INFLAMMATORY PROFILES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND ASTHMA

THESIS SUBMITTED FOR THE DEGREE OF

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BY

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Inflammatory Profiles in Chronic Obstructive Pulmonary Disease and Asthma

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Historically, asthma and chronic obstructive pulmonary disease (COPD) represent polar ends of the spectrum of airways disease defined in part by distinctive profiles of airway inflammation; in practice, overlap can exist between asthma and COPD. This thesis examined the pattern of inflammatory cell infiltration and cytokine expression within the bronchus in COPD and asthma with further study of moderate-severe asthma. In addition using sputum, cytokine expression was further assessed in COPD and asthma and its relation to severity. Based on previous studies, this thesis examined the expression of specifically Interleukin (IL)-13 and Granulocyte Macrophage Colony Stimulating Factor (GMCSF).

We demonstrated mast cell myositis in moderate and severe asthma which reflected increased disease symptoms. Preferential localization of inflammatory cells to airway smooth muscle (ASM) was absent in COPD. CD3+ T-cells infiltration of large airway glands was increased in COPD which may influence mucus hyper-secretion.

We demonstrated IL-13 overexpression within the submucosa in moderate-severe asthma with specific increase in the ASM in severe disease. IL-13 expression was related to eosinophilic inflammation. In sputum, IL-13 protein was increased in mild and severe asthma reflecting IL-13 expression in ASM. There was a general absence of bronchus and sputum IL-13 in COPD.

Sputum GMCSF was increased in moderate-severe asthma and mild-severe COPD. Parallel upregulation of GMCSF and associated receptor (GMCSFr) expression in the submucosa and ASM was present in severe asthma. GMCSF/GMCSFr expression did not exhibit preferential expression in the large airway of COPD.

Our findings suggest inflammatory cell infiltration of the airway structures is present in asthma and COPD which may influence the phenotype. In addition IL-13 is important in severe asthma whilst GMCSF is expressed in asthma and COPD across a range of severity, but to a greater degree in severe asthma.

I. Acknowledgments

I would like to thank all the patient and healthy volunteers who were generous to participate in the studies within this thesis.

I am eternally grateful to Professor Christropher Brightling for my initial appointment and strong guidance through my research which continues to influence my everyday practice. In addition I am grateful to Professor Ian Pavord as co-supervisor and Professors Peter Bradding and Andrew Wardlaw as additional mentors for their perceptive and supportive comments through my research.

Sputum processing and supernatant analysis would not be possible without invaluable and endless technical support from Will Monteiro and Debbie Parker. I am grateful to Vijay Mistry, Camille Doe and Natalie Neale for assistance with the immunohistochemical staining of tissue specimens. Patient characterization was an important aspect of each study in this thesis and I owe a huge debt to the excellent research nursing team of Beverley Hargadon, Sue Mckenna, Hilary Pateman and Maria Shelley for their enthusiasm and diligence. I am also thankful to my surgical colleagues for recruiting and consenting patients pre-operatively prior to lung tissue donation.

I am grateful to colleagues Mike Berry, Dom Shaw and Rosh Siva for their general support over my research period and to all members of ILH, who made my time in Leicester so enjoyable. Finally, special thanks goes to my patient wife Sue and daughter Sophie.

II. Statement of work personally performed

Hypothesis generation and design of each study was designed in conjunction with Prof Brightling based on previous studies looking at large airway mast cell infiltration and IL-13 expression in mild asthma.

Large airway studies

Characterization of subjects participating with COPD large airway studies was performed by myself. I was part of a team that recruited and characterized healthy control subjects and subjects with mild-severe asthma for the asthma biopsy studies. Recruitment and characterization involved consent, sputum induction, methacholine challenge testing and sputum processing. I assisted Professor Brightling with the bronchoscopy procedures in 35% of our study population.

I undertook 50% of immunohistochemical staining of lung tissue for inflammatory cells and cytokine expression. I performed all microscopy and enumeration of inflammatory cells and IL-13 and GMCSF expression within lung tissue. All data analysis was performed by myself.

Sputum studies

As above, I was part of a team that recruited and characterized healthy control subjects, subjects with mild-severe asthma and COPD. Characterization is described as above.

The ELISA process to measure IL-13 and GMCSF was performed by myself with supervision from Debbie Parker, clinical scientist. All data analysis was performed by myself.

III. Publications arising from this thesis

Original papers

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Induced sputum and bronchial mucosal expression of interleukin-13 is not increased in chronic obstructive pulmonary disease. Saha S, Mistry V, Siva R, Parker D, May R, Bradding P, Pavord ID, Brightling CE. Allergy. 2008 Sep;63(9):1239-43.

Increased sputum and bronchial biopsy IL-13 expression in severe asthma. Saha SK, Berry MA, Parker D, Siddiqui S, Morgan A, May R, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. J Allergy Clin Immunol. 2008 Mar;121(3):685-91.

Reviews

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Abstracts

Induced Sputum GM-CSF Concentration Is Raised in Subjects with COPD, S.K. Saha, R. Siva, D. Parker, S. McKenna., B. Hargadon., W. Monteiro, M. Sleeman., C. Drinkwater, A. Wardlaw, I. Pavord,, C.E. Brightling, M., Richmond, American Thoracic Society 2007 A650

Interleukin-13+ Cells Are Present in the Airway Smooth Muscle Bundle of Subjects with Severe Asthma, S.K. Saha, M. Berry, A. Parmaar,, P. Bradding, D.M., A.J. Wardlaw, I.D. Pavord, F., C.E. Brightling, American Thoracic Society 2007 A837

Induced sputum GM-CSF concentration is increased in moderate and severe asthma. S. Saha, D. Parker, M. Berry, W. Monteiro, S Cohen, P. Monk, R Green, A. Wardlaw, I. Pavord and C. Brightling. American Thoracic Society 2006 A70

Interleukin-13+ Cells are increased in the airway smooth muscle bundle of asthmatics but not COPD. S. Saha, N. Neale, M. Berry, P. Monk, P. Bradding, A. Wardlaw, I. Pavord and C. Brightling. American Thoracic Society 2006 A29

Induced sputum GM-CSF concentration is increased in moderate and severe asthma. S.K. Saha, D. Parker, P.D. Monk, E.S. Cohen, M. Berry, W. Monteiro, R.H. Green, A.J. Wardlaw, I.D. Pavord and C.E. Brightling. Supplement Thorax Dec 2005; 60: S30

Increased levels of interleukin-13 concentration in induced sputum of severe asthmatics treated with intramuscular triamcinalone. S. Saha, D. Parker, M. Berry, P. Monk, P. Bradding, R. Green, A. Wardlaw, I. Pavord and C. Brightling. Proceedings of the American Thoracic Society. Vol 2 Abstracts Issue 2005

Induced sputum IL-13, concentration in asthma, COPD & eosinophilic bronchitis – M Berry, D Parker, S K Saha, N Neale, P Bradding, A Wardlaw, I Pavord & C. Brightling.P3642. Eur Resp J Sept 2004

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1. INTRODUCTION

1.1 THE CLINICAL SPECTRUM OF OBSTRUCTIVE AIRWAYS DISEASES

Asthma and COPD are forms of obstructive airway diseases with associated chronic inflammation of the airways. Historically, asthma and COPD represent distinct ends of the spectrum of airways disease separated by multiple features such as smoking exposure, bronchodilator reversibility and airway inflammation with remodeling. In clinical practice, differentiation between asthma and COPD can be difficult (Burge et al. 2003;National Institute for Clinical Excellence 2004). It is likely COPD and asthma share some features of immunobiology. Identification of similar features of airway inflammation may lead to novel therapies to improve patient care in both conditions.

1.1.1 Asthma

The international body known as the Global Initiative for Asthma (GINA) defines asthma:-

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment"(Global Initiative for Asthma 2009)

1.1.2 Epidemiology

It is estimated 300 million people worldwide suffer with asthma. By 2025, an estimated extra 100 million people will be diagnosed with asthma. This increase is expected to be in conjuction with the increase in worldwide globalization (WHO 2009). Within the UK, 5.1 million people suffer with asthma. From this population, Asthma UK report 2.1 million people report symptoms suggestive of severe asthma. More than 1400 people die from asthma annually within the UK. The economic burden to the NHS for people with difficult asthma is £680 million which equates to 3.5 times the cost of an individual with well managed asthma (Asthma UK 2004).

1.1.3 Key inflammatory cells in asthma

The symptoms of breathlessness, wheeze and cough cannot be explained by changes in airway smooth muscle contraction alone. It is likely airway inflammation with key inflammatory cells in co-operation with important airway structures influence the pathophysiological mechanisms that underlie the asthma phenotype.

Historically, mast cells and eosinophils have been associated with allergic asthma. The asthma paradigm changed with recognition of overexpression of TH_2 cytokines by T-lymphocytes (Robinson et al. 1992). Structural cells have also been acknowledged e.g. epithelia, fibroblasts, contributing to the inflammatory milieu that underlies the asthma phenotype. The interaction between inflammatory cells and airway structures will be discussed in section 1.2. Initially, the more pertinent inflammatory cells to the asthma paradigm will be discussed in this section.

Mast cells release a range of autocoid mediators, histamine, prostaglandin D2 and leukotriene (LT) C_4 which can precipitate bronchoconstriction, mucus secretion, and mucosal oedema, all features of asthma. The role of the mast cell fell out of favour, when asthma failed to be significantly controlled by antihistamines. Mast cells also release of other important mediators TH_2 cytokines Interleukin (IL)-4, -5 & -13, which influence Immunoglobulin (Ig) E synthesis and the development of pro-inflammatory cytokines e.g. Transforming Growth Factor(TGF)-β. Major mast cell secretory proteases tryptase, chymase, and carboxy-peptidase can interact with various cell types via protease activated receptors (PARs) and by other processes to influence other cells (Bradding, Walls, & Holgate 2006).

Eosinophils, have long been associated with intrinsic and atopic asthma, with evidence of sputum eosinophilia and increased numbers of eosinophils in blood and bronchial tissue. Eosinophils contain active granules which can be toxic upon release e.g. major basic protein, eosinophilic cationic protein. Airway epithelial damage can be replicated with in vitro release of major basic protein (Frigas, Motojima, & Gleich 1991). Also eosinophils have been shown to produce considerable amounts of leukotrienes (LTC4/D4 and E4) and platelet activating factor (PAF), which are thought to be involved in causing bronchoconstriction. Tissue eosinophilia reflects phenotypic properties with increased responsiveness to corticosteroid therapy (Saha & Brightling 2006). Historically, eosinophils have been considered to be the end effector cells with release of releasing specific eicosanoid lipids, i.e. leukotriene C4, and cationic granule-derived proteins. The eosinophil can be viewed having an immunoregulatory role with influence over T-lymphocytes through the secretion of cytokines e.g. IL-4,-13 and chemokines (Akuthota et al. 2008).

