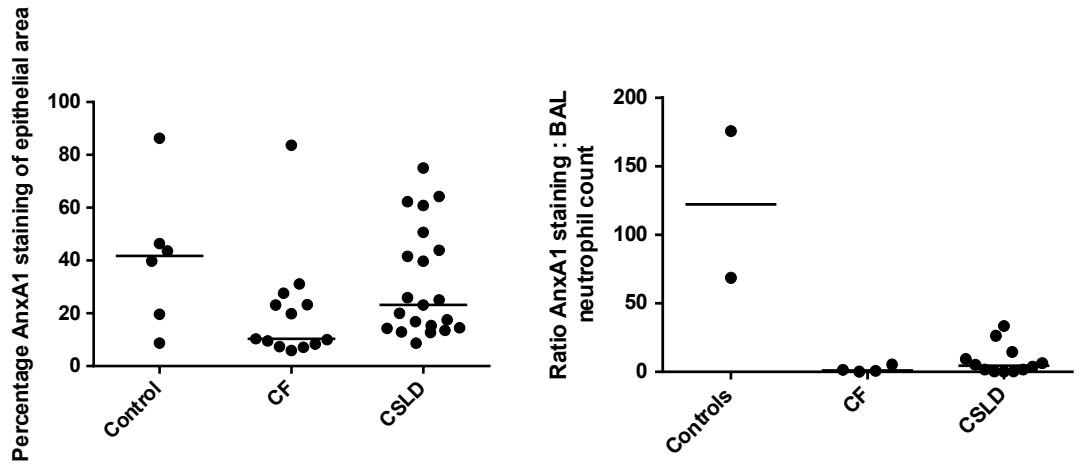


Figure 4.18: Graph A: Percentage AnxA1 staining of the epithelial area of EBB in different patient groups. Graph B: Data normalised for BAL neutrophil count. No statistically significant differences were seen between groups.



A **B**

Figure 4.19: Graph A: AnxA1 staining in controls, established CF and CSLD. Graph B: ratios of AnxA1 staining:BAL neutrophil count in controls, established CF and CSLD. There were no statistically significant differences between groups, and it was not possible to comment on differences in normalised data between controls and other groups due to small control numbers.

The next section investigates levels of ALX mRNA in EBB.

4.7.5 EBB ALX mRNA

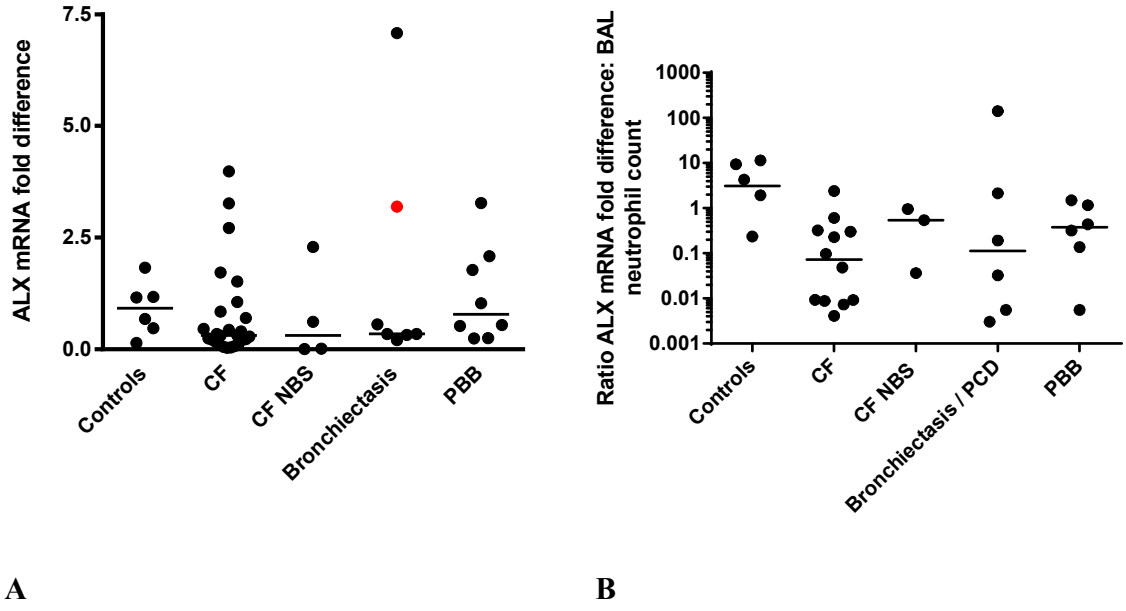


Figure 4.20: Graph A: EBB ALX mRNA levels. Graph B: data normalised for neutrophilic inflammation. Data has been Log_{10} transformed to expand the axis. Horizontal bars represent medians, which did not vary significantly between groups. Red dot represents the PCD patient (not seen in graph B as no cytology result).

There were no significant differences in the levels of ALX mRNA between patient groups (figure 4.20). One CF NBS patient was removed due to a particularly high level of AnxA1 mRNA (685 fold difference), which was markedly out of the range of the other results. When results were expressed as a ratio of BAL neutrophil count to normalise for inflammation, again no statistically significant difference was seen between groups (figure 4.20).

EBB ALX mRNA levels were compared between controls, established CF and CSLD patients. There was no statistically significant difference between groups (figure 4.21), and no difference when normalising for neutrophilic inflammation.

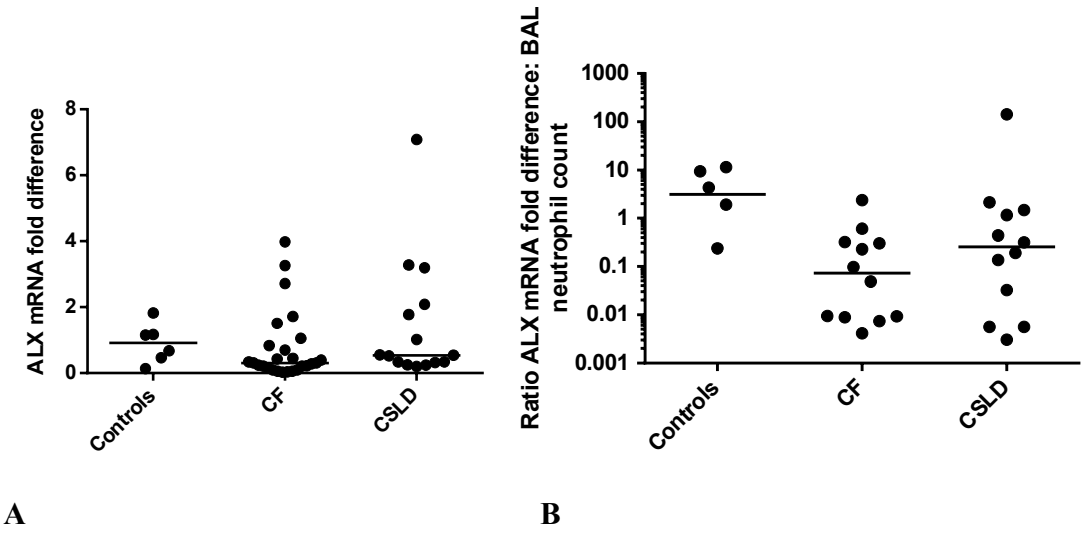


Figure 4.21: Graph A: EBB ALX mRNA in controls, established CF and CSLD. Graph B: ratios of EBB ALX mRNA:BAL neutrophil count in controls, established CF and CSLD. Data has been Log_{10} transformed to expand the axis. There were no statistically significant differences between groups.

AnxA1 mRNA levels were compared between all CF patients and CSLD (figure 4.22). No significant difference was seen between groups, and no difference when normalising for neutrophilic inflammation.

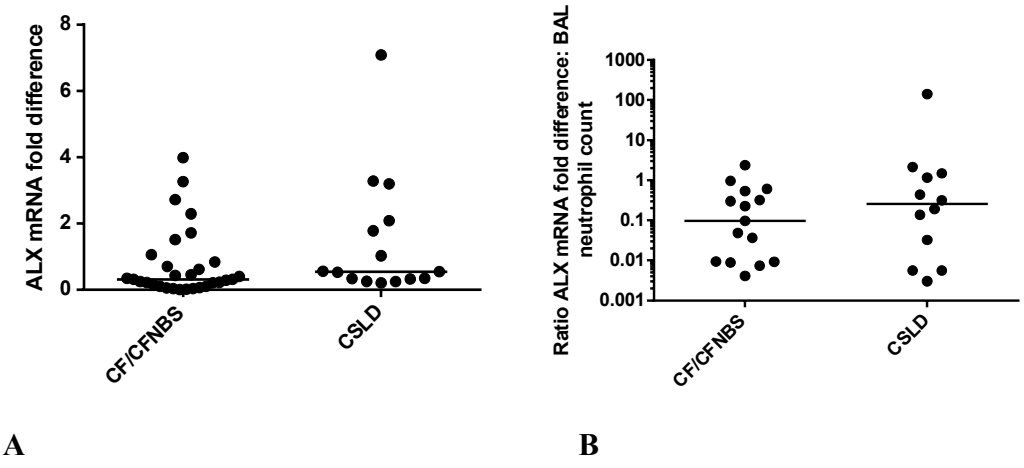
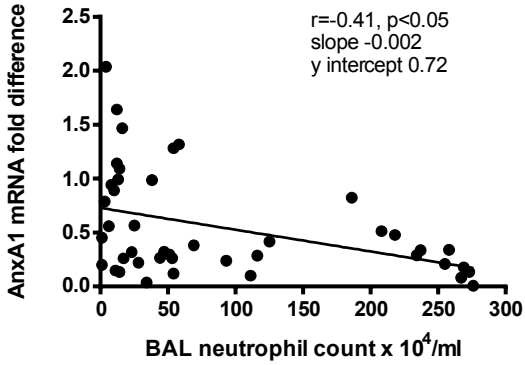


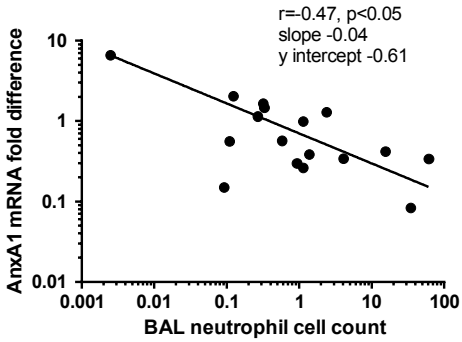
Figure 4.22: Graph A: EBB ALX mRNA in all CF and CSLD. Graph B: ratio of BAL cell pellet ALX mRNA:BAL neutrophil count in all CF and CSLD. There was no significant difference between groups.

The next section examines the effect of other makers of inflammatory severity (BAL neutrophils, CXCL8 and IL-10) on BAL and EBB AnxA1 / ALX mRNA levels and EBB AnxA1 staining.

4.8 Relationship between AnxA1 / ALX and BAL neutrophil count

The relationship between AnxA1 / ALX mRNA and BAL neutrophilia was examined. It would be expected that AnxA1 levels would increase in response to increasing neutrophilia, in an attempt to resolve inflammation. However, it was also possible that higher inflammatory states may overwhelm this inflammatory resolution process. There was a negative correlation between BAL AnxA1 mRNA and neutrophil cell count ($r=-0.41$, $p<0.01$) (figure 4.23). A similar negative correlation was seen on combining CF and CF NBS patients. However, no significant correlation was seen within the CSLD. Statistically the slopes in CF and CSLD did not vary significantly.





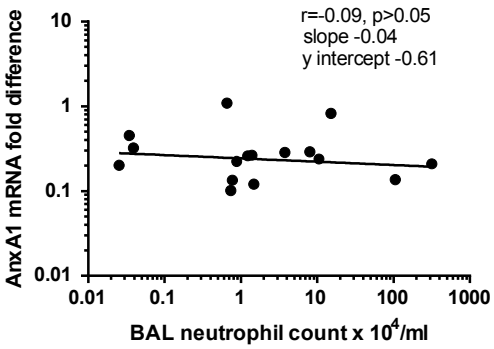


Figure 4.23: Graph showing a negative correlation between BAL AnxA1 mRNA and neutrophil count in all patient groups (A), all CF (B) and CSLD (C). Data have been Log_{10} transformed to expand the axes.

No correlation was seen between BAL ALX mRNA or EBB AnxA1 / ALX mRNA and neutrophil count in CF or CSLD groups, or when grouping all patients together. There was also no correlation seen between BAL neutrophils and EBB AnxA1 staining.

4.9 Relationship between AnxA1 / ALX and BAL CXCL8 and IL-10

The association between pro- and anti-inflammatory cytokines was examined to investigate the role AnxA1 may play in the cytokine profile in paediatric neutrophilic lung disease. There were 47 BAL cell pellet and 41 EBB samples with corresponding cytokine data. The cytokines of particular interest were CXCL8 (given its known pro-inflammatory functions and effects on neutrophils) and IL-10 (traditionally thought to be anti-inflammatory). Levels of IL-10 have been shown to be increased by AnxA1 [188]. In BAL cell pellets an inconsistent picture was seen – with no correlation between AnxA1 mRNA and CXCL8, and only a weakly negative correlation with IL-10 – seen in all patients together and the CF group. With cell pellet ALX mRNA, a weak but significant positive correlation was seen with BAL IL-10 levels ($r=0.40$, $p<0.01$) in all patients (the opposite to AnxA1), and CXCL8 ($r=0.54$, $p<0.05$) in the CSLD group. No correlation was seen between EBB AnxA1 or ALX and either CXCL8 or IL-10 (data not shown).

4.10 Association between AnxA1 / ALX BAL and EBB mRNA

No correlation was seen between AnxA1 / ALX mRNA from the airway wall and lumen.

4.11 Association between EBB AnxA1 mRNA and protein expression measured by immunofluorescent staining

Given that it would be expected that AnxA1 mRNA production should lead to protein synthesis, further analysis was conducted of patients where both EBB AnxA1 mRNA levels and AnxA1 immunofluorescent staining had been conducted. This included a total of 35 patients, with specific group numbers as follows:

- 4 control subjects
- 15 children with established CF
- 4 children diagnosed with CF by NBS
- 4 children with bronchiectasis (of which 1 PCD)
- 8 children with PBB

Where comparable results were available, there was no correlation between biopsy AnxA1 mRNA levels and epithelial protein expression. This includes no correlation within the CF or CSLD groups.

4.12 Overall AnxA1 and ALX results summary

Table 4.4 summarises the results from the analysis of BAL and EBB AnxA1 and ALX mRNA levels.

	Significant differences in median levels between groups	Significant differences in median levels between groups (normalised to BAL neutrophil count)	Differences between controls, established CF and CSLD	Differences between all CF and CSLD	Correlation with BAL neutrophil count
BAL AnxA1	**With outliers** CF NBS > bronchiectasis ($p<0.05$) CF NBS > CF ($p<0.05$) **Without outliers** CF NBS > bronchiectasis ($p=0.05$)	None – although controls and CF NBS levels similar	CF and CSLD similar – and CSLD statistically lower than controls ($p<0.01$, and <0.05 when normalised for neutrophil count)	None	Negative correlation ($r=-0.41$, $p<0.01$) Negative correlation in CF ($r=-0.67$, $p<0.01$) No correlation in CSLD
BAL ALX	None	None	None	None	None
EBB AnxA1	CF > PBB ($p<0.05$) CF NBS > PBB ($p=0.01$) Staining: None	None – although controls and CF NBS levels similar	None	CF > CSLD ($p<0.05$) – but no difference when normalised for neutrophil count	None
EBB ALX	None	None	None	None	None

Table 4.4: Overall summary of BAL / EBB AnxA1 / ALX mRNA and AnxA1 staining results. The greatest differences seen were in AnxA1 mRNA levels, which differed between groups in both BAL cell pellets and EBB, although statistical significance was lost when normalised to BAL neutrophil count. BAL AnxA1 levels were lower in CF and CSLD as compared to controls (although this was only significant for CSLD). There was no difference between BAL AnxA1 mRNA levels between CF and non-CF diseases, but a negative correlation with BAL neutrophil count in CF but not CSLD patients. Thus overall there was not a consistent picture seen, but evidence to suggest that AnxA1, in particular in BAL, may have a role in airway inflammatory resolution, but that this is not disease or CFTR- specific.

4.13 Discussion

Levels of AnxA1 and ALX mRNA were examined in the paediatric neutrophilic airway through the acquisition of BAL and EBB at clinically indicated bronchoscopies. AnxA1 expression was also analysed by immunofluorescent staining of EBB. This research and the methodology will be discussed in the following sections, alongside limitations of the study and future directions.

4.13.1 Principle findings

Overall no clear pattern emerged from analysis of AnxA1 and ALX mRNA in BAL or EBB, or from analysis of AnxA1 protein expression in the airway. Despite this some important differences were seen – particularly in BAL AnxA1 mRNA expression. Firstly in both BAL cell pellets and EBB, AnxA1 mRNA levels were higher in CF NBS patients than some other patient groups (cell pellets CFNBS > CF and bronchiectasis / PCD; EBB CF NBS > PBB). Equally, when normalised for inflammation, AnxA1 levels in BAL and EBB were similar in controls and CF NBS subjects, and lower in other neutrophilic lung diseases, although this was not statistically significant. Levels of BAL AnxA1 mRNA were lower in CF and CSLD than controls. However, there was only a statistically significant difference between CSLD and controls in BAL AnxA1 mRNA, implying AnxA1 mRNA levels had been overwhelmed in these patients. There was not a consistent picture to suggest that there was a difference in AnxA1 / ALX levels between CF and CSLD patients, and levels were similar in these groups. Therefore there was insufficient evidence to suggest a CFTR-specific effect. However, a negative correlation was seen between BAL AnxA1 mRNA levels and neutrophil count in all CF patients, which was not seen in other neutrophilic lung diseases. This therefore shows that in CF alone, BAL AnxA1 mRNA levels are lower with increased inflammation – potentially due to the inflammatory process being overwhelmed.