Lymphocytes are present in large numbers in the mucosa of subjects with asthma. Lymphocytes are rich in cytokines influential in the asthma paradigm; TH_2 cytokines IL-4 &

-13 influence B-lymphocyte release of IgE production which can promote atopic asthma. T-lymphocytes can be divided between CD4+ and CD8+ subsets with further subdivision of the former into T-helper type 1 (TH_1) & 2 (TH_2) subsets. Cross-sectional assessment of BAL derived cytokines in mild atopic asthma demonstrates a predominant TH_2 cytokine profile from CD4+ T-lymphocytes (Robinson et al 1992). Secretion of IL-12 from antigen presenting dendritic cells predisposes CD4+ cell into TH_1 lineage (Kuipers et al. 2004). In more severe disease, TH_1 T-cells secreting tumour necrosis factor and interferonγ and CD8+ cells become prominent, though the pathways that lead to such T-cell promotion is unclear (Hamzaoui et al. 2005). The importance of all T-lymphocytes has been demonstrated with the improvement of lung function in severe asthma with infusion of anti-CD3+ (Kon et al. 1998).

Neutrophils have been suggested to play a role in severe asthma. One group found increased neutrophils in BAL, large airway submucosa and transbronchial biopsies in severe asthma (Wenzel et al. 1997) but this has not been confirmed in other bronchoscopic studies of severe asthma (Vrugt et al. 1999). Cytokines associated with neutrophilic inflammation have been elevated in sputum (IL-8 & myeloperoxidase)(Jatakanon et al. 1999) and large airway epithelium (IL-8) (Shannon et al. 2008). The confounding factor in severe asthma is the use of high dose inhaled and oral corticosteroids which may potentially increase neutrophil population.

Therefore, the current evidence does support the hypothesis that airway inflammation in asthma can be driven by TH_2 type cytokines. However, the relationship between airway inflammation and disordered airway physiology in asthma is unclear. Thus, to describe asthma as a TH_2 disease is oversimplistic. The lack of a relationship between airway inflammation and disordered airway physiology is exemplified by the condition eosinophilic bronchitis, which is the focus of the next section in this introduction.

1.1.4 The clinical syndrome and the associated pathophysiology of Asthma

As previously stated, asthma represents a combination of symptoms, disordered airway physiology and underlying airway inflammation. The relationship between symptoms, abnormal airway physiology and airway inflammation is unclear (Wardlaw et al. 2002). There is a clinical need to better define these relationships to improve therapy especially in severe asthma.

Different taxonomies define asthma severity. GINA employs a five step classification based upon maintenance pharmocotherapy with GINA 1 (mild asthma) entailing the use of as required short acting beta-agonists, through to GINA 5 (severe asthma) requiring oral glucorticoid or anti-IgE therapy (see table 1.1) (Global Initiative for Asthma 2009). The American Thoracic Society (ATS) recognizes the impact of severe refractory asthma upon the individual patient, healthcare resources, and the need to recognize these individuals from the general asthma population (table 1.2.; ATS 2000). A multi-faceted approach is taken to defining refractory asthma with major criteria detailing corticosteroid use and a collection of minor criteria including reliever use, exacerbations and lung function (table 1.2). Acknowledgement of both criteria when assessing severe disease is important as use of the GINA system alone does not recognize a proportion of (GINA 4) patients using inhaled corticosteroid therapy (>1260μg beclomethasone or equivalent) will suffer refractory asthma as defined by the ATS.

Cough, sputum production and dyspnoea are common symptoms but the pathophysiological mechanisms that underlie these features in asthma are complex. Cough can result from the

Table 1.1 GINA classification of Asthma by treatment

GINA	1	2	3	4	5
PRN Use	SABA	SABA	SABA	SABA	SABA
		Select one	Select one	Add one or more	Add one or both
	PRN use	Low-dose ICS	Low dose ICS & LABA	Med-High dose ICS & LABA	Oral glucorticoid
		Leukotriene modifier	Med-High ICS	Leukotriene modifier	Anti-IgE
			Low dose ICS & theophylline	Sustained release theophylline	

PRN=as required S(L)ABA=short (long) acting beta agonist ICS=inhaled corticosteroid

Table 1.2 ATS classification of refractory asthma (1 or 2 major criteria and 2 minor criteria)

Major Characteristics

In order to achieve control to a level of mild–moderate persistent asthma:

1. Treatment with continuous or near continuous (>50% of year) oral corticosteroids

2. Requirement for treatment with high-dose inhaled corticosteroids:

Drug	Dose (m*g/d*)
Beclomethasone dipropionate	>1,260
Budesonide	>1,200
Flunisolide	>2,000
Fluticasone	>880
Triamcinolone	>2,000

Minor Characteristics

1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting β-agonist, theophylline, or leukotriene antagonist
2. Asthma symptoms requiring short-acting β-agonist use on a daily or near daily basis
3. Persistent airway obstruction (FEV1<80% predicted; diurnal PEF variability>20%)
4. One or more urgent care visits for asthma per year
5. Three or more oral steroid “bursts” per year
6. Prompt deterioration with<25% reduction in oral or inhaled corticosteroid dose
7. Near fatal asthma event in the past

stimulation of receptors in the proximal airways. Stimulation of such receptors may be due to a combination of air trapping/hyper-inflation activation as a result of blockage of the distal airways. Examination of bronchial tissue in mild asthma indicates preferential localization of mast cells and neutrophils to submucosal glands with increased mucus occupying the luminal airway compared to controls. This relationship is seen again in severe asthma but in addition to increased glandular area which may account for sputum production (Carroll, Mutavdzic, & James 2002b). This process of luminal narrowing may also contribute to overall airflow obstruction contributing to the symptom of dyspnoea. Computer tomography (CT) and morphometric studies of proximal airways suggest increases in the thickness of the airway wall and potentially with disease severity in asthma (Awadh et al. 1998;Aysola et al. 2008;Niimi et al. 2000). Increases in airway thickness in severe asthma can be attributed to increases in submucosal collagen deposition, smooth muscle area and glandular areas (Benayoun et al. 2003).

Airway hyper-responsiveness (AHR) is a significant physiological feature in asthma but attempts to correlate features of airway remodeling have been inconsistent. Increased ASM and reticular basement membrane thickening have been suggested to influence AHR but these specific features have been identified in eosinophilic bronchitis, a condition of chronic cough characterized by an absence of AHR (Siddiqui et al. 2008). The close relationship of mast cells infiltrating the ASM is likely to underlie AHR with two studies demonstrating positive correlation between mast cell infiltration of the ASM and AHR (Brightling et al. 2002; Siddiqui et al 2008).

The action of inflammatory cells influencing adjacent structures through the lung is likely through the action of locally secreted cytokines and chemokines. Identification of the

secretion of important cytokines and their relationship to inflammatory cells and airway structures could lead to better therapies especially in severe asthma.

1.1.5 COPD

The National Institute for Clinical Excellence (NICE) define COPD but omits the role of airway inflammation(National Institute for Clinical Excellence 2004):-

COPD is characterised by airflow obstruction. The airflow obstruction is usually progressive, not fully reversible and does not change markedly over several months. The disease is predominantly caused by smoking.

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) recognizes the persistence of airway inflammation in the definition of COPD (Global Initiative for Chronic Obstructive Lung Disease 2009):-

COPD is characterized by chronic airflow limitation and a range of pathological changes in the lung, some significant extrapulmonary effects and co-morbidities which may contribute to the severity of the disease in individual patients. Thus, COPD should be regarded as a pulmonary disease, but these significant co-morbidities, must be taken into account in a comprehensive diagnostic assessment of severity and in determining appropriate treatment.

Spirometric confirmation is required with post-bronchodilator Forced Expiratory Volume in 1 second (FEV_1)<80% with a ratio of FEV1 to Forced Vital Capacity(FVC) <70%.

1.1.6 Epidemiology

In 2005, 3 million deaths were attributed to COPD, accounting for 5% of deaths globally. Predictions suggest by 2030, COPD will be the 3rd leading cause of mortality worldwide. The World Health Organisation estimates 80 million worldwide suffer moderate to severe COPD (WHO 2009). Across European cities, prevalence of COPD has been measured as 6.2% (Boutin-Forzano et al. 2007).

Historically, COPD has been considered a disease affecting men considerably more than women. However, in 2000 the number of female deaths attributed to COPD was approaching the number of male deaths in USA (Kazerouni et al. 2004). A UK based study of primary care centres, indicated the prevalence of COPD was now comparable between men and women (Smith et al. 2008).

UK based national guidelines suggest consideration of the diagnosis in patient older than 35 years (National Institute for Clinical Excellence 2004). Data taken from the European Community Respiratory Health Survey indicated a prevalence of 2.5% (95% CI 2.2-2.7) in subjects with mild COPD between the ages of 20-44 across Europe (de Marco 2004). Traditionally beliefs expected 15% of smokers to develop smoking. In an older population (>70 years) that continues to smoke, COPD has been identified in 50-60% of smokers (Lundback et al. 2003)

1.1.7 Key inflammatory cells in COPD

Identifying the fundamental processes involved in the inflammatory changes in COPD is difficult. COPD represents an umbrella term for a collection of conditions which historically

represented chronic bronchitis, a clinical description of sputum production over 3 months over 2 consecutive years and emphysema, a pathological description of alveolar destruction. There is no clear consensus as whether airflow obstruction is related to small airway narrowing through inflammation/remodeling or loss of peripheral alveolar architecture. Normal spirometry can be illustrated in patients with emphysema. COPD can represent a combination of changes in the large and small airways and parenchyma.

Smoking has been identified as the main aetiological factor in 95% COPD (National Institute for Clinical Excellence 2004). Historically, epidemiological studies have highlighted pollution as contributing factors in addition to occupation (Viegi et al. 2006).

COPD involves several inflammatory cell types with high incidence of macrophages, neutrophils and CD8+ lymphocytes seen in the lung in COPD. Macrophages are increased in the large and small airways, parenchyma, BAL and sputum of smokers and COPD. Increased numbers of macrophages were seen specifically in severe COPD. The processes behind increased macrophage frequency are unclear; increased recruitment and proliferation of monocytes has been suggested in conjuction with reduced apoptotic mechanisms. Macrophages release inflammatory cytokines in response to cigarette smoke e.g. IL-8, Tumour Necrosis Factor and even more so in COPD. Macrophages can release anti-inflammatory proteins e.g. Transforming Growth Factor-β and tissue inhibitors of matrix metalloproteinases though this capacity is attenuated in COPD (Tetley 2002).