4.13.2 Strengths and weaknesses of the study

Not all patients had BAL white cell differentials or spirometry results. In particular patients with CF were more likely to have a degenerate BAL sample meaning an accurate neutrophil count was possible in 40% of the cell pellet group and 52% of the biopsy group. Biopsy samples were limited in the CF NBS group.

Immunofluorescent staining patient group

Two control patients did not have BAL samples taken and so BAL neutrophil results or the presence of pathogens are unknown. These patients were included as the numbers of control subjects were limited, the bronchoscopies were macroscopically normal, and the results consistent with other control subjects. Only 50% of CF patients in this cohort had a BAL differential available. It was not possible to acquire enough suitable biopsies from CF NBS patients, as these samples were very small and the morphology poor. Therefore it was not possible to analyse if there were differences in AnxA1 airway expression between those with established CF lung disease, and those diagnosed very young and clinically well.

Methodology

Immunofluorescent staining

It was not possible to successfully stain EBB or BAL cells for ALX, and so conclusions cannot be drawn about how expression of ALX relates to AnxA1 and the inflammatory status of the airway. At the time of performing the experiment very few commercially available ALX antibodies existed that had been used in immunohistochemistry staining and there were no published studies looking at expression of ALX in the airway. In addition it was not possible to double stain BAL cells to examine which cells were expressing AnxA1, further limiting analysis, as the antibodies used did not successfully stain adult PBMCs.

Analysis method

Conventionally immunofluorescent images are interpreted based on the perceived intensity levels of the objects of interest in the tissue. However, this practice is labour-intensive and suffers from intra- and inter-observer variability. A method was therefore devised to quantify the tissue immunofluorescent staining and thus to quantify AnxA1 expression.

To calculate the percentage AnxA1 staining, the green AnxA1 staining was expressed as a factor of another measurement of the total epithelial area. Using the outline of the delineated epithelial area by eye and by hand is not an accurate enough measurement of

the true epithelial area. Therefore DAPI staining was used as a proxy measurement of the epithelial area. Clearly this has limitations as DAPI only stains nuclear material and therefore can never be a true measurement of the whole epithelium. The other significant issue with attempting quantitative analysis of immunofluorescence is the effect of background staining and autofluorescence. This was not corrected for but visually this was not evident in the images in this study. A final criticism of the methodology is that sample analysis was not repeated by the author or by an independent observer. Therefore data are not available for intra- or inter-observer variability.

4.13.3 Strengths and weaknesses in relation to other studies

AnxA1 / ALX protein levels were not measured in this thesis due to limited sample sizes. One previous study showed that levels of BAL AnxA1 protein are reduced in adult CF patients as compared to healthy controls - and suggested that levels were reduced due to cleavage by neutrophil elastase [97], While this is plausible, alternative possibilities exist including a lack of AnxA1 initial production in these patients with more severe lung disease, or degradation by alternative means. However, the work in this thesis did not investigate the effects of neutrophil elastase, and thus it is not possible to comment on whether this enzyme may have affected AnxA1 levels in the airway lumen. The previous published study also showed reduced BAL AnxA1 levels with increased BAL neutrophilia [97], which was a finding in this thesis, but only with BAL AnxA1 mRNA in CF patients. This finding is particularly interesting given paediatric patients generally have less severe lung disease than adult patients. This thesis is the first study to look specifically at the AnxA1 axis in newly diagnosed young CF patients. These novel findings suggest that CF NBS patients have similar BAL AnxA1 levels to controls, and that this is overwhelmed in more severe neutrophilic lung disease. However, the lack of statistical significance warrants cautious interpretation and at best these findings can be described as hypothesis generating.

In vitro work has suggested that the CFTR defect may have an effect on AnxA1 production [198]. This means that there may be an inherent defect in AnxA1 production in CF patients. The data from this thesis has not shown a consistent picture to suggest differences are due to the CFTR defect.

The only previous study specifically looking at AnxA1 immunofluorescent staining in the airway involved a *cftr* $^{-/-}$ mouse model and nasal epithelial cells from adult CF patients [96]. The lungs of *cftr* $^{-/-}$ mice do not stain for AnxA1, as compared to *cftr* $^{+/+}$ mice, implying the lungs of CF patients may also have reduced AnxA1 expression as compared to healthy subjects. However it is recognised that the *cftr* $^{-/-}$ mouse model is a poor phenotypic model of human disease. This group also showed reduced nasal epithelial expression of AnxA1 in adult CF nasal epithelial cells.

Children with CF as a group have less severe lung disease than adult, and no significant difference in epithelial AnxA1 staining was seen between groups. However, while nasal epithelial cells should be representative of the airway as a whole, they are not the same as EBB (although these are only representative of the proximal airway). Small patient numbers also limit further analysis of this data.

As regards the association with BAL cytokines, published studies have only previously looked at the association of AnxA1 and IL-10. *In vitro* work has shown that AnxA1 stimulates the production of IL-10 [188]. No clear relationship was seen in this thesis between the AnxA1 axis and BAL IL-10.

4.13.4 Interpretation of the results

The results from this thesis have not shown a consistent picture within the AnxA1 / ALX axis across disease groups. In particular there was no consistent evidence to suggest that CF patients had an impaired ability to produce AnxA1 as compared to other disease groups, although the negative correlation seen with BAL neutrophils in CF alone suggests there may be an important CFTR effect. However, there is evidence to suggest that increased inflammatory severity, irrespective of the underlying cause, may lead to impaired AnxA1 mRNA production. The finding of generally higher levels of AnxA1 mRNA in CF NBS patients as compared to other diseases is a reflection of milder neutrophilic lung disease in these young children, but not a CF-specific phenomenon.

The variation in results between BAL cell pellets and EBB highlights the compartmentalisation of pathology within the airway. In addition given the expression of

AnxA1 is greatest in haematopoietic cells, this may explain why differences were more evident in cell pellets rather than EBB. The lack of correlation between EBB AnxA1 expression and mRNA levels potentially suggests that protein degradation may be important in the paediatric neutrophilic airway.

In summary, there was no overwhelming evidence to confirm the hypothesis that the AnxA1 / ALX axis is impaired in CF as compared to phenotypically milder diseases. However, there was evidence to suggest that for BAL cell pellet AnxA1, mRNA production was impaired in increasing neutrophilic inflammation irrespective of the underlying disease. In addition there was no evidence to confirm the hypothesis that AnxA1 and ALX mRNA levels are reduced in CF NBS due to a direct effect of CFTR dysfunction, and the results from this thesis showed higher BAL and EBB AnxA1 mRNA in these patients.

Chapter 5

Lung Krüppel-Like Factor expression in EBB

5.1 Analysis of EBB for LKLF

Immunofluorescent staining of airway samples for LKLF has been successfully performed by others [212]. However this previous work utilised explanted lungs from adult CF patients at the time of lung transplantation. These patients are at the most severe end of the CF disease spectrum and had absent LKLF staining as compared to samples from healthy controls. In addition absent staining was also seen in end-stage chronic obstructive pulmonary disease (COPD) samples, again from patients at the time of lung transplantation, implying this is not a CF-specific finding. Previous work has also not quantified LKLF expression in order to formally compare staining between different disease groups. Histological staining for LKLF has not been performed in paediatric airway samples. The hypotheses of this chapter were as follows:

- The failure of LKLF to actively terminate the acute inflammatory process potentially contributes to the severity of childhood CSLD.

- There is a CFTR related defect in the resolution of inflammation by LKLF which contributes to the severity of CF.

5.2 Establishment of an immunofluorescent staining protocol for LKLF using paraffin-fixed paediatric endobronchial biopsies

A method was devised for immunofluorescent staining of paediatric EBB by the author, utilising the pre-existing published method and the AnxA1 staining method as templates. Slides were dewaxed and antigen retrieval was performed using Tris EDTA as previously described. Avidin-biotin blocking steps were included and the slides flooded with protein block as before. Cell permeabilisation was incorporated using Triton-X 100.

Slides were incubated with LKLF antibody (rabbit IgG polyclonal, Abcam, Cambridge, UK) overnight. A control slide for each patient was incubated with rabbit polyclonal IgG (Jackson Immunoresearch, Stratech Scientific, Suffolk, UK). A series of experiments were repeated with varying dilutions of LKLF antibody (1:50, 1:300 and 1:1000). Staining was equally successful at a dilution of 1:50 and 1:300 (figure 5.1), and therefore a dilution of 1:300 was used. All antibodies were diluted with PBS, 0.3% Triton-X 100

and 1% human serum. Following overnight incubation, slides were washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes. They were then incubated in a secondary goat anti-rabbit biotin-conjugated antibody (Jackson Immunoresearch, Stratech Scientific, Suffolk, UK) at a dilution of 1:250 at room temperature for one hour. Finally slides were incubated with streptavidin antibody conjugated to Alexa Fluor 488 at a dilution of 1:250 at room temperature for one hour in the dark. Slides were washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes in the dark. They were then mounted with DAPI and stored at 4^{o}C in the dark until ready for analysis. Once the staining method had been developed it was repeated on two occasions with two slides each from control, CF, bronchiectasis and PBB patients, to ensure staining was reproducible.

5.3 Analysis of LKLF immunofluorescent staining

LKLF staining was predominately and most consistently in the epithelium. For this reason analysis of immunofluorescent staining was performed using the same technique as described in the analysis of AnxA1 staining (see section 4.3.6). However, staining appeared to be within the cytoplasm and also the nuclei in samples, which would be expected given LKLF is a transcription factor (figure 5.2). Results were therefore expressed as the ratio of LKLF:DAPI staining.

5.4 LKLF results

5.4.1 Patient characteristics (table 5.1)

One CF patient was clinically stable at the time of bronchoscopy and underwent Portacath placement. Two control patients did not have a BAL sample but were included in the analysis. These were the same two control patients used in the AnxA1 immunofluorescent staining experiment. There were no morphologically suitable biopsies obtained from CF NBS patients.

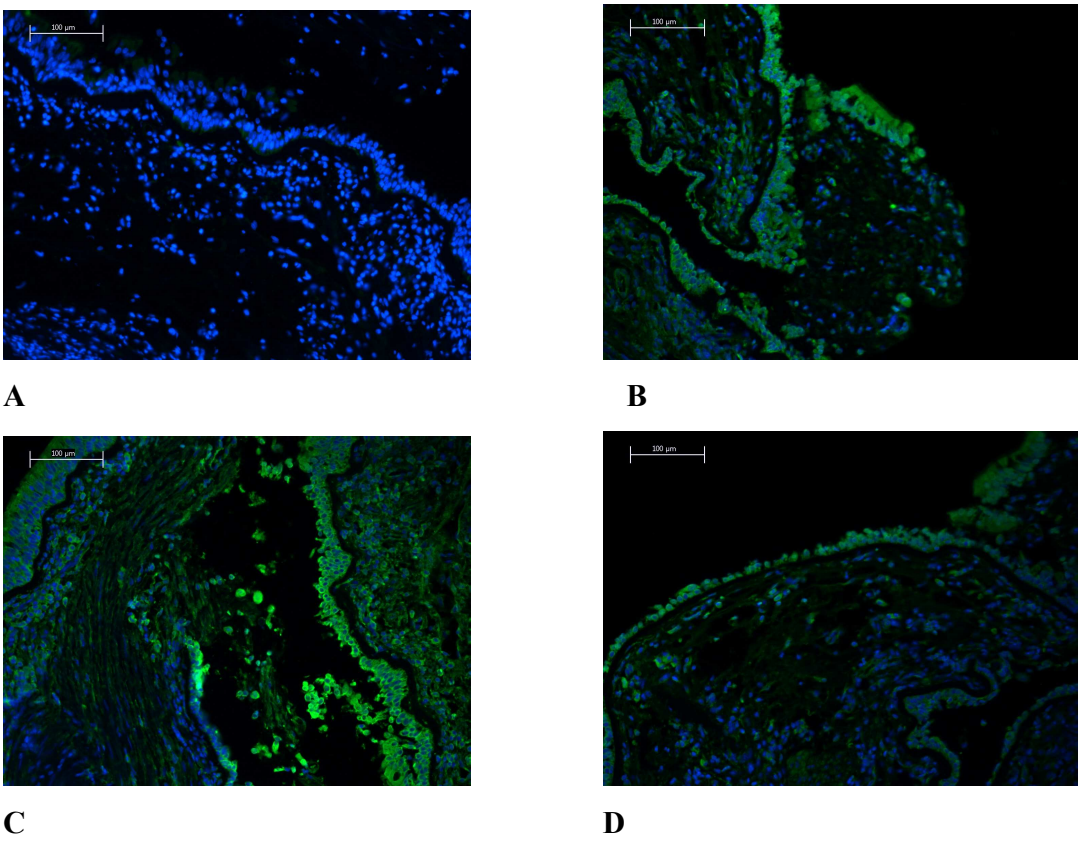


Figure 5.1:: Examples of LKLF staining of EBB using different concentrations of AnxA1 antibody, and isotype control (A). LKLF antibody fluoresces green. Slides were counterstained with DAPI. The primary antibody was used at dilutions of 1:50 (B), 1:300 (C) and 1:1000 (D). A final dilution of 1:300 was used. Magnification X 20.

Nuclear staining

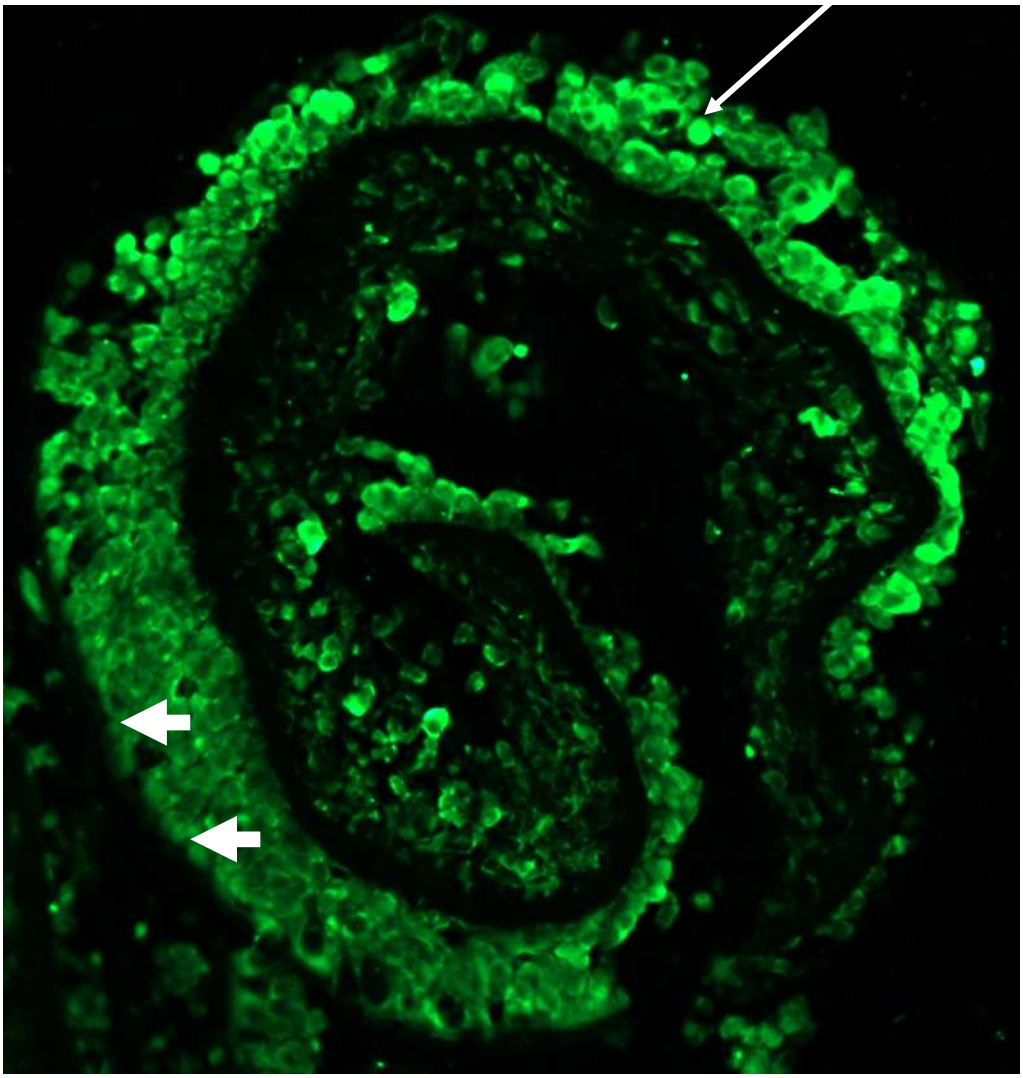


Figure 5.2: Image showing endobronchial epithelial cytoplasmic and nuclear staining of LKLF. Large arrow heads show cytoplasmic staining. Original magnification X 20.

Table 5.1: LKLF immunofluorescent staining patient characteristics (total n=43)

	Controls	CF	Bronchiectasis	PCD	PBB
Number	7	15	7	7	7
Age yrs (IQR)	**11.3 (8.3-13.5)**	**12.3 (5.9 – 13.1)**	**7.8 (5.3-13.3)**	**11.6 (11.1-14.5)**	**3.4 (2.8-4.3)**
Sex male (%)	2 (29)	8 (53)	6 (86)	2 (29)	5 (63)
Number with recent spirometry (%)	_____	11 (73)	4 (57)	7 (100)	_____
FEV1 % predicted (IQR)	_____	73 (64-78)	68.5 (44.5-81)	61 (46-71)	_____
Number with BAL cytology results (%)	5 (71)	9 (60)	6 (86)	7 (100)	7 (100)
BAL % neutrophilia (IQR)	**1 (0-2.4)**	**74 (25.7-87.5)**	**34.9 (6-83.9)**	**50.5 (12-79.3)**	**17 (7-37.4)**
Pathogens isolated on BAL (%)	**0**	**12 (80)**	**4 (51)**	**3 (43)**	**7 (100)**
Infected with *P. aeruginosa* (%)	0	11 (73)	0	4 (57)	0
Respiratory exacerbation	0	1	0	3	0
Inhaled steroids	0	6	6	0	3
Oral steroids	0	0	0	0	0
Azithromycin	0	2	1	0	1
Oral antibiotics	0	7	0	0	0
Nebulised antibiotics	0	7	0	3	0

PBB patients were significantly younger than those with CF ($p<0.05$) and PCD ($p=0.01$). The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.01$) although there were no individual differences between groups. The percentage BAL neutrophilia was significantly higher in CF as compared to control patients ($p<0.01$). Samples from 3 control and 5 PCD subjects had been previously collected by colleagues. IQR=interquartile range.

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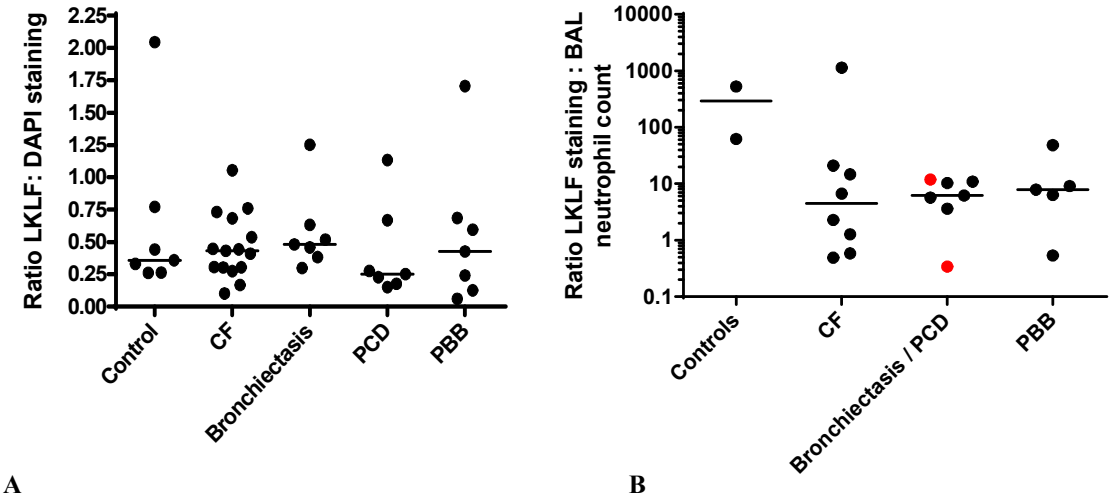
5.5 LKLF expression in EBB measured by immunofluorescent staining

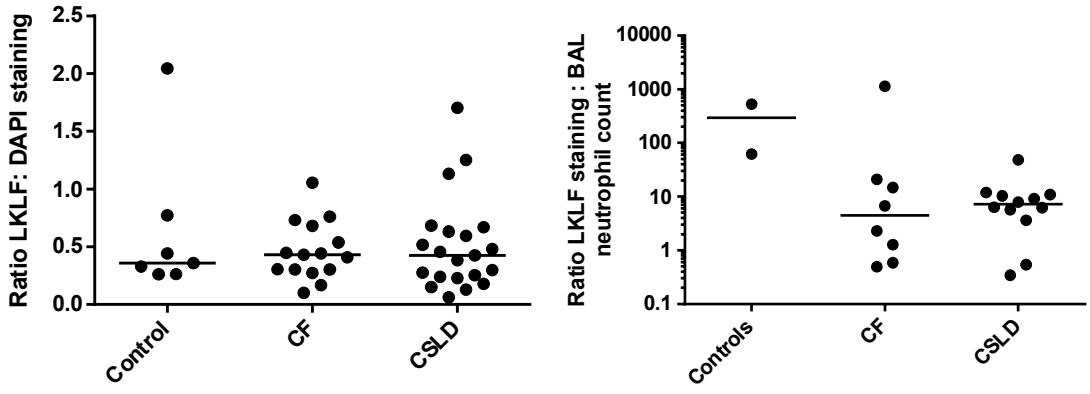


A B

Figure 5.3: Graph A: ratio of LKLF:DAPI staining of the epithelial area of EBB in different patient groups. Horizontal lines represent medians which did not vary significantly between groups. Graph B: LKLF staining normalised for BAL neutrophil count. Data has been Log_{10} transformed to expand the axis. Red dots represent PCD patients. There was no significant difference between groups.

Median LKLF immunofluorescent staining did not vary between groups, and this remained the case when the data was normalised for BAL neutrophil count (figure 5.3). The control patient with the highest LKLF staining underwent bronchoscopy to investigate reported haemoptysis. However, the bronchoscopy findings were entirely normal. As LKLF stains both the cytoplasm and nucleus, in some patients this gave a greater number of LKLF stained pixels than DAPI alone, which only stains nuclear material. For this reason results are presented as ratios rather than percentages as in some patients the ratio LKLF:DAPI was greater than one.

To assess whether the LKLF expression was reduced in CF and other neutrophilic lung diseases (labelled again as CSLD), results were examined in these two groups and compared to controls. There was no difference between these two groups and controls, although when normalising for BAL neutrophil count, there were too few patients in the control group to assess this further as a number had neutrophil counts of zero (figure 5.4). Due to the lack of suitable CF NBS biopsies, it was not possible to assess whether there may be a CFTR-related effect on LKLF expression.



A **B**

Figure 5.4: Graph A: LKLF expression expressed as a ratio of DAPI staining in controls, established CF and CSLD. Graph B: data normalised for BAL neutrophil count. Data has been Log_{10} transformed to expand the axis. There were no statistically significant differences between groups.

The next section examines the association between BAL inflammatory makers and LKLF expression.

5.6 Relationship between LKLF expression and BAL neutrophil count

The association with BAL neutrophil count was examined as it would be expected that LKLF expression would increase in response to increasing neutrophilia, in an attempt to resolve inflammation. However, it was also possible that higher inflammatory states may overwhelm this process leading to lower LKLF expression. A correlation with BAL neutrophil count was only seen in the CSLD group, and not the CF group or when grouping all patients together (figure 5.7). As no correlation was seen with the CF group, it was not possible to compare graph best-fit line slopes and intercepts between these groups. In addition, there were only 8 patients in the CF group which limits the results.

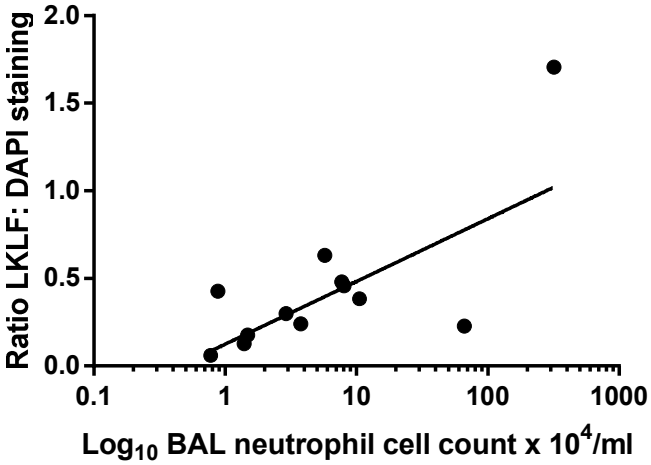
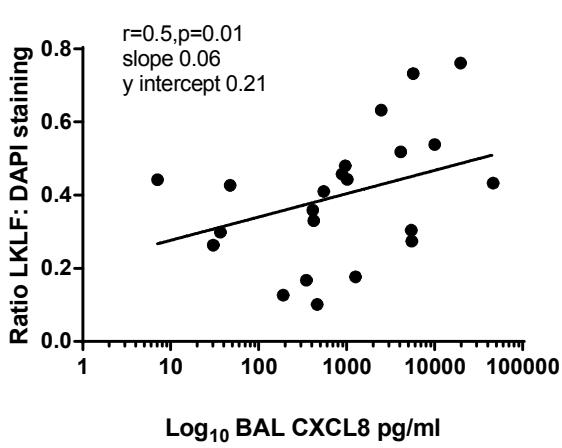


Figure 5.5: Graph showing positive correlation between EBB LKLF expression and BAL neutrophil count in CSLD patients ($r=0.59$, $p<0.05$).

5.7 Relationship between LKLF expression and BAL CXCL8 and IL-10

A total of 28 patients had data for both EBB LKLF expression and BAL cytokine levels. The association with CXCL8 and IL-10 was examined. Previous groups have not examined the role of the traditional anti-inflammatory role of IL-10 in LKLF expression, and no association was seen from the work in this thesis. In contrast LKLF has previously been reported to reduce CXCL8 levels. In this thesis there was a positive correlation seen between LKLF expression and BAL CXCL8 – in all patients together, CF and CSLD groups. This was only statistically significant in all patients together and the CF group (figure 5.8). The graphs were similar between these groups, but the slopes differed significantly between CF and CSLD ($p<0.05$).



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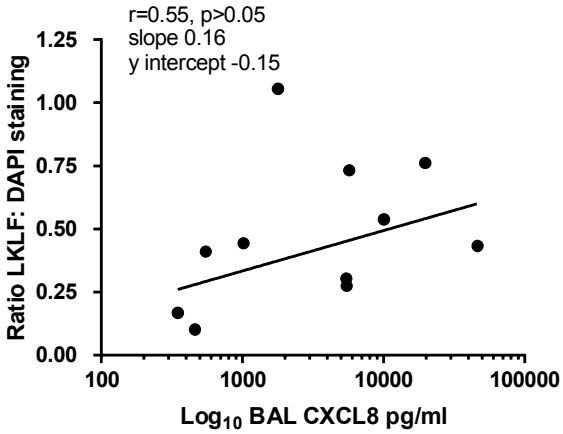

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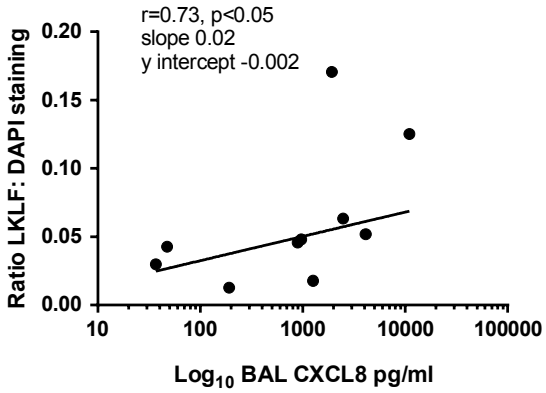


C

Figure 5.6: Graphs showing correlations between airway epithelial expression of LKLF and BAL CXCL8 levels in (A) all patients together, (B) CF patients and (C) CSLD patients. BAL CXCL8 levels have been Log_{10} transformed to expand the axis. A positive correlation was seen in each of the groups, although this was not statistically significant in the CF group. The slopes differed significantly between CF and CSLD ($p<0.05$).

5.8 Discussion

Endobronchial biopsies were obtained from clinically indicated paediatric bronchoscopies and an immunofluorescent staining protocol was developed by the author for these small and precious samples.

5.8.1 Principle findings

It is possible to stain for LKLF in paediatric EBB using immunofluorescence. Staining was predominately seen in the epithelium, and was present both in the cytoplasm and

nuclei. However, there was no statistical difference in airway expression of LKLF between disease groups or controls. There was a positive correlation with BAL neutrophil counts in the CSLD group only. A positive correlation with BAL CXCL8 was seen in all groups – the slopes and intercepts of the best-fit lines were similar in CF and CSLD, although CF did not reach statistical significance. The relationships appear to be different between LKLF and CXCL8 compared with LKLF and neutrophil count. It may be that other, non-LKLF modulated neutrophil chemoattractants affect the relationship, and LKLF only interacts with CXCL8 and possibly other but not all chemoattractants.

5.8.2 Strengths and weaknesses of the study

Analysis method

There may have been errors using the method of analysing expression of epithelial LKLF by expressing data as a ratio of DAPI staining. This required delineating the epithelial area by eye, and utilised the same method as for analysis of AnxA1 staining. In addition, as discussed previously, the lack of data on intra- and inter-observer variability also limits the robustness of these results. As LKLF stains both the cytoplasm and epithelium, this may explain why the ratio was greater than one in some patients. An alternative analysis method is needed to allow for normalisation of staining. One approach would be an improved method of delineating the epithelial area – as discussed in chapter 7.

5.8.3 Strengths and weaknesses in relation to other studies

The only previous study to examine airway expression of LKLF showed that airway epithelial expression is reduced in the airways of adult patients with inflammatory airway disease including CF, however differential expression between cytoplasm and nuclei was not formally quantified [212]. This study also showed that LKLF is constitutively expressed in the small airways of adult subjects with normal lungs. The significant difference between previous published work and this thesis is the age and type of patients included. The published study firstly used adult and not paediatric subjects, and children with CF would be expected to have milder lung disease than adults. Secondly samples from adult CF patients came from lungs removed at transplantation, and therefore represent the most severe end of the disease spectrum. Adult control subjects came from organ donors whose lungs were not used at transplantation, either due to lack of suitable match, age or

smoking history. Additional patient samples came from non-CF bronchiectasis patients who had undergone wedge resection and were also infected with *Mycobacterium avium intracellulare* and patients with end-stage chronic obstructive pulmonary disease (COPD) who were also peri-transplantation. Thirdly there were small numbers of patients in the adult study – only three patients in each group. This group looked at samples from both the large and small airways, and found that LKLF expression was greatest in the small airways. For ethical reasons it was not possible to obtain distal airway tissue from paediatric patients. In the published work immunofluorescent staining was greatest at the luminal aspect of bronchiolar cells in normal lungs. In the non-CF bronchiectasis samples, LKLF had translocated to the epithelial nuclei. In end-stage CF and COPD samples, LKLF staining was absent.

In vitro work with primary airway epithelial cells from healthy lungs showed LKLF predominately stained in the cytoplasm [85]. This thesis has shown that staining for LKLF in paediatric subjects is greatest and most consistent within the airway epithelium. Staining was seen both in the cytoplasm and nuclei of the epithelium in all patient groups including controls, which is in contrast to the adult data published. However, differential cytoplasm and nuclear staining was not quantified further in this thesis, and is an area for future work. There was no significant difference in expression of LKLF between patient groups and controls. Again this is in contrast to the adult data. There may be several reasons for this. Firstly the adult CF patients had end-stage lung disease. Similarly the adult non-CF bronchiectasis patients are likely to have more severe disease than children. In addition these patients were also infected with non-tuberculous mycobacteria which may have affected the results, as none of the paediatric patients were infected with this pathogen. Theoretically adult patients with more severe neutrophilic inflammation within the airway have impaired inflammatory pro-resolution capability, which may explain why staining was absent in end-stage adult CF and COPD patients. In addition, LKLF staining is greatest in small airways, although the previous published work did not say how far distally along the bronchial tree samples were taken. It is possible that the paediatric biopsies taken were too proximal to show significant differences between groups, although this was an unavoidable weakness for ethical reasons.

Experimental work in this thesis showed a positive correlation with LKLF epithelial expression and BAL neutrophil counts in CSLD only. There was a positive correlation with BAL CXCL8 levels in all groups, but particularly all patients together and the CSLD group. This is in contrast to published work which has shown that LKLF expression is downregulated by TNFα and activated neutrophils. They also showed that LKLF inhibits the release of CXCL8 and also inhibits NFκB -driven transcription in the presence of *Pseudomonas aeruginosa* [212]. However, this was *in vitro* work and may not be representative of the paediatric airway, either in health or disease.

5.8.4 Interpretation of the results

This study has shown that previous data on expression of LKLF in the adult airway in health and end-stage disease cannot be extrapolated to paediatric subjects. This may be due to the anatomical site of the biopsies. Alternatively it is possibly due to different disease stage, severity and overwhelming of the inflammatory pro-resolution process in more severe neutrophilic lung disease. However, there was no evidence from this thesis to support this hypothesis. In particular a positive correlation was seen with BAL neutrophil count in CSLD patients, and a positive correlation with BAL CXCL8 levels in all patient groups. A lack of suitable CF NBS biopsies meant it was not possible to assess the role of CFTR in LKLF expression. However levels in all groups including controls were similar implying that LKLF expression is not overwhelmed or upregulated in neutrophilic lung diseases as compared to controls. The positive correlation seen between LKLF and BAL neutrophil count in CSLD patients may imply that LKLF is more important in CSLD as compared to CF, and that the CFTR-defect prevents LKLF expression to resolve inflammation. However this is merely speculative.

In conclusion, in relation to the original hypothesis, expression of LKLF did not vary between paediatric disease groups, and there was no evidence to suggest that LKLF expression was reduced in neutrophilic diseases as compared to controls, or CF as compared to other neutrophilic lung diseases. However, results did show increased expression in some groups with BAL neutrophils and CXCL8, suggesting that LKLF expression may increase with increasing inflammation under certain circumstances. Thus

there may be a role for LKLF in resolving inflammation in paediatric neutrophilic lung disease, but given the inconsistent results, limited conclusions can be drawn.

As regards the second hypothesis, that there is a CFTR-related defect in the resolution of inflammation by LKLF which contributes to the severity of CF, again there was limited evidence to prove this. The only positive finding was that LKLF expression did not increase with BAL neutrophilia in CF as compared to CSLD patients, which may suggest a CFTR-related effect. However, again there was insufficient further evidence to prove this.