Neutrophil accumulation is noted in COPD within BAL and sputum. In the severe COPD phenotype, increased neutrophil presence was seen in the large airway submucosa. In smokers, neutrophilic inflammation of large airway mucosa, small ASM and sputum relates inversely to airflow obstruction (Baraldo et al. 2004; Di Stefano et al. 1998; Stanescu et al.

1996). Neutrophils store proteins which can promote tissue damage e.g. neutrophil elastase, MMPs and oxygen radicals. Chemotactic factors released from macrophages, epithelial cells and neutrophils e.g. IL-8 promote neutrophil recruitment.

Through the parenchyma and small and large airways, there is increased numbers of T-lymphocytes espceically CD8+ cells. T-lymphocytes are rich in cytokines which can recruit other inflammatory cells and granzymes which can cause tissue destruction. Alveolar destruction and severity of airflow obstruction has been related to the frequency of T-cells (Saetta et al. 1998). B-cells have been identified within small airway follicles (Hogg et al. 2004).

Eosinophils will be discussed in section in section 1.1.8

1.1.8 The clinical syndrome and the associated pathophysiology of COPD

The relationship between clinical features and the pathophysiological changes in COPD is complex. As previously discussed, COPD comprises of chronic bronchitis which presents with cough and sputum but is defined by airflow obstruction which is attributed to small airways occlusion and emphysema.

Sputum production has been related with increased large airway glandular size in chronic bronchitis (Reid 1954), but this has not always been a consistent features (Saetta et al. 1997a) though association of inflammatory cells with submucosal glands has been a more consistent features (Saetta et al 1997a; Zhu et al. 2007). Increased mucus production is seen within glands in chronic bronchitis as reflected by mucin expression which has been related to the secretion of local cytokines (IL-4) from inflammatory cells (Zhu et al 2007). Interestingly,

longitudinal studies have not supported chronic bronchitis to be a consistent predictive feature of progressive airflow obstruction (Vestbo 2002).

Breathlessness in COPD displays correlations with parameters of lung function as measured by Forced Expiratory Volume in 1 second (FEV_1) (Stoller, Ferranti, & Feinstein 1986). Airflow obstruction is primarily associated with small airways disease and emphysema rather than large airway chronic bronchitis. Small airway obstruction is evident with luminal narrowing and small airway inflammation associated with fall in FEV_1 (Cosio et al. 1978; Hogg et al 2004). Increased volume of the small airway wall and inflammatory mucus exudates within the small airway contribute to airflow obstruction. The volume of airway wall occupied by a variety of inflammatory cells also is inversely related to airflow obstruction (Hogg et al 2004).

Emphysema contributes to airflow obstruction via a different meachanism with through a loss of elastic recoil from alveolar destruction. This process is again associated with inflammatory cells with cells particularly macrophages and CD8+ cells being increased within emphysematous lung compared to lung tissue from smokers (Retamales et al. 2001).

COPD especially its severe form, has recognized systemic features with extra-pulmonary organ damage. Lower bone mineral density, cardiovascular disease, cachexia and skeletal muscle abnormalities are found in patients suffering severe disease (Larsson 2007). Whether these features are associated with the ill-health of chronic disease and smoking or related to systemic inflammation has not been fully clarified. A meta-analysis reported elevated serum levels of C-reactive protein (CRP), and Tumour Necrosis Factor (TNF) in COPD, suggesting a concurrent systemic inflammatory response (Gan et al. 2004). CRP a recognized marker of atherosclerosis inflammation, displays an even bigger risk of ischaemic heart disease in

association with airflow obstruction. Interestingly, therapies directed to against TNF have not revealed beneficial effects in moderate-severe COPD (Rennard et al. 2007). Identification of novel therapies directed at alternate cytokines may have beneficial effects in COPD.

1.1.9 History of Asthma and COPD

The emphysema component of COPD has been detailed since the 18th century with realization of hyper-expanded lungs. In the 19th century when smoking was rare Laennec realized with post-mortem examination, emphysema co-existed or was the underlying cause for his patients originally labeled with *nervous* asthma (Petty 2006). At this time, such pathological changes would have attributed to genetic predisposition and environmental triggers. Consequent computer tomography studies have confirmed such observations in asthma (Yilmaz et al. 2006). The chronic bronchitis aspect had been detailed in the early 19th century suggesting chronic bronchitis to be a debilitating disorder. The first descriptions of asthma have been found in Ancient Egypt 3000BC. The term of asthma was used in Ancient Greece circa 400BC to describe the feeling of dyspnoea rather than a description of a disease entity(Cohen 1992). Looking at these definitions, it is feasible patients were likely defined under both terms of asthma and COPD, (chronic bronchitis and emphysema) may have shared similar pathologies. The Dutch hypothesis (1961) suggests there is in fact an overlap between asthma and COPD in terms of symptoms, airway physiology, genetic predisposition and AHR. Part of the hypothesis suggests COPD may be an advanced form of asthma (Bleecker 2004). To date, the Dutch hypothesis has not been disproven so attempting to identify common immunobiology in present day, may add some validity to historic observations.

1.1.10 The overlap between Asthma and COPD

In patients with a significant smoking pack year history, the diagnosis of asthma or COPD can be difficult. Lung function decline is a recognised feature of COPD despite monotherapy inhaled corticosteroid therapy (Burge et al. 2000; Lung Health Study Research Group 2000). Lung function decline is also recognised in asthma (Ulrik & Lange 1994). Interestingly in children, the CAMP study suggested inhaled corticosteroid treatment did not impact upon FEV1 decline in children. In older populations, smoking can be a confounding factor in obstructive airways disease with difficulty in dissecting asthma associated asthma apart from COPD (Castro 2008). Interestingly, smoking cessation reduces neutrophilic inflammation in sputum (Chaudhuri et al. 2006).

Airway hyper-responsiveness is considered a feature of asthma. The Lung Health study, a multi-center trial monitoring subjects with mild COPD, demonstrated airway hyper-responsiveness (defined as <5mg) in 47% and 24% of women and men respectively (Tashkin et al. 1992). A smaller study (n=22) with subjects demonstrating more significant airflow obstruction was able to demonstrate AHR with a dose of methacholine 4.29 +/- 5.49 cumulative units (Ramsdell, Nachtwey, & Moser 1982).

The shared clinical features of asthma and COPD may be related to common features of airway inflammation and remodelling; this will be discussed in the next 2 sections.

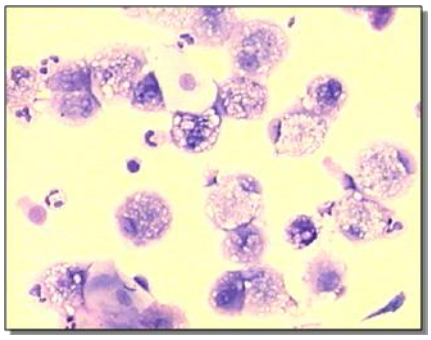
1.1.11 Inflammation in asthma & COPD

As previously stated, airway inflammation and remodeling changes are recognized in airways disease but establishing a clear role within the obstructive airways disease paradigm has been

difficult. Examination of the airways has been extensively examined using non-invasive methods e.g. induced sputum (see figure 3.2), and bronchial tissue obtained from via fibreoptic bronchoscopy and surgical specimens. The identification of separate and similar trends in airway inflammation within disease phenotype can lead to better directed therapy and improve outcomes in both asthma and COPD (Brightling et al. 2005c; Green et al. 2002; Siva et al. 2007).

Airway inflammation within sputum has been used to categorise disease phenotype. Sputum eosinophilic inflammation has been considered the hallmark of asthma but increasing use of sputum induction has led to the recognition of non-eosinophilic inflammation in asthma (figure 1.1). In up to 80% of corticosteroid naïve subjects and up to 50% of corticosteroid treated subjects with asthma, eosinophilia has been detected with converse figures of between 25-50% of corticosteroid naïve subjects displaying an absence of eosinophilia. Patients with sputum eosinophilia display improved responsiveness to corticosteroid therapy (Green et al 2002; Berry et al. 2007). Historically, COPD is associated with neutrophilic inflammation, which has been negatively related with FEV1 and longitudinal FEV1 decline. Less attention has been paid to the presence of eosinophilic airway inflammation in stable COPD although a sputum eosinophilia has been observed in 20-40% of patients with COPD (Saetta et al. 1994; Confalonieri et al. 1998; Pizzichini et al. 1998; Brightling et al. 2000a; Brightling, et al 2005c;). One bronchial biopsy study has reported an increased number of eosinophils in patients with chronic bronchitis and COPD but lower bronchoalveolar lavage (BAL) concentrations of eosinophilic cationic protein (ECP) than in asthmatics suggesting that eosinophils are present but are less activated in COPD (Lacoste et al. 1993). However, sputum ECP concentrations were increased to a greater level than seen with asthma in moderate to severe COPD (Gibson et al. 1998a)

Figure 1.1 Example sputum cytospins illustrating different phenotypes of airway inflammation



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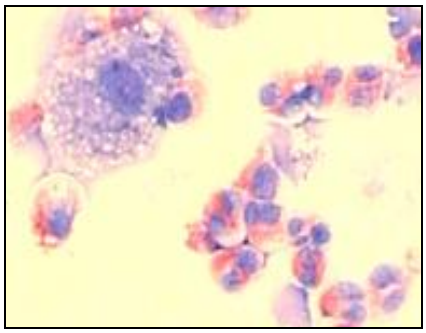
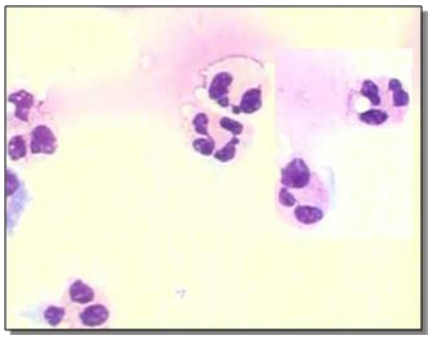
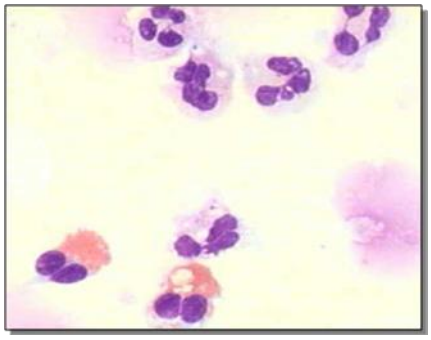


Eosinophilic inflammation



Neutrophilic inflammation



Mixed eosinophilic neutrophilic inflammation

(Brightling et al 2000a; Brightling et al 2005c) suggesting that eosinophils are activated in more severe disease.