Chapter 6

Lipid analysis of BAL and expression of 15-LOX in EBB

6.1 Analysis of BAL for inflammatory resolution lipid mediators by LC/MS/MS

In order to analyse levels of inflammatory resolution lipid mediators in the paediatric neutrophilic airway, BAL supernatant was sent to Professor O'Donnell's laboratory, Institute of Infection and Immunity, School of Medicine, Cardiff University, for lipid analysis by liquid chromatography / mass spectrometry (LC/MS/MS). Professor O'Donnell's laboratory has established expertise in the area of lipid research which was not available in the Lloyd laboratory. The hypotheses of this chapter were as follows:

- Lipid mediators important in the resolution of inflammation are lower in CF than in other paediatric CSLDs
- There is a CFTR specific reduction in pro-resolution lipid mediators, accounting in part for the worse prognosis of CF compared with other CSLDs.

Samples were stored at -80 ºC prior to lipid extraction. Lipid mediators were measured on a Shimadzu high performance liquid chromatographer (HPLC) coupled to ABSciex 4000 Q-Trap MS, using reverse phase LC/MS/MS, with multiple reaction monitoring detection [300]. Standards existed for the following lipids: LxA_4, RvD_1, PD_1, LTB_4, PGE_2, PGD_2, 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE. Paediatric BAL samples were sent in two consignments to the Cardiff laboratory.

6.2 Lipid analysis results

6.2.1 Patient characteristics (table 6.1)

Three CF patients were clinically stable at the time of bronchoscopy which was performed opportunistically during venous access placement. One PCD patient had previously isolated non-tuberculous mycobacteria (NTM) and was receiving nebulised amikacin and oral antibiotics directed against NTM. BAL was not performed in one control patient, who underwent bronchoscopy for reported haemoptysis. No abnormality was found on bronchoscopy.

Table 6.1: BAL lipid analysis patient characteristics (total n=103)

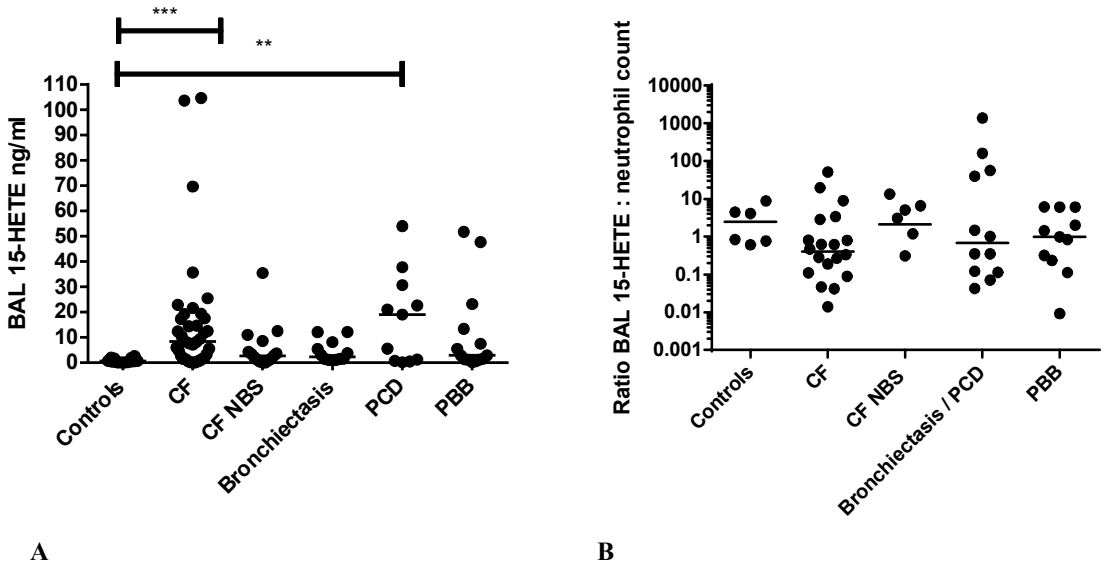
	Controls	CF	CF NBS	Bronchiectasis	PCD	PBB
Number	14	38	14	12	11	14
Age yrs (IQR)	**10.7 (2.8-12.9)**	**10.7 (2.8-12.9)**	**0.3 (0.2-0.3)**	**7.8 (4.3-12.6)**	**11.3 (9.9-14.5)**	**2.9 (2.2-4.3)**
Sex male (%)	7 (50)	21 (55)	10 (71)	10 (83)	6 (55)	6 (43)
Number with recent spirometry (%)	5 (36)	22 (58)	_____	7 (58)	11 (100)	_____
FEV1 % predicted (IQR)	**113 (84-124)**	**69 (57-75)**	_____	**73 (56-89)**	**62 (43-71)**	_____
Number (%) with BAL cytology results	13 (93)	22 (58)	8 (57)	9 (75)	7 (64)	14 (100)
BAL % neutrophilia (IQR)	**2.7 (0.3-2.7)**	**24 (15.6-52)**	**8.3 (1.8-17.9)**	**45.3 (6-83.9)**	**15.7 (1.7-21)**	**21.8 (8.7-36.3)**
Pathogens isolated on BAL (%)	**0**	**20 (53)**	**4 (29)**	**8 (67)**	**3 (27)**	**14 (100)**
Infected with *P. aeruginosa* (%)	0	25 (66)	1 (7)	1 (8)	5 (45)	0
Respiratory exacerbation	0	6	0	0	4	0
Inhaled steroids	0	13	0	9	8	5
Oral steroids	0	1	0	0	0	1
Azithromycin	0	7	1	1	3	2
Oral antibiotics	0	25	13	1	3	0
Nebulised antibiotics	0	22	0	0	1	0

CF NBS patients were significantly younger than controls, CF, PCD and PBB patients ($p<0.0001$), as well as bronchiectasis patients ($p<0.001$). Percent predicted FEV_1 was significantly higher in controls as compared to CF ($p<0.05$) and PCD ($p<0.01$). Percent BAL neutrophilia was significantly higher in CF ($p=0.0001$), bronchiectasis ($p=0.001$) and PBB ($p=0.001$) as compared to controls. The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.0001$). IQR=interquartile range.

LXA_4, LTB_4, resolvins, protectins and prostaglandin D_2 were not detected in BAL samples. Of these mediators, only LXA_4 was detected in mouse BAL sent at the same time as the paediatric BAL samples, and thus served as a useful positive control. The samples were from a mouse model of allergic airways disease from another researcher, but provide evidence that, using the same processes, LXA_4 could be detected in BAL samples.

The following lipids were detected in paediatric BAL samples: 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE. The levels detected of 8-HETE, 11-HETE and PGE_2 were low (<1.7, <1.0 and <2.3 ng/ml respectively). Results from 15-HETE are discussed in the following sections, as this mediator has effects on inflammatory resolution, via LXA_4 production. Levels of 15-HETE were related to markers of airway inflammation and disease severity.

6.3 15-HETE



A **B**

Figure 6.1: Graph A: BAL levels of 15-HETE. Horizontal bars represent medians which varied significantly ($p<0.001$) Levels were significantly higher in CF ($p<0.001$) and PCD ($p=0.01$) patients as compared to controls. Graph B: data normalised for neutrophilic inflammation; no significant differences were seen between groups. Data in graph B have been Log_{10} transformed to expand the axis.

Given 15-HETE is important for both pro- and anti-inflammatory actions, the results could not be predicted. 15-HETE has been detected by others in sputum from adults with

CF, although levels were not related to markers of disease severity. In this thesis, BAL 15-HETE levels were significantly higher in CF ($p<0.001$) and PCD ($p=0.01$) patients as compared to controls (figure 6.1). However no differences were seen on normalising for neutrophilic inflammation suggesting the initial differences were related to disease severity. Overall, of all the measured HETEs, 15-HETE was present in the highest concentrations in BAL.

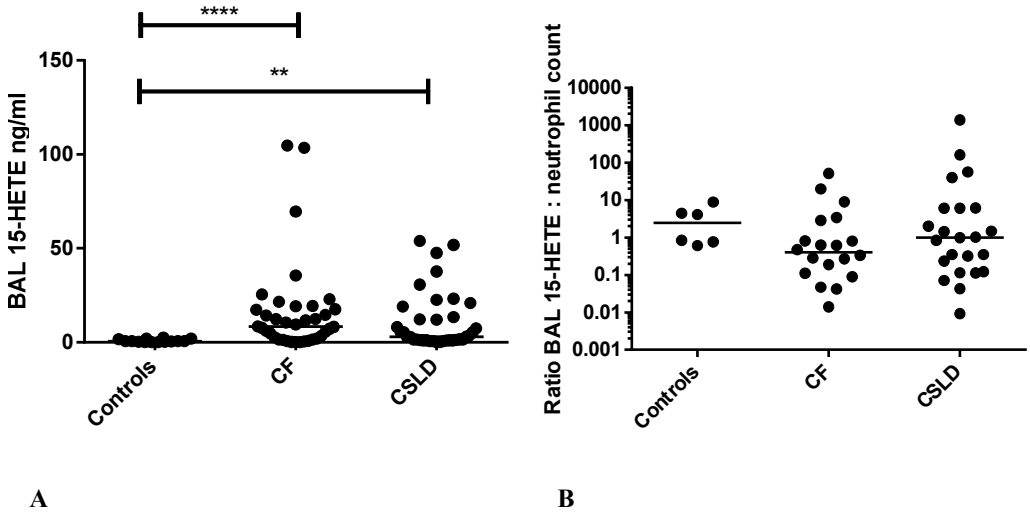


Figure 6.2: Graph A: BAL levels of 15-HETE in controls, established CF and CSLD. Levels were significantly higher in CF ($p<0.0001$) and CSLD ($p<0.01$) patients as compared to controls. Graph B: data normalised for neutrophilic inflammation; no significant differences were seen between groups. Data in graph B have been Log_{10} transformed to expand the axis.

15-HETE levels were significantly higher in established CF and all other neutrophilic lung diseases (grouped as CSLD) as compared to controls, but again this difference was lost on normalisation of the data for neutrophilic inflammation (figure 6.2).

Finally, on comparing all CF (established CF and CF NBS) with CSLD – no differences were seen (figure 6.3).

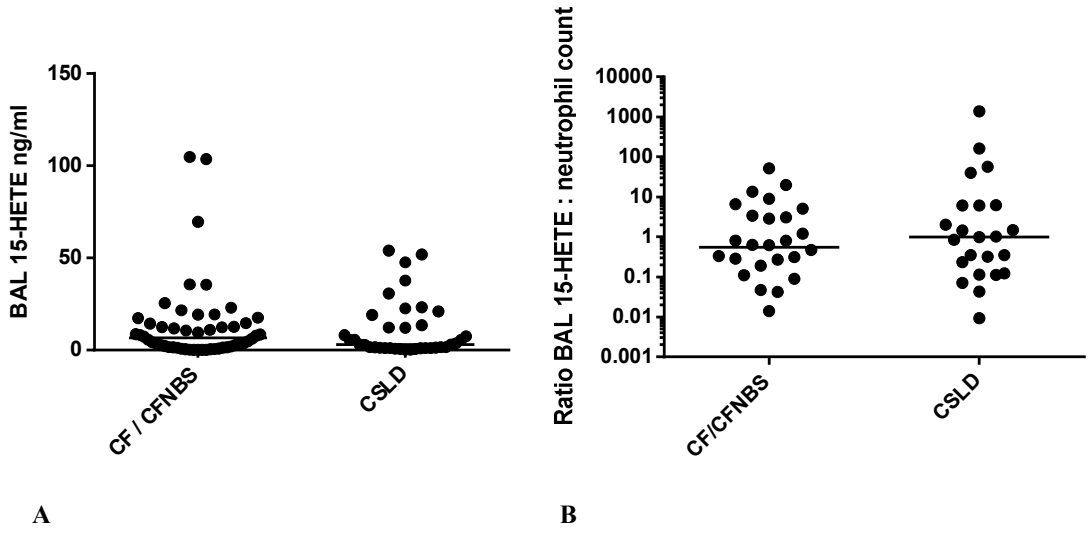


Figure 6.3: Graph A: BAL levels of 15-HETE in all CF and CSLD. Graph B: data normalised for neutrophilic inflammation. No significant differences were seen between groups. Data in graph B have been Log_{10} transformed to expand the axis.

No differences were seen with prescribed therapies apart from nebulised antibiotics for *Pseudomonas aeruginosa* in the CF group. These patients had statistically higher BAL 15-HETE levels than those not prescribed nebulised antibiotics ($p<0.05$). However, by definition this is a self-selecting group with more severe disease, suggesting association rather than causation.

The next section examines the relationship between 15-HETE and markers of airway inflammation and disease severity.

6.4 Relationship between BAL 15-HETE and neutrophil count

15-HETE is reported to have a less marked effect on neutrophil chemotaxis, as compared to the other HETEs. No correlation was seen with BAL neutrophil count in all patients together, all CF or CSLD.

6.5 Relationship between BAL 15-HETE, IL-4, CXCL8 and IL-10

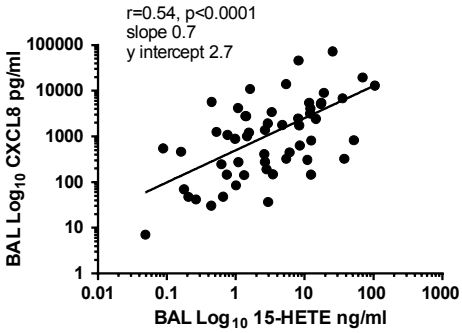
In total there were 66 samples with both HETE and cytokine results.

6.5.1 IL-4

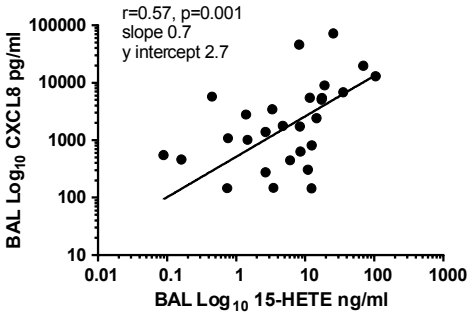
Levels of 15-HETE are associated with increased levels of IL-4 [284]. It would be expected that there would be a positive correlation between BAL 15-HETE and IL-4. However, virtually all IL-4 results were below the assay lower limit of detection and no correlation was evident.

6.5.2 CXCL8

Positive correlations were seen between 15-HETE and BAL CXCL8 in all patients together and all CF patients. There was a weaker non-significant correlation in the CSLD group (figure 6.4).



A



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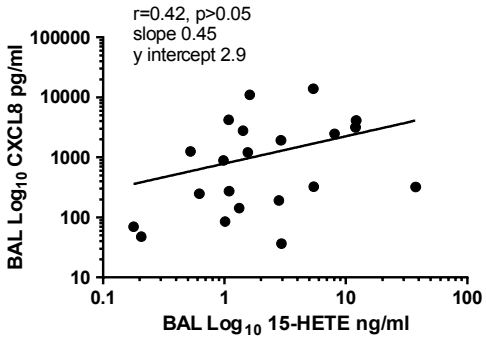


C

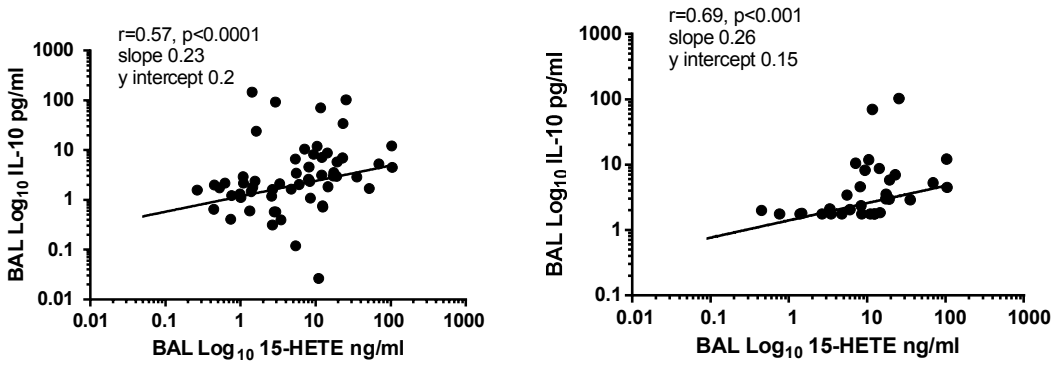
Figure 6.4: Graphs showing association between BAL 15-HETE and CXCL8 in all patients (A), all CF patients (B) and CSLD (C) Data has been Log_{10} transformed to expand the axes. There was no significant difference between slopes in CF as compared to CSLD.

However, the linear regression slopes did not differ significantly between CF and CSLD. Given 15-HETE may therefore mediate pro-inflammatory functions through CXCL8, the

ratios of BAL 15-HETE:CXCL8 were examined. However no differences were seen between groups.

6.5.3 IL-10

Positive correlations were seen between 15-HETE and BAL IL-10 in all patients together and all CF patients (figure 6.5). No correlation was seen with the CSLD group.

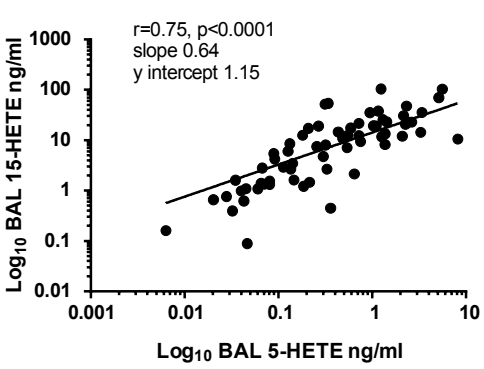


A **B**

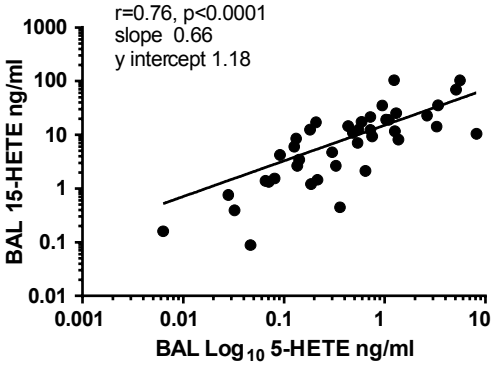
Figure 6.5: Graphs showing association between BAL 15-HETE and IL-10 in all patients (A), all CF patients (B). Data has been Log_{10} transformed to expand the axes.

6.6 Association between BAL 15- and 5-HETE

Lipoxins are mostly produced by the enzymatic action of 15-LOX and the production of 15-HETE. 15-HETE is converted by 5-LOX in neutrophils and eosinophils to produce lipoxins, with a reduction in 5-HETE production. Therefore if 15-HETE is involved in inflammatory resolution and the production of LxA_4, an inverse relationship between 15- and 5-HETE would be expected. Although LXA_4 was not detected, the relationship between 5- and 15-HETE was examined to establish whether there was evidence to suggest that 15-HETE may be acting to resolve inflammation. However, the opposite was seen in this cohort – with a positive correlation between these two lipid mediators, as shown in figure 6.6. A significant positive correlation was seen in all disease groups, and the slopes did not differ significantly between CF and CSLD. Thus the positive correlation between BAL 5- and 15-HETE occurs irrespective of expected phenotypic severity / disease group.



A



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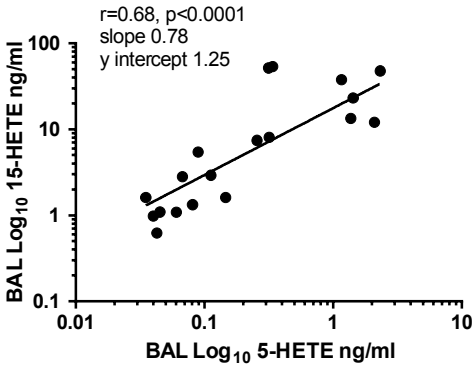


C

Figure 6.6: Graphs showing correlations between 5- and 15-HETE in all patients (A), all CF patients (B) and CSLD (C). Data has been Log_{10} transformed to expand the axes. The slopes did not differ significantly between CF and CSLD.

6.7 15-HETE and pathogen status

Levels of BAL 15-HETE were higher in those patients with positive microbiological cultures at the time of bronchoscopy, when grouping all patients together. However there was no association seen in the CF or CSLD groups, and on normalising for airway inflammation by expressing as a ratio of BAL neutrophils, this association was lost. Likewise levels were higher in CF patients with *Pseudomonas aeruginosa*, but again no association was seen on correcting for neutrophilic inflammation. Thus the differences seen were a reflection of disease severity and not purely pathogen status.

6.8 Analysis of EBB for 15-Lipoxygenase (15-LOX) expression

The next section discusses the results from immunofluorescent staining of EBB for 15-LOX. EBB were stained to visualise expression of 15-LOX within the airway. This enzyme was examined since 15-HETE was detected in higher quantities in BAL as compared to the other HETEs. In addition 15-HETE levels were highest in CF patients and thus if the source of 15-LOX for 15-HETE production was from the airway wall, it might be expected that expression of this enzyme would be highest in these patients. The hypotheses of this section were:

- 15-LOX airway expression is increased in neutrophilic lung diseases as compared to controls

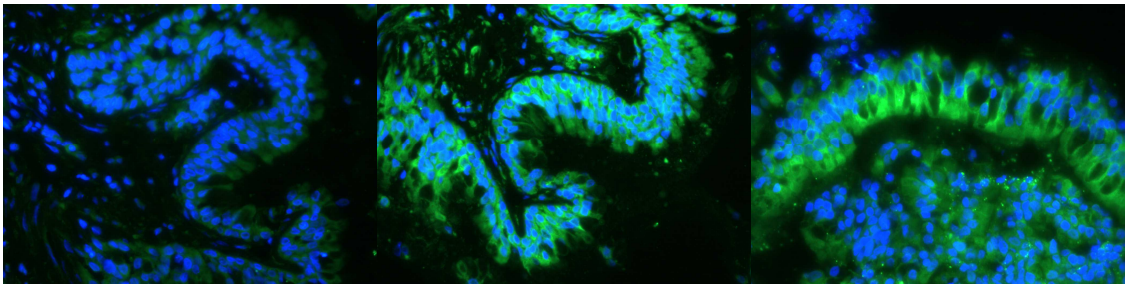
- Airway expression of 15-LOX is highest in established CF patients as compared to other neutrophilic lung diseases

6.9 Establishment of an immunofluorescent staining protocol for 15-LOX using paraffin-fixed paediatric endobronchial biopsies

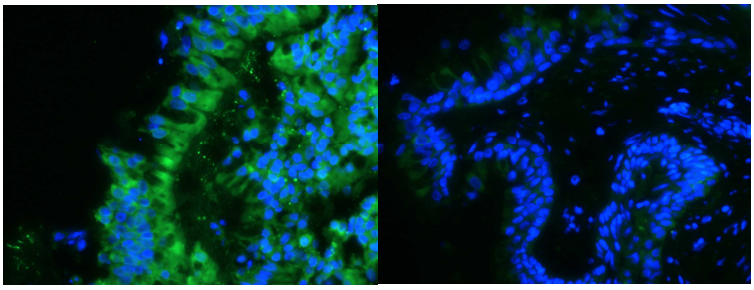
In adult asthmatic patients, 15-LOX has been found by immunostaining in the epithelium and submucosa of endobronchial biopsies [301]. As far as the author is aware, 15-LOX expression by immunofluoresence has not been performed in paediatric endobronchial samples, or neutrophilic lung disease. Slides from paediatric CF patients were used to provide positive controls, on the assumption that expression would be increased in more severe airway disease. If this staining was negative a plan was made to acquire samples from severe asthmatics to develop the method. However, staining was successful and therefore the use of samples from asthmatic subjects was not necessary. Previous published work discussing staining of samples for 15-LOX have not used paediatric samples, nor have they utilised paraffin-fixed biopsies. A method was therefore devised by the author, utilising experience with immunofluorescent staining for AnxA1 as a basis.

Slides were dewaxed and antigen retrieval was performed using Tris EDTA. Avidin-biotin blocking steps were included and the slides flooded with protein block. Cell permeabilisation was incorporated using Triton-X 100, on the knowledge that 15-LOX is

intracellular. 15-LOX mouse anti-human monoclonal antibody (Lifespan Biosciences, Stratech Scientific, Suffolk, UK) was used initially at dilutions of 1:50, 1:100, 1:250 and 1:500. A mouse IgG2 isotype control slide was prepared for each patient. Staining was seen using all dilutions, but was less effective at 1:500. As there was no difference between the other three staining dilutions, 1:250 was used for further experiments (figure 6.7). Following overnight incubation, slides were washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes. They were then incubated in a secondary rabbit anti-mouse biotin-conjugated antibody as described above, at a dilution of 1:250 at room temperature for one hour. All slides were again washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes. They were then incubated with streptavidin antibody conjugated to Alexa Fluor 488 at a dilution of 1:250 at room temperature for one hour in the dark. Slides were finally washed in 3 changes of PBS and 0.3% Triton-X 100, each for 5 minutes in the dark. Slides were then mounted with DAPI and stored at $4^{o}C$ in the dark until ready for analysis, using the same technique as previously described for AnxA1 and LKLF, by expressing staining as a percentage of DAPI staining.



A **B** **C**



D **E**

Figure 6.7: Examples of immunofluorescent staining of EBB for 15-LOX (green). Slides were counterstained with DAPI (blue). A: Isotype control slide. Other images represent staining with different dilutions of 15-LOX. B: 1:50, C: 1:100, D: 1:250, E:1:500. A dilution of 1:250 was used in further experiments. Magnification x 100.

6.10 15-LOX results

6.10.1 Patient characteristics EBB (table 6.2)

BAL was not performed in one control patient, and this was the same patient discussed in the BAL lipid analysis cohort. One CF patient was clinically stable and underwent bronchoscopy during venous access placement.

Table 6.2: 15-LOX immunofluorescent staining patient characteristics (total n=38)

	Controls	CF	Bronchiectasis	PCD	PBB
Number	6	13	6	6	7
Age yrs (IQR)	**11.7 (6.3 – 14.2)**	**12.3 (6.4 – 13.2)**	**9 (4-14.4)**	**11.4 (9.4-13.8)**	**3.8 (2.4- 4.3)**
Sex male (%)	**2 (33)**	**8 (62)**	**5 (83)**	**1 (17)**	**4 (57)**
Number with recent spirometry (%)	_____	10 (77)	4 (67)	6 (100)	_____
FEV1 % predicted (IQR)	_____	74 (60-81)	69 (45-81)	58 (41-75)	_____
Number with BAL cytology results (%)	5 (83)	7 (54)	5 (83)	6 (100)	7 (100)
BAL % neutrophilia (IQR)	**1 (0-2.4)**	**74 (12.7-89)**	**31 (6-83.9)**	**31.5 (6.9-84.2)**	**19.3 (9.3-38.7)**
Pathogens isolated on BAL (%)	**0**	**10 (77)**	**3 (50)**	**3 (50)**	**7 (100)**
Infected with *P. aeruginosa* (%)	0	9 (69)	0	3 (50)	0
Respiratory exacerbation	0	1	0	3	0
Inhaled steroids	0	5	5	5	3
Oral steroids	0	0	0	0	0
Azithromycin	0	1	0	1	1
Oral antibiotics	0	6	0	1	0
Nebulised antibiotics	0	6	0	2	0

PBB patients were significantly younger than those with CF ($p<0.05$). The percentage BAL neutrophilia was significantly higher in CF as compared to controls ($p=0.01$). The median number of patients who isolated pathogens on bronchoscopy varied significantly ($p<0.01$).

6.11 15-LOX expression in EBB measured by immunofluorescent staining

A total of 39 EBB were of suitable quality to be made into paraffin-fixed slides. Samples were acquired from newly recruited patients and also from slides previously acquired by colleagues. There were insufficient CF NBS patients with biopsies suitable to be made into paraffin-fixed slides, due to poor morphology and the very tiny size of the biopsies.

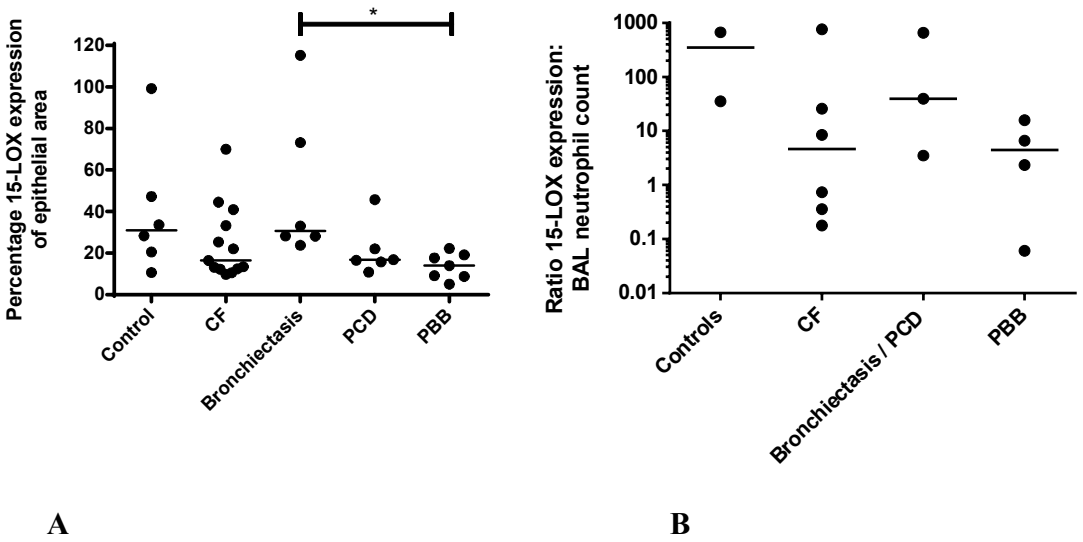
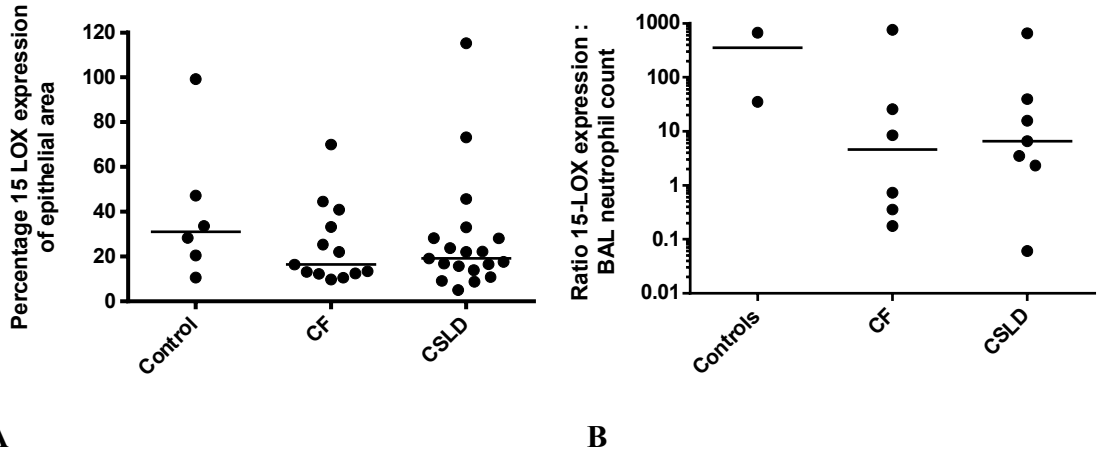


Figure 6.8: Graph A: percentage 15-LOX staining of the epithelial area of EBB in different patient groups. Levels in bronchiectasis were higher than PBB ($p<0.05$). Graph B: data normalised for neutrophilic inflammation. No significant differences between groups. Data in graph B were Log_{10} transformed to expand the axes.

15-LOX staining was predominately in the airway epithelium. There was no significant difference in median 15-LOX expression between groups, including on normalising data for neutrophilic inflammation (figure 6.8). In addition there were no suitable CF NBS biopsies. In one bronchiectasis patient the expression of 15 LOX was measured as > 100%, which highlights some issues with the analysis method, discussed previously. On normalising for inflammatory status, a number of samples in the control group did not have a ratio result as the neutrophil count was zero. No differences in 15-LOX expression were seen between CF and CSLD (figure 6.9). On normalising data for BAL CXCL8, ratios were higher in controls as compared to CF, but there were only 4 patients

in the control group. No significant correlation was seen between expression of 15-LOX and levels of BAL 15-HETE.



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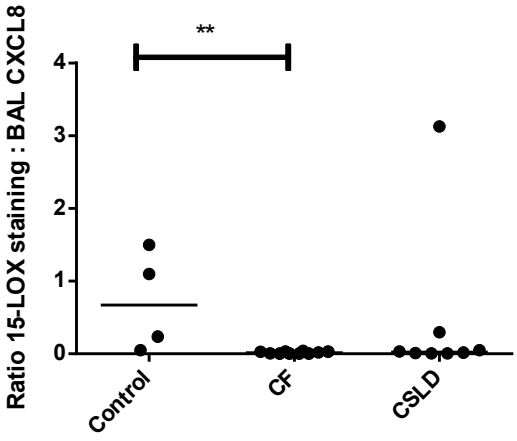

C

Figure 6.9: Graph A: percentage 15 LOX staining of the epithelial area of EBB in controls, CF and CSLD. Graph B: data normalised for neutrophilic inflammation. There were no significant differences between groups. Graph C: data normalised for BAL CXCL8 level. The ratio was higher in controls as compared to CF in graph C ($p<0.01$). Data in graph B were Log_{10} transformed to expand the axes.

The next section examines the effect of other makers of disease severity on 15-LOX expression.

6.12 Relationship between 15-LOX expression and BAL neutrophil count

When grouping patients together, no correlation was evident between epithelial 15-LOX expression and BAL neutrophil count. Analysis of individual disease groups was limited by patient numbers, but no correlations were seen (data not shown).

6.13 Relationship between 15-LOX expression and BAL macrophage count

15-LOX type B mRNA has been reported to correlate positively with percentage BAL macrophages and negatively with percentage BAL neutrophils in control patients, although no association was seen in CF [244]. Therefore the correlation between airway 15 LOX expression and BAL macrophages was analysed, but no significant correlation found when grouping all patients together or within patient groups (data not shown).

6.14 Relationship between 15-LOX expression and BAL cytokines (IL-4, CXCL8 and IL-10)

There were 20 patients with both 15-LOX immunofluorescent staining and BAL cytokine results. No significant association was evident between airway 15-LOX expression and any of the measured BAL cytokines. In particular there was no significant correlation with BAL IL-4 levels, although most IL-4 levels were below the LLD (data not shown).