The relationship between lung function decline and eosinophilic inflammation is unclear. A negative correlation between FEV_1 and the ratio of activated eosinophils to total eosinophils in endobronchial biopsies from subjects with COPD was demonstrated (Lams et al. 2000) and a similar negative correlation between FEV_1 and sputum eosinophils and ECP was found (Balzano et al. 1999). However, in contrast in another study no relationship between small airway eosinophilia and severity of COPD defined by GOLD criteria (Hogg et al 2004).

The origin of eosinophilic airway inflammation in COPD is unclear although it is widely assumed that it indicates an asthmatic component to the fixed airways obstruction (Barnes 1998). This is unlikely to be the case as most studies on patients with COPD rigorously exclude subjects with variable airflow obstruction and those with clinical features suggesting asthma. It is more likely that smoking and other mechanisms that recruit neutrophils into the airway mucosa in COPD may in turn cause a minor degree of eosinophil influx. However, it is difficult to explain the very high levels of sputum eosinophilia observed in some of our subjects. An alternative and intriguing possibility is that eosinophilic COPD starts as eosinophilic bronchitis. This is a common cause of chronic cough in middle age characterised by a sputum eosinophilia but no symptoms and functional evidence of variable airflow obstruction or airway hyperresponsiveness (Gibson et al. 1989). Although characterised by normal spirometric values at the time of diagnosis this has been associated with an accelerated decline in FEV_1 and the development of COPD (Brightling et al. 1999b; Berry, et al 2007; Birring et al. 2002).

COPD exacerbations are associated with sputum and bronchoscopic bronchial biopsy evidence of eosinophilic inflammation (Lacoste et al 1993; Saetta et al 1994). Bronchial biopsies taken from patients during acute exacerbations and compared with stable COPD show a 30-fold increase in the total number of eosinophils and only a 3-fold increase in neutrophils (Saetta et al 1994). The presence of high concentrations of TNF-α (a pro-inflammatory cytokine which activates adhesion molecules on endothelial cells influencing eosinophil chemotaxis) and the eosinophil products ECP and EPO in induced sputum also supports a role for the eosinophil in COPD exacerbations (Pizzichini et al. 1996; Keatings et al. 1997a; Keatings & Barnes 1997; Gursel et al. 1997).

Traditionally, asthma has been considered to be an eosinophilic condition driven by TH2 cytokines. Sputum neutrophilia has been described as a feature of smoking related airflow obstruction (Stanescu et al 1996). Study of neutrophil sputum markers in asthma and COPD indicate that neutrophil granular protein myeloperoxidase, is present in similar quantities in both conditions (Keatings & Barnes 1997). Subjects with severe asthma as defined as requiring high dose inhaled +/- oral corticosteroids in addition to demonstrating eosinophilic activity, have also shown increased amounts of sputum neutrophils and myeloperoxidase compared to mild asthmatics and healthy controls. The confounding factor of concurrent corticosteroid treatment is has to be appreaciated.

In addition to recognition of an overlap of eosinophilic and neutrophilic phenotypes between asthma and COPD, there is an overlap of cytokine expression between these conditions. TNF-α has been recognised to be part of the severe asthma paradigm (Berry et al. 2006) but this cytokine has also been implicated in the cachexic phenotype of COPD (Di Francia et al. 1994). TH_2 cytokines IL-4 and IL-13 have been recognised to be involved in mucus secretion

in chronic bronchitis and COPD (Miotto et al. 2003; Zhu et al 2007). This will be discussed in greater depth in later sections.

1.1.12 Remodeling in asthma & COPD

Establishing a relationship between morphometric structural changes in the large and small airways and disease phenotype potentially reflects an over simplified approach. Compared to controls, asthma and COPD share similar trends in airway structural changes – goblet cell hyperplasia within thickened epithelia, increased smooth muscle mass, increased vascularity (Hashimoto, Tanaka, & Abe 2005) and mucous gland areas but both COPD and asthma broadly display different phenotypic features. It is likely the phenotypic expression of such structures relates closely to infiltrating and adjacent inflammatory cells and their local cytokine release (Brightling & Pavord 2004). For example, mast cells within the ASM in asthma contain TH2 cytokines, IL-13 and IL-4 but not IL-5 (Brightling et al. 2003c). The potential effect of this has been seen with in vitro assessments of human ASM co-cultured with IL-13, displaying increased contractility with acetylcholine and reduced relaxation with beta-agonists (Grunstein et al. 2002). Potentially neutrophil localization within the small ASM plays a significant role with airflow obstruction of the small airway with the degree of infiltration in controls and COPD, being inversely related to FEV1 (Baraldo et al 2004). Inflammatory cell microlocalisation will be discussed further in section 1.2.

Reticular basement membrane (RBM) thickening another hallmark feature of remodeling, has been extensively assessed in asthma and its relationship to severity and in COPD and eosinophilic bronchitis (EB). EB represents a condition manifesting with chronic cough usually responsive to corticosteroid therapy, with similar immunopathological features to

asthma but an absence of airway hyper-responsiveness and mast cell myositis (Brightling et al 2002; Brightling et al. 2003b). Studies have suggested RBM thickening is related to airway hyper-responsiveness, but this seems unlikely with similar degrees or rbm thickening seen between asthma and EB, which is part defined by absent AHR (Brightling et al 2003b; Siddiqui et al 2008). There are conflicting findings regards RBM thickness with asthma severity and COPD. RBM thickness is increased in both COPD and asthma compared to controls, but recent findings suggest greater thickness is a differentiating feature of severe asthma compared to COPD and mild asthma (Bourdin et al. 2007); this contrasts directly with Pretolani et al (Benayoun et al 2003) who suggested using smaller subject groups, intermittent asthma represented increased RBM thickening compared to severe asthma and COPD, with no difference between the latter two subgroups. Establishing the functional significance of RBM thickening remains unclear. Using RBM measurements from asthma between GINA 1-5, univariate analysis did not identify a correlation with FEV1 (Siddiqui et al 2008). This contrasts with analysis using subjects with mild and severe asthma but interestingly not the full spectrum of disease, by Bourdin et al which demonstrated negative correlation with rbm thickness (R_s=-0.56, $p<0.0001$) (Bourdin et al 2007). RBM thickening has been correlated with log sputum eosinophil percentage (Siddiqui et al 2008), suggesting this feature may be consequent feature of airway inflammation alone.

The inflammatory milieu of extracellular matrix surrounding airway structures in the airways has been studied in asthma and less so in COPD. The ECM is derived of fluids, proteoglycans and glycosaminoglycans containing inflammatory cells. The ECM provides structural strength, osmotic activity, assistance to cellular migration and cytokine/growth factor elaboration. In asthma, increased collagen deposition has been recognized. One study did suggest increased collagen (III) deposition in COPD but only in comparison to subjects with

mild-moderate asthma not healthy controls (Benayoun et al 2003). Increased deposition of tenascin and laminin has been seen in smokers without evidence of airflow obstruction (Amin et al. 2003) suggesting a potential contribution to later COPD.

The role of other structures (vessels, myofibroblasts and nerves) will be discussed in section 1.2.

Despite similar trends in structural changes, asthma and COPD display different phenotypic qualities. It seems likely the abnormal airway structures are influenced by the inflammatory cells and local specific secretory products localized within these structures and this relationship requires detailed evaluation. This will be addressed in the following section 1.2.

1.2 INFLAMMATORY CELL MICROLOCALISATION TO STRUCTURAL COMPONENTS WITHIN THE AIRWAY

1.2.1 Inflammatory cell localisation to airway structures

To date, little attention has been paid to the localisation of inflammatory cells within structural compartments of the airway wall and whether there is preferential distribution of inflammatory cells related to disease phenotype. Communication between cells within the airway predominately occurs across distances of only a few microns, as many inflammatory mediators are rapidly inactivated once they leave the cell. Thus, direct cell–cell interactions are likely to be critical in modulating cellular function. It is therefore likely that microlocalisation between inflammatory and structural cells is a fundamental organising principle of airway inflammation and repair.

Localisation of inflammatory cells within airway submucosa and the epithelium have been extensively examined but have not revealed any convincing linkage between the disorded airway physiology seen in obstructive airways disease. Examination of inflammatory cell infiltration within other structures may explain the clinical features of asthma and COPD and yield better understanding. There is an increasing recognition that inflammatory cells selectively localise to different airway structures in disease (as summarised in table 1.3) and it is biologically plausible that this microlocalisation has important functional consequences. The importance of microlocalisation of inflammatory cells has been demonstrated with studies identifying inflammatory cell infiltration of the ASM. For example, bronchoconstriction is a consequence of ASM contraction. Therefore, the localisation of

Table 1.3 Localisation of inflammatory cells to large airway structures

	Mast cells	T-cells	Neutrophils	Eosinophils	Macrophages
Epithelium					
Asthma	Moderate:+[1],[2-6];-[7-9], [10]#, [11]	High:+ [2];- [3], [9;12], [10]#	Low:-[9]	Moderate:+ [2;4], [12], [10]¶, [11], [13]¶];- [9], [14]	Rare:+ [2];- [9]
COPD	Low + [15], [16]¶;- [14], [17]	High:+ [18]§;- [14], [16]#	High:+ [14], [18]§;- [16]#,[17]	Rare:+ [18]§;- [16]#,[17]	Moderate:+ [18]§, [19];- [16]#
Glands					
Asthma	Moderate:+ [20;21]	Not studied	Low:+ [21]	Not studied	Not studied
COPD	Low:+ [15];- [14], [22]	Low:- [14]	Low:+ [23]	Rare:- [14]	Low:+ [14]
Nerves					
Asthma	Not studied	Not studied	Not studied	Low:+ [24]	Not studied
COPD	Not studied	Not studied	Not studied	Not studied	Not studied
Smooth muscle					
Asthma	Moderate:+ [1;7;25], [26-28], [20];- [29], [30]	Rare:+ [28];- [7], [12]	Not present	Very rare	Not present
COPD	Moderate:- [31;32]	Not studied	Not Studied	Not Studied	Not Studied

+: increased compared with control; -: not increased compared with control.