6.15 Discussion

This is the first study to examine BAL lipoxins, resolvins, protectins and HETEs in children with neutrophilic airway disease. It is also the first such study to examine airway 15-LOX expression. Given previous published research with LXA_4 it would be expected that levels of this lipid mediator in particular would be lower in those with the most severe airway neutrophilic inflammation, potentially due to the inflammatory resolution capability being overwhelmed. There is a suggestion in the literature that the ability of LXA_4 to resolve inflammation may be related specifically to the CFTR-defect. The inclusion of CF NBS patients in this thesis as well as those with established CF should aid further clarification of this uncertainty. Given the high levels of BAL 15-HETE seen, it was hypothesised that 15-LOX expression would also vary between groups, and that expression would be positively related to BAL 15-HETE and LXA_4 levels. The relationship between 15-LOX and airway neutrophilic inflammation is less

clear and thus whether there are specific differences in neutrophilic lung diseases is uncertain. Given the findings of previous research a positive correlation was expected between 15-HETE, 15-LOX expression and BAL IL-4. This research and the methodology will be discussed in the following sections, alongside limitations of the study and future directions.

6.15.1 Principle findings

BAL

LxA_4, RvD_1, PD_1, LTB_4 and PGD_2 were not detected in any BAL samples. Levels of 15-HETE were higher in established CF patients as compared to the other patient groups, including CF NBS. These differences were however lost on normalising for inflammation, where no differences were seen between disease groups and controls, or CSLD and all CF patients. No correlation was seen between 15-HETE and BAL neutrophil count. Positive correlations were seen with CXCL8, IL-10 and 5-HETE, to a greater extent in CF as compared to CSLD. No correlation was seen with IL-4, although the majority of IL-4 results were below the LLD.

EBB

There was no statistically significant difference in airway expression of 15-LOX between disease groups or controls once data had been normalised for neutrophilic inflammation. However, the ratio of 15-LOX:BAL CXCL8 was higher in controls as compared to CF, but there were limited patients in the control group. There was no correlation between 15-LOX expression and BAL 15-HETE. Epithelial expression did not correlate with BAL neutrophilia or measured cytokines.

6.15.2 Strengths and weaknesses of the study

A potential weakness is the fact that the main pro-resolving lipid mediators of interest were not detected in any of the BAL samples. The O'Donnell laboratory has considerable experience in this area and therefore it would be unlikely that there were errors in the laboratory technique. However sample storage, as discussed below, may have played a part.

This study is also the first to examine 15-LOX expression in paediatric neutrophilic airway disease. One particular weakness is that there were no morphologically suitable CF NBS biopsies available and therefore it was not possible to establish how airway epithelial expression of 15-LOX may vary between young babies newly diagnosed with CF, and those with established disease.

The same method for analysis of immunofluorescent images was used as described in previous sections. Again a significant issue with this method is that following analysis the percentage epithelial expression of 15-LOX was calculated as greater than 100% in one patient.

6.15.3 Strengths and weaknesses in relation to other studies

HETEs

There are clearly several differences between the published data and the results in this thesis. The first significant finding was that in contrast to published papers, neither LXA_4 nor LTB_4 were detected in any paediatric BAL samples. This may be related to methodology as the published groups utilised ELISA to detect these lipid mediators, whereas the BAL samples in this thesis were analysed using LC/MS/MS. It is generally reported that LC/MS/MS is a more accurate method of lipid analysis with greater specificity than ELISA. However LTB_4 has previously been detected by HPLC, and it was found that 90% of LTB_4 measured by ELISA corresponded to LTB_4 detected by HPLC [302]. This group also showed that prolonged frozen storage of BAL samples leads to degradation of LTB_4 and possibly other lipid mediators. LTB_4 levels fell significantly to 20% in 14 days. They reported storage of concentrated BAL in methanol at -70^{o}C resulted in almost no loss of LTB_4. It is feasible that the storage of BAL samples in this thesis may have led to degeneration of lipid mediators as BAL samples were stored for greater than 14 days and for the most part several months at -80^{o}C. Previous studies examining LXA_4 in BAL have varied in their methodology as regards storage of BAL samples for lipid analysis. A number of groups did not comment on this issue when investigating LXA_4 in BAL or sputum by ELISA [13, 244, 245, 303]. In contrast Planagumà stored BAL samples in methanol at -80^{o}C prior to analysis by ELISA [304]. Yang added butylated hydroxytoluene (an antioxidant) and triphenylphosphine (a reducing agent) and

a broad spectrum COX inhibitor (indomethacin) prior to freezing and storing sputum samples [13]. They did not use methanol. This group detected a number of lipid mediators, including LXA_4 and RvE_1 by LC/MS/MS. They also reported lower levels of PGE_2, $PGF_{2\alpha}$, TXB_2 and LTB_4 in sputum as compared to previous groups who had utilised radioimmunoassay (RIA) or ELISA. This may be due to the fact that RIA and ELISA use antibodies which may detect similar metabolites, for example metabolites of LTB_4 and thus overestimate the amount of lipid mediators present. There may therefore be two methodological issues as to why lipoxins, resolvins, protectins and LTB_4 were not detected in BAL samples in this thesis; problems with long term frozen storage leading to degradation of mediators and that LC/MS/MS is more accurate than ELISA and therefore may lead to lower levels being detected, or indeed no mediator detected.

Methodological issues aside there may be other reasons why LXA_4 was not detected in paediatric samples in this thesis. Firstly the CF patients in the studies discussed above may represent differing disease severity, although given LXA_4 has been detected in paediatric samples by others this is probably unlikely. In addition lipoxins were detectable in adult CF patients, albeit at low level. Given children with CF generally have milder disease than adult subjects it would be expected that BAL lipoxin levels should be higher in these paediatric patients.

Knowledge of the biochemical pathways leading to the production of LXA_4 provides further avenues for consideration. Lipoxins are mostly produced by the enzymatic action of 15-LOX [226]. Upregulation of 15-LOX favours LXA_4 production at the expense of leukotriene synthesis. 15-LOX was detected in endobronchial biopsies in both disease groups and controls in this thesis, although there was no significant difference in 15-LOX expression between these groups. However, the expression of 15-LOX in BAL cells, such as neutrophils, has not been examined, and it is possible that 15-LOX in BAL cells has a greater effect on BAL LXA_4 production than epithelial 15-LOX. 15-LOX mRNA has previously been reported in BAL cells from paediatric CF patients and inflammatory controls [244]. However, mRNA presence does not necessarily equate to protein production, and therefore the role of BAL 15-LOX, and its cellular location remain unanswered questions which may impact on LXA_4 expression in cells retrieved from

BAL. It is also uncertain whether there may be as yet unknown in vivo inhibitor that could affect enzyme activity.

A broad selection of lipid mediators has been found in the sputum of adult patients with CF. There were associations between pro-inflammatory LTB_4 and reduced lung function as well as pro-resolution RvE_1 and improved lung function [222]. RvD_1 and PD_1 were not detected in any of the BAL samples in this thesis, including control subjects. Both RvD_1 and PD_1 have not previously been sought in the paediatric airway, or in neutrophilic lung disease. Therefore it is uncertain whether they are expressed at all under these circumstances.

15-LOX

This study examined airway expression of 15-LOX protein in paediatric neutrophilic lung disease, utilising samples from children with a range of ages, diseases and disease severity, There are limited previous studies looking specifically at 15-LOX expression in adult CF patients, and none involving other adult neutrophilic lung diseases.

There is one previous study in children, which reported BAL 15-LOX mRNA levels, rather than airway protein expression, in children with CF and disease controls [244]. The other main difference compared with this thesis was the control patients, as the controls in this previous published work had non-CF diseases requiring clinical bronchoscopy. Some of these control patients isolated pathogens at bronchoscopy and had positive bacterial cultures. Accepting these differences, this group reported a trend for lower 15-LOX mRNA in CF patients, which differs to the findings in this thesis, but this did not reach statistical significance. 15-LOX type B (an iso-enzyme of 15-LOX) mRNA was significantly lower in CF patients as compared to controls. There was no association with 15-LOX type B mRNA and the ratio LXA_4: LTB_4 in CF patients, but a positive correlation in control patients. 15-LOX type B also correlated positively with percentage BAL macrophages and negatively with percentage BAL neutrophils in their diseased control patients, although no association was seen in CF patients. LXA_4 and LTB_4 were not detected in the BAL from patients in this thesis; therefore it was not possible to assess any association between 15-LOX expression and these lipid mediators. In contrast to the

published work no association was seen between 15-LOX and percentage BAL neutrophils or macrophages in either controls or CF patients.

Sinonasal expression of 15-LOX has previously been examined in adult CF patients, and no statistical difference in expression was seen between those with CF and healthy controls, although there was incomplete information on patient clinical status [197]. These results are therefore the same as the findings in this thesis. However, their methodology was different. Firstly samples were not from the lower airway, although nasal samples are likely to be similar. Secondly immunohistochemical staining was utilised and the results scored by a pathologist. This group reported staining of the columnar epithelium in both CF and control patients, which was predominately seen in the staining in this thesis. These adult CF patients also had positive staining in apical and basal cell layer cytoplasm. Control specimens exhibited staining in a variety of locations: apical and basal cell cytoplasm

Another study looked at expression of 15-LOX in CF peripheral blood mononuclear and neutrophil cells [198]. This study measured 15-LOX mRNA levels and found no expression in CF patients, but increased expression in asthmatic subjects as compared to controls.

Mild asthmatics are reported to have increased 15-LOX expression in airway submucosal eosinophils, but not in the bronchial epithelium, as compared to healthy controls. 15-LOX also amplifies IL-4 levels. However, no association was seen with BAL IL-4 levels in this thesis.

6.15.4 Interpretation of the results

HETEs

Given LXA_4, resolvins and protectins were not isolated in any BAL samples it has not been possible to provide answers to whether the ability to resolve airway neutrophilic inflammation varies between different patient groups in this thesis. LTB_4 was also not detected. These findings are in contrast to previous published work whereby lipoxins, resolvins and LTB_4 have been detected from BAL and sputum from subjects with neutrophilic airway disease. Most of this previous work has involved adults but there

have been limited paediatric studies as outlined above. The most probable explanations for these findings are related to methodology as discussed above.

This thesis has shown that HETES can be detected using LC/MS/MS in BAL from paediatric subjects with neutrophilic airway disease, and control subjects with isolated upper airway disease. However, the exact role of a number of the HETEs remains uncertain. It is also uncertain what the biological significance is of low level HETEs. Given that 15-HETE was higher in CF patients, and there was a positive correlation with some markers of increased inflammation (CXCL8, 5-HETE but not neutrophils), this shows an association with an increased airway inflammatory state. The lack of association with BAL neutrophilia is possibly due to the fact that of all the HETEs, 15-HETE is reported to have the least effect on neutrophil chemotaxis.

Although 15-HETE levels were highest in CF patients as compared to other groups, given there were no differences between groups on normalisation of data for BAL neutrophil count, then this is not a disease-specific phenomenon. The higher levels in CF may be a reflection of the increased airway inflammatory severity in CF patients. In addition if 15-HETE was functioning in an inflammatory resolution capacity, with concurrent promotion of LXA_4 production, then it would be expected that there would be an inverse relationship between BAL 5- and 15-HETE, which was not seen. This may imply that 15-HETE does not have an inflammatory resolution action in the paediatric neutrophilic airway. However, given the cross-sectional nature of this thesis it is not possible to comment further on this.

Again, given the cross-sectional nature of the study, the meaning of the positive correlation between BAL 15-HETE and IL-10 remains uncertain. It is possible that IL-10 could be acting in a pro- or anti-inflammatory capacity. Equally there may be an additional factor driving up both these mediators. However, this particular finding was specific to CF and implies that this may be related to CFTR dysfunction.

Together this evidence suggests that raised BAL 15-HETE is associated with increasing airway inflammation. Overall there was no evidence to show that raised levels were

disease-specific or related to the CFTR defect, although the positive correlation with BAL IL-10 was specific to CF. Higher levels seen in established CF patients are a reflection of more severe inflammation in the airway in this cohort. However, without concurrent measured lipoxin levels, the potential effects of 15-HETE on inflammatory resolution mechanisms is uncertain.

15-LOX

While care must be taken in extrapolating results from peripheral blood cells to the airway, and between adults and children, and having regard to the limitations of measuring mRNA rather than protein, this thesis and previously previous published work together suggest that in CF subjects peripheral mononuclear and neutrophil cells, sinonasal cells from polyps (published work) and endobronchial biopsies (this thesis) do not show elevated 15-LOX expression in response to inflammation. This may be consistent with dysregulation of inflammatory resolution, or alternate sources of 15-LOX may be important (such as from airway inflammatory cells). Results from this thesis suggest that this is not a CF-specific phenomenon, as elevated 15-LOX expression was also not seen in other neutrophilic diseases. Unfortunately the lack of suitable CF NBS biopsies meant it was not possible to ascertain further whether those with milder CF disease have altered expression in 15-LOX. The lack of correlation between airway 15-LOX and BAL 15-HETE suggests that there may be alternative sources of 15-LOX in the airway contributing to 15-HETE production. Finally as 15-LOX appears to be involved in the pathophysiology of the asthmatic airway, and given the finding by others of 15-LOX expression in submucosal eosinophils, this enzyme may not play a significant role in the neutrophilic inflamed airway.

In summary, and principally due to the most significant pro-resolution lipid mediators not being detected in any of the BAL samples, it was not possible to ascertain whether lipid mediators are important in the pathophysiology of neutrophilic lung diseases; or whether differences in lipid inflammatory resolution mediator levels are related to the ability to resolve inflammation in these diseases.

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Chapter 7

Final discussion and conclusions

7.1 Principle results in relation to the original hypotheses

The hypotheses tested in this thesis were:

1. Failure to actively terminate the acute inflammatory process is important in the pathophysiology of childhood neutrophilic airway disease

2. The differences in prognosis between these diseases are related to the extent to which neutrophilic inflammation can actively be resolved.

In order to test these hypotheses the aims were to recruit children with neutrophilic lung diseases and controls undergoing flexible bronchoscopy and collect BAL and EBB samples to measure mechanisms important for the resolution of airway inflammation. The mechanisms and mediators chosen for further analysis were: CD25+FoxP3+ T regulatory cells, the AnxA1 / ALX axis, LKLF and lipid mediators. Three novel immunofluorescent staining protocols were established by the author – staining EBB for AnxA1, LKLF and 15-LOX. The findings were then related to markers of airway neutrophilic inflammation, BAL cytokines (in particular IL-10) and markers of disease severity.

The first section in this chapter evaluates how well these hypotheses were proven and whether the specific objectives for each mediator were achieved.

Hypothesis 1: Failure to actively terminate acute inflammation is important in the pathophysiology of childhood neutrophilic airway disease.

While there was not a consistent pattern seen across all analysed mediators, there was evidence to suggest that some are important in neutrophilic airway disease.

IL-10

IL-10 was measured primarily to establish whether significant correlations existed between this cytokine and measured inflammatory resolution mediators. This was performed as IL-10 is traditionally reported to be an anti-inflammatory cytokine. A significant number of results were below the assay detection limit. However, levels were

higher in established CF as compared to other neutrophilic diseases and controls, although this difference was lost once data was normalised for neutrophilic inflammation, suggesting no CFTR effect. Conversely, the ratio of CXCL8:IL-10 was higher in all CF (established and CF NBS) as compared to CSLD, thus implying a CF-specific effect. Therefore inflammatory resolution of CXCL8 by IL-10 appears to be important in paediatric neutrophilic lung disease and appears to be less effective in CF patients. However, the discordant findings between neutrophilic inflammation (no CFTR effect) and an important neutrophilic chemokine (CXCL8) are not explained by this work.

BAL Tregs

The principle objective was to establish a flow cytometry protocol for paediatric BAL to investigate numbers of different lymphocyte subsets, and in particular Tregs. Using a method devised by the King's laboratory it was possible to identify these lymphocyte subsets in paediatric BAL. There was not a clear picture to suggest that Tregs are important in inflammatory resolution in the paediatric neutrophilic airway. The findings of note were that the ratio Tregs: BAL neutrophils was higher in controls as compared to CSLD, the positive correlation between Tregs and BAL neutrophils and the negative correlation between Tregs and BAL IL-10 in CF but not CSLD.

AnxA1 / ALX

The main objectives were to measure AnxA1 / ALX mRNA levels in BAL and EBB and to establish an immunofluorescent staining protocol for AnxA1 / ALX in BAL cells and EBB. These objectives were achieved other than staining of EBB and BAL cytospins for ALX. Although it was possible to stain BAL cytospins for AnxA1, it was not possible to further quantify the cells of origin. EBB AnxA1 staining was predominately localised in the airway epithelium. No overall clear pattern was seen in BAL and EBB, possibly due to inflammatory compartmentalisation. However, there was some evidence to suggest that AnxA1 may be important in resolving neutrophilic inflammation in the paediatric airway lumen, and that levels are overwhelmed in neutrophilic lung disease irrespective of the underlying cause. Given the AnxA1 axis is predominately active in haematopoietic cells, this may explain why differences were seen in the airway lumen (BAL) but not the airway wall (EBB).

LKLF

The principle aim was to develop and establish an immunofluorescent staining protocol for LKLF using EBB. This was successfully achieved and staining was seen in both the cytoplasm and nuclei of the airway epithelium. Although a positive correlation was seen with BAL CXCL8, particularly in the CF group, the correlation with BAL neutrophil count was only seen in the CSLD group. It is therefore possible that the resolution of inflammation by LKLF is more important in CSLD than CF, and that the CFTR defect impairs the ability of LKLF to resolve inflammation. However, this is merely speculative and overall it was not clear that LKLF is important in resolving neutrophilic inflammation in the paediatric airway. This is in contrast to published adult data, and highlights the importance of not extrapolating adult data to the paediatric population – given children as a group tend to have milder lung disease.

Lipid mediators and 15-LOX

A large number of BAL samples were sent to the Cardiff laboratory but none of the key pro-resolution lipid mediators were detected. A number of HETEs were however detected and levels of 15-HETE correlated positively with markers of increased airway inflammation (BAL CXCL8, 5-HETE and to a lesser extent BAL neutrophilia). There were also positive correlations with IL-10, the meaning of which is uncertain. 15-HETE is reported to be involved in both pro-inflammatory and inflammatory resolution pathways. Given the high levels of 15-HETE detected in these paediatric samples, the expression of 15-LOX was further examined. The objective to successfully immunofluorescent stain EBB for 15-LOX was successfully achieved. Staining was mainly seen in the airway epithelium. However, no significant association was seen between expression of 15-LOX in the airway wall and markers of luminal inflammation. Levels of BAL 15-HETE did not correlate with EBB expression of 15-LOX, and therefore there may be alternative sources of 15-LOX within the airway lumen. Therefore, principally because none of the key lipid mediators were identified in BAL, it was not possible to comment on whether these mediators are important in resolution of neutrophilic inflammation in the paediatric airway.

Hypothesis 2: The differences in prognosis between these diseases are related to the extent to which neutrophilic inflammation can actively be resolved.

The principal findings are presented in table 7.1. Overall there was not a clear picture to show that the ability to resolve neutrophilic inflammation differed between CF as compared to other neutrophilic lung diseases. Thus there was not clear evidence to show that the differing prognosis between these groups was related to the ability to resolve airway neutrophilic inflammation

	Established CF *vs* CSLD *vs* controls *(are there differences due to disease severity?)*	**All CF (including CF NBS) *vs* CSLD** *(are there differences due to the CFTR defect?)*
BAL IL-10	Levels higher in established CF and CSLD as compared to controls, but not on normalising data for neutrophilic inflammation Ratio CXCL8:IL-10 higher in established CF as compared to controls and CSLD	No difference between all CF and CSLD, including on normalising for neutrophilic inflammation Ratio CXCL8:IL-10 higher in all CF as compared to CSLD Ratio CXCL8:IL-10 higher in all CF with positive BAL microbiological cultures Positive correlation between BAL neutrophil count and CXCL8:IL-10 in all CF but not CSLD
BAL Tregs	No significant differences between groups On normalising for neutrophilic inflammation, higher levels in controls as compared to CSLD	No difference between all CF and CSLD, including on normalising for neutrophilic inflammation Positive correlation between percentage Tregs and BAL neutrophil count in CF only Negative correlation between percentage Tregs and BAL IL-10 in CF only
BAL and EBB AnxA1 / ALX	BAL AnxA1 mRNA levels similar in CF and CSLD – and CSLD statistically lower than controls, including when normalised for neutrophil count No differences in EBB AnxA1 staining between groups	EBB AnxA1 mRNA higher in CF as compared to CSLD, but no difference on normalising data for neutrophilic inflammation No differences in mRNA levels of BAL AnxA1 or ALX in BAL / EBB between CF and CSLD. No differences on normalising for neutrophilic inflammation Negative correlation between BAL AnxA1 mRNA and BAL neutrophil count in CF but not CSLD No differences in EBB AnxA1 staining between groups
EBB LKLF	No significant differences between groups, including on normalising data for neutrophilic inflammation. Positive correlation between BAL neutrophil count and LKLF expression in CSLD only Positive correlation between BAL CXCL8 and LKLF in CF	No CF NBS biopsies

	CF *vs* CSLD *vs* controls *(are there differences due to disease severity?)*	**All CF (including CF NBS) *vs* CSLD** *(are there differences due to the CFTR defect?)*
BAL 15-HETE	Higher in CF and CSLD as compared to controls, but no differences when normalising for neutrophilic inflammation	No significant differences between groups, including on normalising data for neutrophilic inflammation Positive correlation between BAL CXCL8 and 15-HETE in CF and CSLD only, but only significant in CSLD Positive correlation between BAL IL-10 and 15-HETE in CF only Positive correlation between BAL 5-HETE and 15-HETE in CF and CSLD
EBB 15-LOX	No differences between groups and none on normalising for neutrophilic inflammation. Higher ratios 15-LOX: BAL CXCL8 in controls as compared to CF	No CF NBS biopsies

Table 7.1: Summary of results analysing differences in inflammatory resolution mediators between different disease groups. Overall while there was some evidence to show that levels of pro-resolution mediators differed with disease severity, there was not clear evidence that the ability to resolve neutrophilic inflammation differed between CF and other CSLD, or accounted for differences in disease prognosis. CF=cystic fibrosis, CF NBS=CF newborn screened, CSLD=chronic suppurative lung disease, BAL=bronchoalveolar lavage, EBB=endobronchial biopsies, IL=interleukin, Tregs=T regulatory cells, AnxA1=annexin A1, ALX=annexin A1 receptor, LKLF-lung krüppel-like factor, 15-HETE=15- hydroeicosatetraenoic acid, 15-LOX=15 lipoxygenase.

7.2 Strengths and weaknesses

The strengths and weaknesses of specific sections are discussed in the relevant chapters. In this chapter strengths and weaknesses pertinent to all chapters are discussed and summarised.

7.2.1 Patient groups

One of the main strengths of this thesis is that this is the first study to examine a number of inflammatory resolution mediators in the paediatric airway. It included a large number of children with a range of neutrophilic lung diseases of varying severity, as well as control subjects. The control subjects were however not true healthy controls as ethically this was not possible. Although they did not show evidence of lower airway inflammation, the relationship between the mediators studied and upper airway disease is unknown. The inclusion of non-CF neutrophilic diseases allowed the distinction between CFTR-specific findings and those related purely to infection and inflammation irrespective of the underlying cause. The other neutrophilic disease groups were however not necessarily representative of all patients with these diseases, given that there had to be significant concerns for a bronchoscopy to be considered, and thus only children at the severe end of the spectrum were studied. This is particularly true of the PBB group.

This was a cross-sectional study, and allowed the comparison of inflammatory resolution mediators across a range of ages and indications for bronchoscopy. However, the cross-sectional methodology also means that conclusions can only be drawn about association and not causation. Data was limited for some areas, and not all patients had data available on BAL neutrophilia or percent predicted FEV_1. A number of patients within the neutrophilic disease groups had BAL neutrophil levels within the same range as control patients, and therefore not all subjects within patient groups had evidence of airway neutrophilia at the time of the study. However, group levels of BAL neutrophils are similar to other studies. In particular CF NBS patients showed evidence of BAL neutrophilia and pathogen isolation, which has been reported by others [25, 26]. This further shows that even though these young CF patients were asymptomatic, they had evidence of airway disease and are therefore not perfect controls for established CF patients. The ideal time to assess the airways of these babies would be at birth. However, this would

not be routinely possible except in babies needing surgery for meconium ileus, where non-bronchoscopic lavage could be performed. Furthermore, ideally babies with other neutrophilic lung diseases would be included to compare with CF NBS subjects; age is an unavoidable confounding factor in these babies.

It was not possible to assess the true effect of treatments such as oral steroids and oral azithromycin on patients due to limited numbers and the cross-sectional nature of the study, and also treatment could not ethically be stopped. It would also be difficult to disentangle the effects of treatment from the effects of the indication for which the treatment was prescribed. Use of IV antibiotics at the time of the bronchoscopy or started immediately after the bronchoscopy was used as a marker of an acute CF respiratory exacerbation. However, the fact that some patients received IV antibiotics at the time of bronchoscopy and some started afterwards may also have affected the results, by treating infection and thus altering inflammatory status. Other treatments such as nebulised antibiotics may also have had a similar effect. In addition all PCD and bronchiectasis patients had a pulmonary exacerbation at the time of bronchoscopy. This meant that although as a group these patients have milder disease than CF patients, they were not a true direct comparison with stable CF patients. Biopsies from CF NBS patients were morphologically unsuitable for immunofluorescent staining and thus analysis of the expression of AnxA1, LKLF and 15-LOX was not possible in this group. Finally, despite the large number of patients compared to other studies, some of the assays may have been underpowered. However, a power calculation could not be performed given there was no similar research prior to this thesis.

7.2.2 Pathogen status

The definition of pathogen isolation at the time of bronchoscopy relied on culture and did not utilise molecular methods. Sampling errors may also be important and the lobes lavaged may not be representative of pulmonary infection throughout the lung [305, 306]. It is possible therefore that pathogens were not detected in some patients. This may have biased the study against detecting differences in those with pathogens and thus although any differences that were detected are likely to be real, some may have been missed. Patient numbers were too small to delineate CF patients into those that had never isolated

Pseudomonas aeruginosa as compared to those that had isolated this pathogen previously and those that were chronically infected. Increased patient numbers may have helped to delineate any possible associations with *Pseudomonas aeruginosa* infection and mediator levels and would enable the Leeds criteria to be utilised to further define Pseudomonas status [265]. Levels of the studied mediators were compared between those with positive and negative microbiological cultures. However those with evidence of infection may have generally more severe airway disease. Thus the presence of infection *per se* may not be the main determining factor in levels of mediators.

7.2.3 Bronchoscopic methods

There may potentially have been bronchoscopic sampling errors which may have affected the results. Taking samples from only one or two lobes may not be representative of the entire airway [305, 306], with evidence to suggest that cytokines in particular may show regional variation [306]. A number of samples had cytokine levels below the assay detection limit, possibly in part due to dilution. Dilutional effects may have also differed between groups and individual patients, but it was not possible to control for this.

A number of previous studies have raised the issue of correcting for dilution of the extracellular fluid that BAL contains. Dilution is variable and depends on a number of factors. These include the size of the bronchoscope, volume and number of aliquots of lavage fluid instilled into the airway, the airway surface area that the lavage fluid is in contact with and the dwell time [307]. Some groups have used the concentration of urea [308] or albumin [309] in the BAL fluid as a proxy measurement of the dilution factor. The difficulty with such methods is that the diffusion of urea into BAL is time dependent; and underlying pulmonary disease affects the diffusion of albumin. Therefore neither of these methods is reliable [271]. Current guidelines therefore do not advise correcting for a dilution factor, and suggest reporting cellular and non-cellular factors as the concentration per ml of BAL [271, 272]. The volume of 0.9% sodium chloride in patients was adjusted to body weight, which has previously been shown to lead to recovery of a constant proportion of extracellular fluid [310]. Finally, there are likely to be a large number of proteases, such as neutrophil elastase in BAL, particularly from CF patients, which may degrade some BAL inflammatory mediators [311]. Thus the use of anti-

proteases may prevent such degradation leading to results that more accurately reflect the inflammatory drive, although detailed further work is needed to determine the optimal types and concentrations of anti-proteases. However, most published studies do not use anti-proteases in such analyses.

The biopsies taken unavoidably were not necessarily representative of the whole airway and may not reflect distal disease. Biopsy sampling from the proximal airway therefore may not be representative of distal disease, but was an unavoidable weakness, since obtaining distal airway tissue by blind transbronchial biopsy is unethical for research purposes.

7.3 Unanswered questions and future research

Due to the cross-sectional nature of this research it has not been possible to draw conclusions about the association of the analysed mediators and prescribed treatment, or the effect of an infective exacerbation and clinical stability in neutrophilic lung disease. Longitudinal studies would be required to investigate these associations further. Interventional studies would also yield further information, but multiple bronchoscopies for research purposes would not be ethical in paediatric patients. Therefore one approach would be collect research samples through collaboration with other groups (e.g AREST CF – who perform annual bronchoscopies on children with CF), although this would not aid acquisition of non-CF neutrophilic lung disease patients. These patients may need to be collated over a longer period, by continuing to approach those needing clinical bronchoscopies. Results could then be related to treatment and status in a longitudinal fashion. Control patients could be acquired from paediatric patients undergoing surgery without airway disease. An alternative approach would be the use of induced sputum although this is more challenging, but not impossible, in very young children. A number of specific questions have arisen from the work in this thesis which would be addressed and are outlined below.

Is IL-10 pro- or anti-inflammatory in the paediatric neutrophilic airway?

Given the ratio of BAL CXCL8:IL-10 was higher in all CF as compared to CSLD and controls, an inflammatory resolution function appeared to be apparent, which was

impaired in CF. However, the negative correlation between BAL Tregs and IL-10, and the positive correlation between BAL 15-HETE and IL-10 in CF only is intriguing. The cross-sectional nature of this study has not provided answers as to whether IL-10 in this situation is acting in a pro or anti-inflammatory manner. This would be addressed by interventional studies examining the effect of treating pulmonary exacerbations with IV antibiotics on BAL IL-10, neutrophil and CXCL8 levels, in both CF and other neutrophilic lung diseases. By including CF NBS patients it would be possible to see whether changes in BAL IL-10 levels in response to a treated exacerbation differ between younger CF patients and those with more established disease. Further delineation of the role of IL-10 could be examined using animal models. The effects of infection on inflammation in CFTR $^{-/-}$ IL-10 $^{-/-}$ double knock-out mice would be examined to observe the role of IL-10 in CF. Further examination of the inflammatory profile in these mice following the administration of IL-10 would also delineate the effects of this cytokine.

What is the lymphocyte profile in the normal paediatric airway?

The full characteristics of the lymphocyte profile within the normal paediatric airway remain uncertain, and future studies with increased patient numbers are needed. Future Treg cell analysis by flow cytometry in BAL would utilise more accurate methodology to delineate live cells and lymphocytes, by the methods previously discussed. The cytokine associations with Tregs also prompt further investigation. In particular given the known importance of TGFβ and IL-35 in the function and development of Tregs, the association with these cytokines should be examined in future work. A number of groups have now shown that subgroups of Tregs are involved in different diseases. Therefore it would be necessary to look at these in more detail rather than the broad approach adopted in this thesis.

Are Tregs of greater importance in the airway submucosa as compared to the airway lumen?

Given the lymphocyte predominance in the submucosa of the airway wall in CF patients [77], future work would be guided towards analysis of Tregs within endobronchial biopsies, using immunofluorescence.

Is resolution of inflammation by AnxA1 important in the paediatric airway lumen?

The full effect of neutrophilic airway inflammation in children on the AxA1 axis remains uncertain. Results from BAL cell pellets are limited by the fact that only mRNA was measured, and therefore the next step would be to look for AnxA1 and ALX protein levels. This would be particularly interesting in the CF NBS group where levels of BAL AnxA1 mRNA are similar to controls. Furthermore coexistent measurement of neutrophil elastase levels would perhaps show an association with AnxA1 in more severe neutrophilic lung disease – whereby lower AnxA1 levels are associated with higher neutrophil elastase levels. In addition given the previous work by others, the role of the pro-inflammatory form of AnxA1 (by mRNA and protein analysis) would be worth pursuing as this has not previously been examined in paediatric neutrophilic lung disease.

Animal models of AnxA1 already exist using mice treated with a CFTR inhibitor. These had an augmented inflammatory response to induced inflammation, associated with reduced levels of AnxA1. The administration of AnxA1 to these mice corrected the increased neutrophilic infiltration with a reduction in inflammation [198]. It would be possible to examine the role of neutrophil elastase in this mouse model to see if this plays a role in determining AnxA1 expression. In addition, there are now other animal models of CF that have been developed. These include the pig [312] and ferret [313]. These models appear to better represent the human CF airway and therefore further studies using these models may more accurately represent the role of AnxA1 in the CF airway and thus provide a suitable model for further laboratory interventional studies of airway inflammation [314, 315].

It is unclear which cells in the paediatric neutrophilic airway lumen express AnxA1 and ALX, and warrants further investigation, to establish whether there is predominant production by neutrophils. This would further strengthen the argument that the AnxA1 axis plays a role in neutrophilic airway disease. Double staining for neutrophil elastase could be used as a neutrophil marker to see if neutrophils are the predominant cells responsible for AnxA1 / ALX production.

Airway neutrophilic inflammation in advanced CF is steroid-resistant [316]. One mechanism by which corticosteroids work is through AnxA1 release, which is likely to be impaired in severe CF disease, given BAL AnxA1 mRNA levels were lower in CF and CSLD as compared to controls. Restoring AnxA1 levels in these patients may be one way of restoring steroid sensitivity in these patients. Clearly further preliminary work is needed to fully delineate this role, and to fully investigate this interventional studies would be needed.

Is resolution of inflammation by AnxA1 important in the airway wall in CF NBS patients?

The ratio of EBB AnxA1:BAL neutrophils was similar in controls and CF NBS. However, the lack of suitable CF NBS biopsies means that it was not possible to examine epithelial AnxA1 expression in young CF patients before they develop overt clinical disease. It has therefore not been possible to see whether AnxA1 expression is increased in these young CF patients, in a similar manner to mRNA levels. In future work protein levels could be measured in EBB through Western blotting, which would be feasible in CF NBS biopsies.

Is ALX present in the paediatric airway?

Given EBB and BAL cell pellet ALX staining was unsuccessful, one approach would be to repeat the experiment using other commercially available antibodies that have been used in other experimental processes (for example Western blotting). It is also possible that ALX is not expressed in the airway epithelium of children, and indeed staining of the adult airway has not previously been published. In addition these biopsies may only be representative of the proximal airway, and not the airway as a whole, and thus ALX may not be expressed throughout the whole airway.

Is there a more accurate method of immunofluorescent analysis of EBB?

There are alternative immunofluorescent quantification methods that would be considered, although whether these alternatives are more accurate that the current method is uncertain. However, future work would employ repeated analysis steps to provide data on intra- and inter-observer variability. One alternative analysis method would be to use

calibration beads to quantify the brightness of staining. These beads can be used to obtain a standard curve, allowing subsequent fluorescence to be quantified by spectrofluorometry.

An alternative immunofluorescent analysis method would be to utilise computer software to quantitatively analyse AnxA1 immunofluorescence. Immunofluorescence quantitative analysis (IFQA) has been previously described [317-319]. This usually requires a two-stage process. Firstly a compartment relevant to the biomarker is detected. The signal of interest is then separated from the background, generally by intensity thresholding. Computer software is then used to quantify the expression of the biomarker of interest. The main disadvantage of these methods is poor performance of histogram thresholding on images with low signal-to-background ratios. One system has been developed which appears to overcome these issues, by modelling the threshold as a function of background characteristics [320]. This may be one method of analysing AnxA1 expression more accurately.