#: corticosteroids did not significantly reduce intra-epithelial cell numbers compared with placebo;

¶: inhaled/oral corticosteroids reduced intra-epithelial cell count compared with placebo;

§: epithelium and submucosa used as denominator for cell counts;

See p.180 for references

inflammatory cells within the ASM bundle is likely to modulate AHR and variable airflow obstruction. In asthma, certain studies have shown mast cell infiltration of the large ASM to be inversely correlated to AHR (Brightling et al 2002; Siddiqui, et al 2008), while in COPD neutrophil infiltration of small ASM is negatively correlated with measures of airflow obstruction (Baraldo et al 2004).

1.2.2 Inflammatory cell localisation to ASM

In asthma and, according to some reports, in nonasthmatic subjects, the ASM bundle is infiltrated by tryptase staining mast cells (Amin et al. 2005; Ammit et al. 1997; Begueret et al. 2007; Berger et al. 2003a; Brightling et al 2002; Carroll, Mutavdzic, & James 2002a; El Shazly et al. 2006; Koshino et al. 1993). Mast cell–ASM interactions in asthma are summarised in figure 1.2. Mast cell number correlated positively with the degree of AHR (Brightling et al 2002; Siddiqui, et al 2008) and with the bronchoconstrictor response to a deep inspiration (Slats et al. 2007), suggesting that mast cell–ASM cell interactions are likely to be central in the development of the disordered physiology in asthma. This finding was strengthened with an absence of mast cells observed within the ASM bundle of subjects with eosinophilic bronchitis, a condition very similar immunopathologically, characterised by a steroid responsive cough but an absence of airway hyper-responsiveness (Brightling et al 2002). Although the majority of studies have reported an increase in the number of mast cells in the ASM bundle in asthma, some have not been able to confirm this observation (Liesker et al. 2007). However, although Balzar *et al.* (Balzar et al. 2005) did not report a significant increase in the number of mast cells in the ASM bundle in asthma, the numbers were double that observed in normal controls and this almost reached statistical significance ($p = 0.06$).

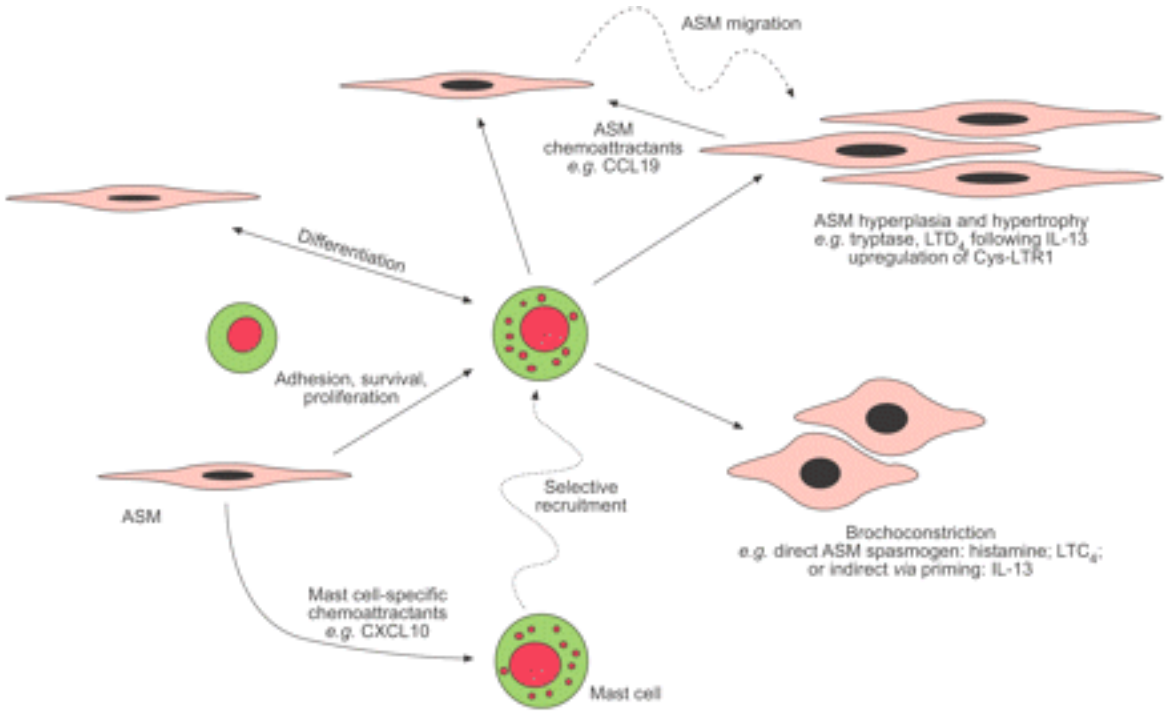


Fig. 1.2 Mast cell interaction with the ASM

Mast cells recruit to the airway smooth muscle (ASM) under the influence of ASM-derived chemoattractants and avidly adhere to ASM. In the ASM bundle there is an appropriate milieu to support mast cell survival and the cells interact resulting in cellular differentiation, ASM hyperplasia, recruitment of ASM progenitors and ASM contraction either directly or indirectly. CCL: CC chemokine ligand; CXCL: CXC chemokine ligand; LT: leukotriene; IL: interleukin.

Potentially the relevance of mast cell infiltration may not be related to the numbers of mast cells infiltrating the unit area of ASM alone, but also the phenotypic expression of mediators is likely to be relevant. In asthma, ASM mast cells expressing TH_2 cytokines IL-4 & -13 but not IL-5 have been identified (Brightling et al 2003c). Release of mast cells mediators from secretory granules has been identified within the airways of fatal and non-fatal asthma (Carroll, Mutavdzic, & James 2002a; Chen et al. 2004). Study of both intact and degranulated mast cells has suggested an influence of ASM shortening in fatal asthma (Chen et al 2004). Interestingly, there was a paucity of other inflammatory cells, with only one study reporting the presence of T-cells in the ASM bundle (Begueret et al 2007), suggesting that predominantly mast cells are selectively recruited to the ASM bundle in asthma.

Inflammatory cell localisation to the ASM bundle is also a phenomenon of COPD. As aforementioned, Baraldo *et al.* found increased numbers of neutrophils and CD8+ cells, but not mast cells, in the small airways of smokers with COPD, and noted that the neutrophil number was inversely related to lung function (Baraldo et al 2004). This is consistent with an earlier study showing that the number of neutrophils in the ASM bundle in small airways in smokers is related to air trapping determined by computed tomography scanning (Berger et al. 2003b). The significance of mast cell infiltration within ASM in the small and large airways is less clear. Bronchioles from subjects with COPD, stained for chymase positive cells reflected a positive correlation between air trapping and infiltration of ASM with mast cells (Berger et al 2003a). Preferential infiltration of the large ASM with mast cells has not been seen in COPD in studies using surgical resection material from subjects with malignancy and concurrent COPD and bronchial biopsies from subjects with COPD alone (GOLD I-II) (Zhu et al 2007; Liesker et al 2007).

Assessment of the infiltration of other inflammatory cells than mast cells has been assessed in asthma using bronchial biopsy studies (Brightling et al 2002). Quantitative examination for other inflammatory cells infiltrating the ASM is lacking. CD4+ & CD8+ T-cells have been demonstrated within large ASM from subjects with COPD but no preferential increase compared to control groups (Zhu et al 2007).

Activation of the inflammatory cells within the ASM bundle would be predicted to have important consequences on ASM function. Mast cell degranulation releases the mediators histamine, prostaglandin (PG)D_2 and leukotriene (LT)C_4 which are all potent agonists for ASM contraction (Brightling et al. 2003a). Mast cell cytokines may further contribute to AHR. As previously mentioned, ASM derived mast cells in asthma express IL-4 & -13. IL-13 has been shown to attenuate relaxation to ß-agonists and augment contractility to acetylcholine with the ASM (Laporte et al. 2001; Grunstein et al 2002). Similarly, the mast cell protease may be important in modulating ASM contractility and AHR (Johnson et al. 1997). The effect of neutrophil-derived mediators on ASM function is less clear, with conflicting reports from animal studies showing that elastase can increase and diminish smooth muscle responsiveness (Christensen et al. 1992; Gray & Mitchell 1996),.

The interactions between inflammatory cells and ASM cells may have more long-term consequences. Mast cells co-cultured with ASM promote ASM differentiation with increased α-smooth muscle actin expression (Woodman et al. 2008). Similarly, mast cell differentiation towards the chymase-positive phenotype observed within the ASM bundle may be mediated by mast cell–ASM interactions. Increased ASM mass is a well-established feature of both asthma (Carroll et al. 1993) and COPD (Saetta et al 1998). A number of mast cell mediators, including histamine (Panettieri et al. 1990), tryptase (Berger et al. 2001) and LTD_4 (Espinosa

et al. 2003), as well as the neutrophil product elastase (Huang et al. 2004), promote ASM proliferation.

Alternatively, increased ASM mass may be a consequence of recruitment of ASM progenitors. This view is supported by the increased number of fibrocytes that migrate into the airway following allergen challenge (Schmidt et al. 2003). Recent evidence suggests that ASM migration towards the ASM bundle is mediated by activation of CC chemokine receptor (CCR)7, ASM- and mast cell-derived CC chemokine ligand (CCL)19 (Kaur et al. 2006b). In asthma, increased ASM mass occurs predominantly in the large airway and in COPD in the small airways. Small ASM mass is inversely associated with post-bronchodilator forced expiratory volume in one second in COPD (Saetta et al 1998). Using a computational model, increased muscle mass has been shown to be probably the most important abnormality responsible for the increased airflow resistance observed in response to bronchoconstricting stimuli in both asthma and COPD (Lambert et al. 1993). Consistent with this view, in a cross-sectional study of the immunopathology of asthma across severity using a multiple regression model, increased ASM mass and ASM hypertrophy were the features of remodelling that were associated most strongly with impairment in lung function (Benayoun et al 2003). The relative contribution of ASM mass to overall airway wall thickness in the small airways is much greater than that in the large airways. Thus, increased ASM mass in the small airways is likely to make a significant contribution to the development of fixed airflow obstruction characteristic of COPD and sometimes seen in persistent chronic severe asthma.

Therefore, mast cell interactions with ASM in asthma are likely to be important in the development of AHR and may play a critical role in the development of increased ASM mass and the development of fixed airflow obstruction seen in severe disease. The role of inflammatory cell–ASM interactions in COPD in the small airway may be related to gas

trapping, but in the large airway the role of ASM–inflammatory cell interactions is uncertain and needs to be further explored.