Is LKLF airway expression less important or impaired in the paediatric CF airway as compared to CSLD?

This study has shown no statistically significant difference in airway LKLF expression between paediatric disease groups and controls. Nevertheless the positive correlation seen between LKLF expression and BAL neutrophils in CSLD but not CF suggests that this inflammatory resolution pathway may be less important or impaired in CF. However, the positive correlation between BAL CXCL8 and LKLF expression does not necessarily support this. Thus no firm conclusions can be drawn but there is evidence to suggest that LKLF is important in the paediatric neutrophilic airway. Further work would be directed towards the inflammatory response to infection in LKLF $^{-/-}$ CF animal models, with subsequent analysis of the effects of administration of LKLF.

The finding that LKLF correlates with BAL CXCL8 levels is in contrast to previously published *in vitro* work. The overall finding of no difference in airway expression between groups is in contrast to previously published adult data, where LKLF expression was lower in CF as compared to other airway diseases. It is possible that LKLF

expression is normal or elevated in paediatric inflammatory airway disease, but in more severe adult disease expression is down-regulated. However the lack of association with BAL neutrophilia does not fit with this hypothesis and indeed potential triggers for this remain uncertain. Future research would be directed towards LKLF mRNA levels in endobronchial biopsies, relating these to protein levels, to establish whether there are post-transcriptional modifications that affect protein production. This method would also enable analysis of CF NBS biopsies as the morphological quality would not be crucial to analysis.

This work has also shown that LKLF expression in the paediatric airway is within both the cytoplasm and nucleus. Given previous published work has demonstrated differential nuclear and cytoplasmic staining which appears to be related to disease severity, this would be further quantified in paediatric subjects. Finally alternative quantification methods could be utilised as described for AnxA1.

Are BAL Lipid mediators important for inflammatory resolution in the paediatric neutrophilic airway?

Unfortunately as none of the key lipid mediators important for resolving inflammation were detected in any of the paediatric BAL samples it was not possible to address this hypothesis. Further work would repeat this analysis using fresh BAL and if there was enough sample then it would be appropriate to store BAL samples both with and without the addition of methanol to see if this prevented lipid degradation.

Is 15-HETE pro- or anti-inflammatory in the paediatric neutrophilic airway?

On normalising for neutrophilic inflammation or BAL CXCL8, no differences were seen in levels of BAL 15-HETE between groups. In addition no correlation was seen between BAL 15-HETE and neutrophil count. Given that of all the known HETEs, 15-HETE has the least effect on neutrophil chemotaxis this is probably unsurprising. However positive correlations were seen with CXCL8 and 15-HETE in all groups, and between IL-10 and 15-HETE in CF. It may be that the pro-inflammatory functions of 15-HETE are mediated through chemoattractants such as CXCL8. Alternatively 15-HETE may increase in an attempt to resolve inflammation, and thus levels increase in line with CXCL8 and IL-10.

However the cross-sectional nature of this work makes this merely speculative. What can be concluded is that there were no differences between groups and therefore the correlations seen are not related to the CF defect. Further longitudinal and interventional studies are needed, examining the effects of pulmonary exacerbations and their treatment on BAL 15-HETE expression.

Are there other sources of 15-LOX within the airway lumen?

The lack of association between airway 15-LOX expression and BAL 15-HETE suggests alternative sources of 15-LOX may be important. Given 15-LOX mRNA has been reported by others in paediatric BAL cells and 15-LOX is involved in LXA_4 production, future work should be directed towards examining the protein expression of 15-LOX in BAL samples, through immunofluorescent staining of BAL inflammatory cells. This is important as although no differences were found in airway epithelial protein expression of 15-LOX, this may not be the case for other airway sources.

The most studied form of 15-LOX, and the enzyme examined in this thesis, is 15-LOX-1. An isoenzyme of 15-LOX also exists, 15-LOX type B. This iso-enzyme is also known as 15-LOX-2. 15-LOX type B has limited tissue distribution, with mRNA detected in prostate, airway epithelial cells, skin and cornea, but not in leukocytes. Both iso-enzymes metabolise AA to 15-HETE. Much of the published literature regarding 15-LOX type B concerns tumorigenesis. However, following the completion of experimental work, a subsequent paper was published which has suggested that 15-LOX type B may be important in the paediatric CF airway. In this instance the role of 15-LOX type B was examined in BAL from paediatric CF patients and paediatric controls [244]. The controls in this instance were disease controls with non-CF lower airway disease. 15-LOX type B mRNA was significantly lower in CF patients as compared to controls. There was a trend for lower 15-LOX-1 mRNA in CF patients, but this did not reach statistical significance. There was no association with 15-LOX type B mRNA and LXA_4/ LTB_4 in CF patients, but a positive correlation in control patients. 15-LOX type B also correlated positively with percentage BAL macrophages and negatively with percentage BAL neutrophils in control patients, although no association was seen in CF subjects.

This suggests that 15-LOX type B may be of greater importance in the paediatric airway, and is an area for future research.

7.4 Conclusion

Much of the published inflammatory resolution work involves animal models or adult subjects, and there is little data on the role of inflammatory resolution in children. In addition, little is known about the immunology of the normal paediatric airway, which needs to be considered when undertaking analysis of the paediatric airway in disease. Given the LKLF results in this thesis, in the same way that there needs to be caution in extrapolating data from mouse to human, one needs to be cautious in extrapolating adult data to children.

This thesis has highlighted the expression of some key molecules that are likely to play a role in the development of resolution of inflammation in the paediatric airway. In particular AnxA1 mRNA levels are reduced in more severe neutrophilic lung disease as compared to controls, but this was not related to the CFTR defect. As regards evidence of a CFTR related defect in inflammatory resolution, there was limited evidence to show that the ability of IL-10 to resolve CXCL8 inflammation in the CF airway is impaired as compared to CSLD, given the ratio of CXCL8:IL-10 was greater in CF as compared to CSLD. However this type of cross-sectional analysis cannot delineate causation.

Further longitudinal studies are needed to advance understanding of the effects of AnxA1, IL-10 and other inflammatory resolution mediators in the paediatric neutrophilic airway. These would be clinically valuable, because if in at least some groups of CF patients, active resolution of inflammation is impaired, then augmenting these pathways could be a novel therapeutic opportunity. It would be essential that such therapies were focussed on those patients in whom inflammation was over-exuberant and damaging, and not those (likely early in the course of the disease) for whom inflammation is protective. Ultimately this question can only be resolved by carefully stratified, placebo controlled, double blind randomised controlled trials.

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Appendix A
01
BAL cDNA quantification data

Group	Patient ID	RT-PCR pellet	cDNA ng/ul	260/280	260/230
CF	555	pellet	1281.5	1.76	1.82
CF	533	pellet	1208.7	1.76	1.79
CF	535	pellet	1113.3	1.77	1.48
CF	537	pellet	1251.5	1.78	1.79
CF	540	pellet	709.4	1.74	1.24
CF	500	pellet	1224.5	1.77	1.64
CF	403	pellet	1230.4	1.78	1.7
CF	454	pellet	1228.7	1.78	1.64
CF	461	pellet	1279.8	1.79	*1.78*
CF	493	pellet	1237.4	1.8	1.82
CF	495	pellet	1295.7	1.79	1.47
CF	504	pellet	1292.3	1.79	1.58
CF	529	pellet	1209.8	1.81	1.76
CF	437	pellet	327.8	1.54	1.7
CF	448	pellet	373.7	1.47	1.55
CF	450	pellet	370.4	1.53	1.61
CF	453	pellet	368.8	1.48	1.74
CF	456	pellet	521	1.46	1.59
CF	458	pellet	521.3	1.66	1.81
CF	472	pellet	290.2	1.49	1.62
CF	476	pellet	435.3	1.49	1.82
CF	478	pellet	316.7	1.51	1.73
CF NBS	550	pellet	1418.9	1.8	2.02
CF NBS	527	pellet	1149.1	1.81	1.14
CF NBS	519	pellet	1301.1	1.8	1.56
CF NBS	509	pellet	1217.8	1.81	1.77
CF NBS	544	pellet	135401	1.81	1.87
CF NBS	541	pellet	451.2	1.8	0.97
CF NBS	556	pellet	1688.3	1.8	1.89
CF NBS	532	pellet	1265.5	1.81	1.61
CF NBS	428	pellet	333	1.5	1.5
CF NBS	436	pellet	957.9	1.43	1.71
CF NBS	459	pellet	362.4	1.51	1.74
CF NBS	470	pellet	329.6	1.49	1.78
CF NBS	477	pellet	293.8	1.5	1.63
CF NBS	479	pellet	274.1	1.5	1.86
CF NBS	490	pellet	309.7	1.5	1.65
Healthy	591	pellet	1440.7	1.8	1.94
Healthy	592	pellet	845.7	1.81	1.63
Healthy	457	pellet	350.7	1.57	1.88
Healthy	464	pellet	271.1	1.48	1.77
Healthy	467	pellet	269.5	1.49	1.82
Bronchiectasis	551	pellet	1293.7	1.81	1.69
Bronchiectasis	536	pellet	1257.2	1.82	1.79
Bronchiectasis	520	pellet	1308.4	1.81	1.81

Bronchiectasis	575	pellet	1279.5	1.81	1.8
Bronchiectasis	405	pellet	1050.8	1.82	1.58
Bronchiectasis	408	pellet	1261.6	1.81	1.81
bronchiectasis	499	pellet	1291.2	1.82	1.82
Bronchiectasis	427	pellet	368.8	1.54	1.76
Bronchiectasis	486	pellet	348.2	1.49	1.72
PCD	439	pellet	317.8	1.48	1.86
PCD	488	pellet	538.1	1.47	1.75
PBB	542	pellet	1243.7	1.76	1.8
PBB	545	pellet	1225.6	1.76	1.89
PBB	402	pellet	1177.9	1.76	1.71
PBB	430	pellet	1295.3	1.76	1.56
PBB	466	pellet	604.1	1.7	1.18
PBB	483	pellet	1230.9	1.76	*1.83*
PBB	432	pellet	923.8	1.74	1.36
PBB	431	pellet	288.5	1.48	1.78
PBB	434	pellet	319.4	1.49	1.75
PBB	440	pellet	279.4	1.5	1.31
PBB	445	pellet	316.5	1.53	1.63
PBB	447	pellet	313	1.53	1.81
PBB	449	pellet	359.7	1.54	1.89

Appendix A
02
EBB cDNA quantification data

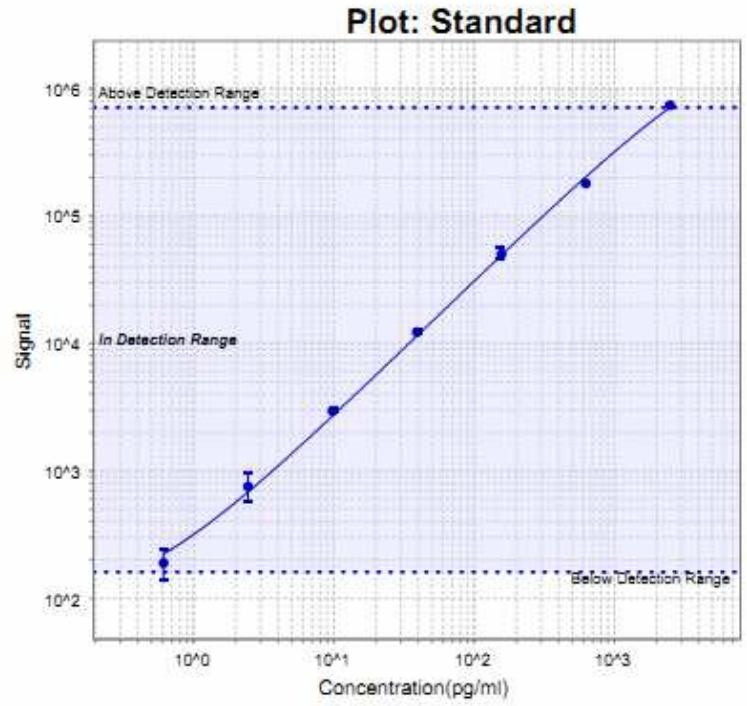
	Patient ID	RT-PCR Biopsy	cDNA ng/Ul	260/280	260/230
CF	555	Biopsy	1340.9	1.79	1.77
CF	533	Biopsy	1908.5	1.77	1.43
CF	535	Biopsy	1909.5	1.77	1.76
CF	537	Biopsy	1813	1.75	1.21
CF	540	Biopsy	1893.5	1.76	1.39
CF	500	Biopsy	2009	1.76	1.69
CF	437	Biopsy	323.02	1.61	1.4
CF	448	Biopsy	417.02	1.57	1.44
CF	450	Biopsy	619.89	1.71	1.3
CF	453	Biopsy	338.42	1.71	1.38
CF	456	Biopsy	406.5	1.69	1.37
CF	458	Biopsy	401.76	1.68	1.45
CF	460	Biopsy	318.67	1.67	1.29
CF	472	Biopsy	477.2	1.69	1.26
CF	476	Biopsy	592.81	1.8	1.43
CF	478	Biopsy	441.98	1.52	1.25
CF	397	Biopsy	2250.4	1.76	1.63
CF	546	Biopsy	1659.5	1.78	1.58
CF	497	Biopsy	1537.6	1.77	1.79
CF	515	Biopsy	2184	1.76	1.73
CF	517	Biopsy	1134.9	1.78	1.74
CF	524	Biopsy	1193.1	1.79	1.57
CF	525	Biopsy	1145	1.79	1.76
CF	573	Biopsy	1564.8	1.76	1.65
CF	583	Biopsy	1296.1	1.79	1.7
CF	475	Biopsy	503.84	1.68	1.29
CF	589	Biopsy	460.2	1.77	1.76
CF NBS	550	Biopsy	1377.2	1.77	1.53
CF NBS	519	Biopsy	1168.1	1.78	1.66
CF NBS	436	Biopsy	421.26	1.68	1.17
CF NBS	479	Biopsy	406.15	1.68	1.35
CF NBS	572	Biopsy	2148.7	1.76	1.77
Healthy	591	Biopsy	819.6	1.79	1.56
Healthy	592	Biopsy	1238.7	1.78	1.56
Healthy	457	Biopsy	611.99	1.79	1.5
Healthy	464	Biopsy	498.57	1.73	1.45
Healthy	523	Biopsy	931.8	1.77	1.54
Healthy	446	Biopsy	443.19	1.69	1.42
Bronchiectasis	551	Biopsy	1175	1.78	1.6
Bronchiectasis	520	Biopsy	2157	1.76	1.75
Bronchiectasis	427	Biopsy	447.54	1.71	1.38
PCD	439	Biopsy	435.87	1.68	1.18
Bronchiectasis	560	Biopsy	1379.7	1.77	1.58
Bronchiectasis	594	Biopsy	1072.9	1.78	1.37

Bronchiectasis	462	Biopsy	440.53	1.72	1.44
PBB	542	Biopsy	2369.7	1.77	1.8
PBB	545	Biopsy	1359	1.77	1.8
PBB	431	Biopsy	463.02	1.7	1.45
PBB	434	Biopsy	407.8	1.69	1.42
PBB	440	Biopsy	420.91	1.68	1.31
PBB	445	Biopsy	392.99	1.65	1.42
PBB	447	Biopsy	389.59	1.69	1.34
PBB	449	Biopsy	380.24	1.62	1.3

Appendix B
01
CXCL8 standard curve

Plate: Plate_*2BC1QA6116X*
Assay: IL-8 (Human)
Group: Standard

Sample ^	Well	Concentration (pg/ml)	Signal	Mean	CV	Calc. Concentration (pg/ml)	Calc. Conc. Mean (pg/ml)	Calc. Conc. CV
S001	A01	2500	748882	742021	1.31	2660	2629	1.67
	A02		735159			2598		
S002	B01	625	183370	180498	2.25	564	555	2.26
	B02		177626			546		
S003	C01	156	54083	50332	10.5	171	159	10.1
	C02		46581			148		
S004	D01	39.1	12503	12283	2.53	42.3	41.6	2.41
	D02		12063			40.9		
S005	E02	9.77	3043	2955	4.21	11	10.6	4.08
	E01		2867			10.3		
S006	F01	2.44	887	755	24.7	3.21	2.71	26.3
	F02		623			2.2		
S007	G02	0.61	151	190	29	0.301	0.468	50.3
	G01		229			0.634		
S008	H02	0	82	93	16.7	0	0.044	141
	H01		104			0.089		



Appendix B
02
IL-4 standard curve

Plate: Plate_*25B8YAQ689X*
Assay: IL-4 (Human)
Group: Standard

Sample	Well	Concentration (pg/ml)	Signal	Mean	CV	Calc. Concentration (pg/ml)	Calc. Conc. Mean (pg/ml)	Calc. Conc. CV
S001	A01	2500	39408	34805	18.7	3240	2890	17.2
	A02		30201			2539		
S002	B02	625	4851	5919	25.5	474	569	23.5
	B01		6986			663		
S003	C02	156	1319	1378	6.01	142	147	5.64
	C01		1436			153		
S004	D01	39.1	356	350	2.63	39.8	39.1	2.67
	D02		343			38.4		
S005	E01	9.77	123	115	10.5	12.2	11.1	13.9
	E02		106			10		
S006	F01	2.44	56	49	21.9	3.26	2.14	74.5
	F02		41			1.01		
S007	G01	0.61	48	50	4.29	2.09	2.31	13.6
	G02		51			2.54		
S008	H01	0	38	31	31.9	0.516	0.258	141
	H02		24			0