1.2.3 Inflammatory cell localisation to (myo)fibroblasts

In asthma, communication between the epithelium and myofibrolasts microlocalised within the lamina propria results in the increased deposition of collagen contributing to a thickened reticular basement membrane (Holgate et al. 2000; Holgate et al. 2001). In asthma, increased numbers of subepithelial myofibroblasts have been demonstrated and correlate with the degree of reticular basement membrane thickening (Brewster et al. 1990). This layer of mesenchymal cells is in close proximity to the epithelium and the activation of the airway epithelium, secondary to epithelial damage, is likely to be integral to the activation of the airway mesenchyme. Whether inflammatory cells are co-localised to subepithelial mesenchymal cells is unclear. However, there is evidence that implicates mast cells, eosinophils and T-cells in playing a key role in perpetuating interactions between epithelial and mesenchymal cells. For example, in co-culture with mast cells, primary bronchial fibroblasts from asthmatics increase their expression of pro-collagen 1 expression mediated by mast cell-derived IL-4 (Plante et al. 2006). Cytokines IL-4 and IL-13 activate epithelial cells *in vitro* to produce transforming growth factor (TGF)-ß2, as well as having a direct effect upon myofibroblasts promoting CCL11 release and eosinophil recruitment to the subepithelium (Richter et al. 2001). Submucosal mast cells, eosinophils (Brightling, et al 2003c; Berry et al. 2004) and T-cells (Bentley et al. 1993) are important sources of these cytokines.

In COPD, subepithelial fibrosis is an important component of small airway obliteration. However, the importance of inflammatory cells localised and interacting with mesenchymal cells in this disease is unclear.

1.2.4 Inflammatory cell localisation to mucus glands

Mucus production is a cardinal feature of asthma and COPD and mucus gland hyperplasia is a feature of both conditions (Dunnill, Massarella, & Anderson 1969; Aikawa et al. 1989; Aikawa et al. 1992; Zhu et al 2007). Patients with asthma across the spectrum of severity, including fatal asthma, demonstrate increased numbers of degranulated mast cells (Carroll, Mutavdzic, & James 2002a; Chen, et al 2004) and neutrophils (Carroll, Mutavdzic, & James 2002b) within submucosal glands compared with controls. However, the degree of mucus-related obstruction expressed as the percentage of the airway lumen occupied by mucus only correlated with mast cell numbers and not with neutrophil numbers (Carroll, Mutavdzic, & James 2002b). In COPD, the submucosal glands are infiltrated by neutrophils and macrophages (Saetta et al. 1997b). Mast cell infiltration of mucus glands has been a feature of COPD but this has not been a consistent finding (Pesci et al. 1994; Saetta, et al 1997a; Zhu et al 2007). Eosinophils and T-cells were not increased in number in submucosal glands in asthma or COPD. A recent report has demonstrated that plasma cells are increased in the submucosal glands in patients with COPD and chronic bronchitis without airflow limitation, compared with smoking controls (Zhu et al 2007). Furthermore, there was a close correlation between the number of plasma cells in glands and the number of IL-4 mRNA cells and protein expressing cells within the glands, and an increase in mucus in the glandular epithelium compared with smoking controls. Mast cells were present but not increased in glands and the ASM in COPD, chronic bronchitis and smoking controls in the study (Zhu, et al 2007).

Mast cell proteases, tryptase and chymase are potent stimuli for mucus secretion and other mast cell mediators, PGD_2 and LTC_4 (Jeffery & Zhu 2002), together with mast cell-derived cytokines, IL-6, IL-13 (Cohn et al. 2002) and IL-4 (Dabbagh et al. 1999), have also been implicated in glandular hyperplasia and mucus production. More recently, mast cell

expression of amphiregullin, a member of the epidermal growth factor family, was increased in asthma and this upregulates mucin gene expression by epithelial cells, implicating amphiregullin in goblet cell metaplasia and mucus hypersecretion (Wang et al. 2005), (Okumura et al. 2005). Similarly, neutrophils may promote mucus hypersecretion by upregulation of MUC5AC by neutrophil elastase (Voynow et al. 1999; Shao & Nadel 2005).

1.2.5 Airway epithelium

A variety of inflammatory cells infiltrate the airway epithelium. The interaction between pathogenic and allergic stimuli with inflammatory cells infiltrated within the epithelium can influence the underlying airway structures. In asthma and healthy subjects, T-cells are the most abundant inflammatory cell in the epithelium (Laitinen, Laitinen, & Haahtela 1993; Djukanovic et al. 1997; Brightling et al 2003b; Ying et al. 2005), but increased numbers of T-cells in the epithelium in disease is rarely reported (Laitinen, Laitinen, & Haahtela 1993). Similarly, in COPD, both granulocytes and T-cell numbers have been shown to be increased in the epithelium in some studies (Saetta et al 1997b; Panzner et al. 2003) but not in all (Saetta et al. 1993; Saetta et al 1997b; Hattotuwa et al. 2002). In contrast, eosinophils and dendritic cells are consistently reported to be increased in the epithelium in asthma (Djukanovic et al. 1990; Foresi et al. 1990; Laitinen, Laitinen, & Haahtela 1992; Laitinen, Laitinen, & Haahtela 1993; Brightling et al 2003b). In most reports (Foresi, et al 1990; Gibson et al. 1993; Pesci et al. 1993; Laitinen, Laitinen, & Haahtela 1993; Pesci et al 1994; Amin et al 2005; Shahana et al. 2005), but not all (Djukanovic et al 1990; Djukanovic et al 1997; Saetta et al 1997b; Brightling et al 2003b; Chen et al 2004; Ying et al 2005), mast cell

numbers are increased in the airway epithelium in asthma and COPD. In addition, neutrophil infiltration into the airway epithelium is a feature of COPD (Ying et al 2005).

Mast cells adhere avidly to airway epithelium *via* a carbohydrate-dependent mechanism (Sanmugalingam, Wardlaw, & Bradding 2000) and mast cell survival is promoted by epithelial cell-derived stem cell factor (Columbo et al. 1992). Mast cells activate epithelial cells through release of IL-4, IL-13 and tryptase (Cairns & Walls 1996; Brightling et al 2003c), but in contrast epithelial cells attenuate both constitutive and immunoglobulin (Ig)E-dependent histamine release from human lung mast cells (Yang, Wardlaw, & Bradding 2006).

Eosinophils adhere to epithelial cells *via* a CD18-dependent mechanism closely regulated by the local cytokine milieu (Godding et al. 1995; Sato et al. 1997), and survival is maintained, in part, by release of nerve growth factor and brain-derived growth factor. Adhesion leads to eosinophil activation, release of eosinophil cationic protein (Takafuji et al. 1996) and promotes epithelial apoptosis (Trautmann et al. 2002). T-cells also promote epithelial apoptosis (Trautmann et al 2002), activation *via* release of cytokines and, reciprocally, epithelial cells augment T-cell proliferation and activation (Mattoli et al. 1990). The increase in epithelial apoptosis may contribute to the increased epithelial fragility and shedding often described in asthma (Jeffery 2001), although whether this is a real feature of asthma or a biopsy artefact remains unclear (Ordonez et al. 2000; Fahy 2001).

Survival of dendritic cells in the airway epithelium is maintained in part by granulocyte-macrophage colony-stimulating factor (GM-CSF) released by the epithelium in response to protease-activated receptor-2 activation (Vliagoftis et al. 2001). Retention is augmented by enhanced epithelial intercellular adhesion molecule-1 expression and activation with Th2

polarisation by release of a variety of mediators, in particular PGE_2 and IL-10 (Soumelis et al. 2002) (De Jong et al. 2002). Reciprocally, the expression of CD40L by dendritic cells enhances production of chemokines, pro-inflammatory cytokines and epithelial defensins by the asthmatic epithelium, and represents an important arm of the innate epithelial immune response (Propst et al. 2000).

1.2.6 Airway nerves

To date, interest has focused on eosinophil–airway nerve interactions. Eosinophils have been shown to cluster around cholinergic nerves in patients with fatal asthma (Costello et al. 1997). There are no reports in human disease that describe the presence or absence of other inflammatory cells co-localised to airway nerves, but a guinea pig model of asthma suggests that there may be selective localisation of esoinophils to airway nerves as there was a paucity of other inflammatory cells.

The dominant nervous innervation of the airways is vagally mediated *via* the parasympathetic nervous system (Colebatch & Halmagyi 1963). Release of acetylcholine from these nerves has a variety of consequences regulated by local muscarinic receptors, including bronchoconstriction (Colebatch & Halmagyi 1963), release of mucus from glands (Baker, Peatfield, & Richardson 1985) and vasodilatation (Laitinen, Laitinen, & Widdicombe 1987). Cholinergic-mediated AHR seems to be related to loss of muscarinic 2 receptor (M2R) inhibition rather than increased expression/altered function of M3R on ASM (Whicker, Armour, & Black 1988), (Haddad et al. 1996). The interaction of eosinophils with M2Rs is important to the pathogenesis of neuronal AHR. Eosinophils co-cultured with human Caucasian neruoblastoma cell line cell leads to increased expression of the M2R, which is mediated *via* an adhesion-dependent release of eosinophil proteins, including major basic

protein and nerve growth factor, increasing acetylcholine release and potentiating vagally mediated bronchoconstriction (Durcan et al. 2006).

Eosinophilic bronchitis, cough, variant asthma and idiopathic chronic cough are associated with increased concentration of mast cell products in sputum (Birring et al. 2004;Brightling et al. 2000b). Possibly these mediators may have influence over the nerve endings involved in the development of cough reflex hypersensitivity and cough but to date, mast cell microlocalisation to airway nerves has not been elucidated. A rather similar interaction is thought to be important in the genesis of itch (Yosipovitch, Greaves, & Schmelz 2003; Gibson 2004). In COPD, the potential role of airway nerve–inflammatory cell interactions has not been investigated.

1.2.7 Vasculature

Vascular remodelling occurs in asthma and COPD (Chetta et al. 2003; Chetta et al. 2005; Feltis et al. 2006; Hashimoto, Tanaka, & Abe 2005) and is associated with airflow obstruction (Hashimoto, Tanaka, & Abe 2005). The role of vascular remodelling in the development of AHR in asthma is contentious. Kanazawa *et al.* (Kanazawa, Nomura, & Yoshikawa 2004) demonstrated that vascular epidermal growth factor (VEGF) was increased in the sputum in asthma, but not in nonasthmatic eosinophilic bronchitis, and correlated with increased airway vascular permeability and AHR. However, the duration of disease in the nonasthmatic eosinophilic bronchitis cohort in the study by Kanazawa *et al.* (Kanazawa, Nomura, & Yoshikawa 2004) is unclear and disease duration is critical in assessing the impact of some features of airway remodelling (Siddiqui & Brightling 2006). Interestingly subjects with chronic nonasthmatic eosinophilic bronchitis (with an absence of airway hyper-responsivness) have been demonstrated to show vascular remodelling in a similar degree to patients with

moderate-to-severe persistent asthma and is therefore unlikely to be related to AHR (Siddiqui et al. 2007b). The increased vascularity in asthma was related to disease duration and airflow obstruction, but not AHR.

Very few studies have examined the association of cellular interactions and vascular remodelling in asthma and COPD. Chetta *et al.* (Chetta et al 2003) demonstrated that the number of mast cells in the subepithelium correlates with the number of vessels in asthma; however, a similar correlation was not observed for eosinophilic airway inflammation. Although studies have described an association with sputum VEGF and percentage of eosinophilia in sputum (Asai et al. 2003), a similar relationship *in vivo* has not been described. Despite the lack of data linking airway inflammation and vascular remodelling in asthma, growth factors (VEGF, ß-fibroblast growth factor and angiogenin) co-localise predominantly to eosinophils, macrophages and CD34+ cells *in vivo*, and the expression of these growth factors correlates with the percentage of vascularity of the airway wall (Hoshino, Takahashi, & Aoike 2001) Furthermore, the expression of the main signalling receptor for VEGF (FLT-1) is increased in vessels in asthmatics (Feltis et al 2006). Taken together, these observations suggest that eosinophils, macrophages and mesenchymal cells may be important in regulating vascular remodelling in asthma. However, it remains unclear whether inflammatory cells are selectively co-localised to vessels in asthma or COPD.

1.2.8 Distribution of inflammatory cells to airway structures between small and large airways

Although a large number of studies have examined cellular localisation to the airway wall in large and small airways, few have assessed the distribution of inflammatory cells to specific structures within the airway wall according to airway size.

Mast cell smooth muscle mysotitis has been consistently observed in proximal ASM and has also been reported in small airway wall smooth muscle in asthma (Brightling et al 2002; (Carroll, Mutavdzic, & James 2002a; Berger, et al 2003a; Chen et al 2004; Amin, Janson, Boman, & Venge 2005; El Shazly et al 2006; Begueret, et al 2007). Although mast cell density was increased in membranous compared with cartilaginous airway in the epithelium and ASM in one study (Carroll, Mutavdzic, & James 2002a), mast cell numbers were not increased in COPD compared with controls in the proximal airway smooth muscle (Zhu et al 2007), and conflicting data exist for the distal airways. One study showed increased mast cells in the ASM (Berger et al 2003b) and another reported that numbers were not increased compared with healthy controls (Baraldo et al 2004). Similarly, T-cells, neutrophils and macrophages (but not eosinophils) have been observed in the ASM in large and small airways in COPD, but only small airway CD8+ T-cells and neutrophils have been shown to be increased compared with healthy controls (Baraldo et al 2004).

Further studies are required to establish whether the localisation of inflammatory cells to airway structures is consistent between large and small airways in asthma and COPD.

1.2.9 Mechanisms involved in selective localisation

Inflammatory cells microlocalise to specific compartments within the airway, with an abundance of some and a paucity of other cells within airway structures. Inflammatory cell localisation is a feature of asthma and COPD, but interestingly the pattern of this microlocalisaion is different. This raises the question: what are the mechanisms controlling the selective recruitment of inflammatory cells to structural compartments within the airway?

Granulocyte trafficking has been extensively studied and characterised (Wardlaw 1999). Cytokines have an integral role in the release from haematopoetic tissue, transfer from vasculature and migration across target tissue. For example, the recruitment of eosinophils into the airway is mediated by a multi-step process directed by T Helper cytokines. The first step is increased production and release of eosinophils from the bone marrow under the influence of IL-5 (Dent et al. 1990; Foster et al. 1996) and specific chemoattractants, such as CCL11 (eotaxin) (Sehmi et al. 1992), CCL5 (RANTES), CCL12 (monocyte chemotactic protein-5) and CCL3 (macrophage inhibitory protein-1α) (Gonzalo et al. 1998). Secondary target organ vasculature has increased adhesiveness for eosinophils through the specific effects of locally generated IL-4 and IL-13. These cytokines induce the expression of vascular cell adhesion molecule (VCAM)-1 that binds to eosinophils through the very late activation antigen-4 receptor, which is not expressed by neutrophils, and P-selectin, to which eosinophils bind with greater avidity than neutrophils (Symon et al. 1996; Edwards et al. 2000; Woltmann et al. 2000). The interaction of the eosinophil with these adhesion molecules is mediated by integrins ($\alpha_4ß_1$ and $\alpha_4ß_7$) (Erle et al. 1994; Walsh et al. 1996), which have been shown to bind to VCAM-1 (Bochner et al. 1991). Following adhesion, transmigration across the endothelium is mediated by complement proteins such as C5a (DiScipio et al. 1999). The final phase of trafficking involves chemotaxis, which is mediated by chemokines, such as

CCL11 (Rothenberg et al. 1997), and involves encryption and passage through the matrix network of the airway wall. CCL11 has been shown to bind to CCR3, a high-affinity receptor expressed by eosinophils, and mediates directional migration through tissue (Ying et al. 1999). Survival of eosinophils is augmented by IL-5 (Yamaguchi et al. 1988) and GM-CSF (Kankaanranta et al. 2000). In contrast, the mechanisms involved in the recruitment of inflammatory cells in tissue to structural components is poorly understood.

Selective recruitment of inflammatory cells to the ASM is likely to be mediated by smooth muscle-derived chemoattractants and by the maintenance of the correct microenvironment to maintain cell differentiation and survival. ASM has a significant secretory capacity, so it clearly has the potential to recruit inflammatory cells (Knox et al. 2000). For example, CXC chemokine ligand (CXCL)8 (IL-8) and CXCL10 (interferon-inducible protein-10) released by activated ASM in COPD may mediate neutrophil and CD8+ cell migration into the ASM bundle in the small airway (Oltmanns et al. 2005), (Hardaker et al. 2004). A plethora of chemotactic factors for mast cells are released by ASM, notably stem cell factor (Berger et al 2003a), CCL11 (eotaxin) (Hirst et al. 2002; Brightling et al. 2005b; Sutcliffe et al. 2006), CXCL8 (IL-8) (John et al. 1998; Hirst et al 2002), CX3C chemokine receptor 1 (El Shazly et al 2006) and TGF-ß (Berger et al 2003a). CXC chemokine receptor (CXCR)3 is the most abundantly expressed chemokine receptor on human lung mast cells within the ASM bundle. Human lung mast cell migration is induced by the CXCR3 ligand CXCL10, which is released preferentially from Th1-stimulated asthmatic ASM cells compared with those from healthy controls (Brightling et al. 2005a) and is released in response to Toll-like receptor-3 activation (Morris et al. 2006). Interestingly, Th2-stimulated ASM from asthmatics is chemotactic for mast cells compared with nonasthmatic ASM mediated *via* activation of CCR3 and CXCR1. This is not due to differential expression of chemokines but is likely to be due to the release of

a factor that inhibits mast cell migration released by the nonasthmatic ASM (Sutcliffe et al 2006). It is also important to consider why some inflammatory cells, notably eosinophils and T-cells, are rarely seen in the ASM in spite of appropriate chemotactic signals. Eosinophil paucity in ASM may be explained by selective cleavage of eosinophil chemoattractants CCL5 (RANTES) and CCL11 (eotaxin) by mast cell ß-tryptase (Pang et al. 2006). Similar interactions may explain the lack of other cells, such as T-lymphocytes from ASM bundles, in asthma as Lazaar *et al.* (Lazaar et al. 2002) demonstrated that mast cell chymase inhibited integrin-mediated T-cell adhesion to ASM cells.

Similarly, the airway epithelium has the capacity to secrete a number of chemokines that are involved in the recruitment of inflammatory cells (Holgate et al. 1999; Message & Johnston 2004). The induced sputum CXCL10 concentration from subjects with nonasthmatic eosinophilic bronchitis is increased and mediates mast cell migration *via* CXCR3 activation (Woodman L 2004). Airway epithelium is an important source of CCR3 ligands, which play a role in the recruitment of mast cells, eosinophils and Th2 cells. CXCL8 is an important neutrophil and mast cell chemoattractant, and its expression is increased in induced sputum from subjects with COPD (Keatings et al. 1996; Gompertz et al. 2001) and neutrophilic asthma (Gibson, Simpson, & Saltos 2001), and is increased in response to cigarette smoke (Chalmers et al. 2001). How chemokines interact to recruit inflammatory cells to the airway epithelium is poorly understood and it is unknown why there is less selection in cell recruitment than observed in the ASM.

Chemokine expression by airway mucus glands has not been extensively examined. CXCL8 mRNA expression by mucus glands is increased in response to Pseudomonas infection in bronchiectasis (Inoue et al. 1994). However, to date, there are no data to explain the selective

recruitment of mast cells and neutrophils to the airway mucus glands in asthma, and neutrophils and macrophages in COPD.

Eosinophil localisation to airway nerves is mediated by the release of CCR3 ligands by nerves (Fryer et al. 2006). It would be predicted that airway nerve-derived CCL11 would also recruit Th2 cells and mast cells, but whether eosinophil localisation to the airway nerves in human disease is selective is unclear.

It is likely that a number of chemokines, other chemotaxins and inhibitory factors play a role in the selective recruitment of inflammatory cells into the airway. As previously described, it may be that the release of chemotaxins by ASM and other airway structural cells varies in response to different stimuli, such as cigarette smoke, infection or allergen exposure. Future studies should explore the relative importance of these triggers and the associated network of chemotaxins that are released in promoting selective inflammatory cell microlocalisation to structural compartments of the airway.

Identification of overexpressed cytokines within lung tissue in obstructive airways disease may yield better understanding of the trafficking of inflammatory cells through affected lung tissue which may hopefully lead to novel therapies to improve symptom control and halt disease progression.

1.2.10 Modulation of compartment inflammatory cells within lung tissue

Animal models have provided insight into the mechanisms that underlie inflammatory cell migration into the airway. There has been little focus on the localisation of cells to structural components of the airway and currently there is no established animal model to study mast

cell localisation to the ASM bundle. Indeed, on the contrary, in a rat model of asthma CD4+ cells were located within the ASM bundle and were implicated in driving ASM proliferation (Ramos-Barbon et al. 2005). However, whether T-cell localisation to the ASM bundle is a feature of asthma is contentious, with one report supporting this view but most unable to identify T-cells in the ASM bundle. Therefore, the relevance of current animal models to human disease is questionable.

In vitro two-dimensional migration assays have been informative about inflammatory cell migration towards primary structural cells from asthmatics (Brightling et al 2005a), but further work studying cell migration in three-dimensional culture systems and in *ex vivo* bronchial biopsies using advanced imaging, for example two-photon microscopy (Huang et al. 2007), may improve current understanding of inflammatory cell migration in more biologically relevant models.

Whether inflammatory cell–structural cell interactions drive airway remodelling is unclear. However, advances in design-based stereological methods in the assessment of bronchial biopsies (Ferrando et al. 2003; Woodruff et al. 2004; Hays et al. 2005; Innes et al. 2006) and improvements in imaging techniques (Niimi et al. 2004; Vignola et al. 2004; Martinez et al. 2005; Hasegawa et al. 2006) have provided new objective tools with reduced bias to assess airway remodelling. These approaches will assist in defining the interplay between inflammatory cell localisation to the airway structures, disordered airway physiology and remodelling in obstructive airways disease. It is important that future studies not only use these techniques in cross-sectional studies but are used to study airway remodelling longitudinally and the effects of pharmacological and nonpharmacological interventions.

There are relatively few studies that have examined the inflammatory cell composition of bronchial biopsies in response to anti-inflammatory therapies in asthma and COPD (Djukanovic et al. 1992; Laitinen, Laitinen, & Haahtela 1992; Trigg et al. 1994; Bentley et al. 1996; Djukanovic et al 1997; Faul et al. 1998; Faul et al. 2002; Gizycki et al. 2002; Hattotuwa, et al 2002; O'Sullivan et al. 2002; O'Sullivan et al. 2004; Barnes et al. 2006). To date, none of these have examined the effect of treatment on cellular infiltration into the ASM bundle, mucus gland or localisation to airway nerves. Therefore, it is unknown whether recruitment of inflammatory cells into these compartments is steroid responsive, or indeed responsive to any current therapy, or whether it is refractory to anti-inflammatory therapy.

In asthma, oral and inhaled corticosteroids consistently reduce the number of eosinophils in the airway epithelium (Djukanovic et al 1990; Laitinen, Laitinen, & Haahtela 1992) and some, but not all, report reductions in T-cells and mast cells (Djukanovic et al 1997). A limited number of bronchoscopy studies that have examined the anti-inflammatory effect of corticosteroids in COPD have not identified a consistent anti-inflammatory effect (Hattotuwa et al 2002; Gizycki et al 2002; Barnes et al 2006). For other anti-inflammatory therapy the picture is also unclear and the data sparse. There are no reports on the effects of anti-leukotriene therapy on cellular localisation to airway structures; anti-IgE in asthma led to a reduction in T-cells in the airway epithelium (Djukanovic et al. 2004) but there was no change in other cell numbers and the phosphodiersterase-4 inhibitor cilomilast had no effect on neutrophil numbers in the epithelium in COPD (Gamble et al. 2003). There is therefore a pressing need to understand the effect of current treatment on modulating inflammatory cell localisation to structural cells.

Recruitment of inflammatory cells into the airway is an important target for treatment of asthma and COPD. In addition to treatments with broad anti-inflammatory actions, a number

of antibody and small-molecule therapies have been developed, or are in development, to target specific aspects of cell trafficking (O'Byrne 2006). For example, anti-IL-5 reduces the number of eosinophils in the airway (Flood-Page et al. 2003) but whether this has an impact on eosinophil infiltration into the epithelium is unknown and, more importantly, whether anti-IL-5 has clinical benefits, *e.g.* in reducing asthma exacerbations, is uncertain. In animal models of asthma, CCR3 antagonists reduce the clustering of eosinophils along cholinergic nerves and AHR secondary to M2R dysfunction (Fryer et al 2006). In COPD, strategies to block CXCR2 and CXCR3 are in development and may offer novel approaches to reducing the inflammatory profile in COPD, which is currently not affected by pharmacotherapy. The potential efficacy of such an approach is supported by a recent safety study that used a monoclonal antibody to IL-8 in COPD, which resulted in small improvements in dyspnoea score (Mahler et al. 2004). Alternative strategies that target events in cellular activation involved in migration may offer novel therapies. For example, the mast cell calcium-activated potassium channel modulates the retraction of the cell body during migration. Specific inhibition of this ion channel by TRAM-34 attenuated CXCL10-mediated mast cell migration (Cruse et al. 2006).

Whether inflammatory cell localisation to structural cells can be modulated by current and future treatments needs to be examined further and the functional consequences of this inhibition need to be fully determined.

CONCLUSION

Emerging evidence suggests that inflammatory cell microlocalisation to structural cells has important functional consequences. Understanding the fundamental steps that are involved in

the migration of inflammatory cells towards structural cells, such as the airway smooth muscle bundle and the interactions between these cells, may provide novel targets for the future treatment of asthma and chronic obstructive pulmonary disease.

1.2.11 UNANSWERED QUESTIONS

To date, mast cell localization has been recognized within the ASM bundle in asthma, but whether there is specific preferential localization in asthma of worsening severity remains unknown. Potentially, the mast cell and it secretory products may represent a therapeutic target in severe asthma if a relationship can be established.

To date, inflammatory cell infiltration of the large airway structures (minus the submucosa) in COPD, has been less well characterized. Recognition of patterns of inflammatory cell microlocalisation to large airway structures may explain the various aspects of the COPD phenotype.

1.3 INTERLEUKIN-13 AND THE RELATIONSHIP WITH OBSTRUCTIVE AIRWAYS DISEASE

Interleukin (IL)-13 is a TH2 cytokine first defined in 1993 after extraction from activated peripheral blood lymphocytes, demonstrating inhibition of cytokines usually induced by lipopolysacchiride (LPS) as part of the TH1 pathway (Minty 1993). IL-13 was discovered to possess actions that would direct cells towards the TH2 pathway, with induction of B-cell production of IgE (Punnonen et al. 1993). The gene position for IL-13 has been mapped in close proximity to IL-4 on chromosome 5q 23-31(Minty et al. 1993).

1.3.1 Animal models implicate IL-13 as critical in the pathogenesis of asthma and COPD

A considerable weight of evidence supporting a role for IL-13 in airways disease is derived from animal models. In 1998, Grunig and colleagues first reported that in a murine model of allergic asthma selective neutralization of IL-13 led to reversal of airway hyper-responsiveness and inflammation. In addition they found that administration of IL-13 conferred an asthma-like phenotype to nonimmunized T cell-deficient mice by an IL-4Rα-dependent pathway (Grunig et al. 1998). Similarly, Wills-Karp et al found that the addition of IL-13 to non-immunised mice was sufficient to induce the pathophysiological features of asthma independent of immunoglobulin E and eosinophils (Wills-Karp et al. 1998).

Subsequent murine studies suggested that IL-13 may exacerbate airway responsiveness via direct effects upon epithelial cells (Kuperman et al. 2002) and airway smooth muscle (Tliba et

al. 2003). Mice lacking signal transducer and activator of transcription 6 (STAT6) were protected from all pulmonary effects of IL-13. Reconstitution of STAT6 only in epithelial cells was sufficient for IL-13-induced airway hyperresponsiveness and mucus production in the absence of inflammation, fibrosis or other lung pathology, highlighting the importance of the effects of IL-13 on epithelial cells. IL-13 also exerts direct effects on airway smooth muscle leading to increased force of contraction as a consequence of augmentation of G protein-coupled receptor (GPCR)-associated calcium signaling (Tliba et al 2003).

In addition to asthma, murine models have highlighted a role of IL-13 in COPD. Zheng and colleagues reported that IL-13 over-expression in the adult murine lung induced emphysema, mucus metaplasia, inflammation and fibrosis (Zheng et al. 2000). These effects were mediated by matrix metalloproteinase (MMP) and cathepsin-based proteolytic pathways and were reversed by the addition of MMP or cysteine proteinase antagonists. Potentially IL-13 regulation in emphysema may also be related to upregulation of IL-18. Transgenic IL-18 murine models resulted in increased IL-13 coupled with pulmonary inflammation and structural changes reflective of emphysema (Hoshino et al. 2007).

Recently, murine models have been used to extend previous concepts that chronic lung disease is a result of an innate immune response to low grade infection (Kim et al. 2008). Mice infected with sendai virus demonstrated development of mucus metaplasia and airway hyper-responsiveness mimicking features of asthma and COPD despite clearance of the virus. In the acute phase, CD4+ T-lymphocytes were the predominant source of IL-13 but in the chronic phase, macrophages stimulated by invariant natural killer cells became the most significant source. These findings provide new insights into a novel iNKT-macrophage

mediated IL-13 overproduction, leading to chronic lung disease and expand the possible role of IL-13 in the onset and chronicity of airways disease.

1.3.2 IL-13 Receptors

The receptor structure and downstream signaling of the IL-4/IL-13 receptor system are illustrated in **figure 1.3**. IL-13 exerts its effects predominately via a dimeric receptor comprising of IL-4Rα and IL-13Rα1 (IL-4RII). IL-13 binds IL-13Rα1 with low affinity and then IL-4Rα binds to form a high-affinity cytokine-binding heterodimer. IL-13Rα1 is expressed by airway epithelium, fibroblasts, smooth muscle and most leucocytes including mast cells within the airway except T-lymphocytes (Murata et al. 1998; Laporte et al 2001; Lordan et al. 2002; Wang et al. 2004; Kaur et al. 2006a). Stimulation of this receptor within airway non-immune cells types leads to activation of the STAT-6 and -1 pathways (Wang et al 2004).

IL-13Rα2 binds IL-13 exclusively and with high affinity. This receptor lacks a signaling motif and exists in soluble and membrane bound forms. These characteristics led to the view that coupling to this receptor disallows binding of the IL-13 protein with IL-13Rα1, and therefore IL-13Rα2 acts as a 'decoy' receptor. Recently, the functional purpose of the IL-13Rα2 subunit has gathered much speculation. In vitro studies with human airway fibroblasts suggest that activation of the IL-13Rα2 subunit may attenuate the actions of IL-13 and -4 (Andrews et al. 2006). In support of this view comparison of the effects of lung-targeted transgenic IL-13 in mice with wild-type and nullRα2 loci demonstrate that IL-13Rα2 is a

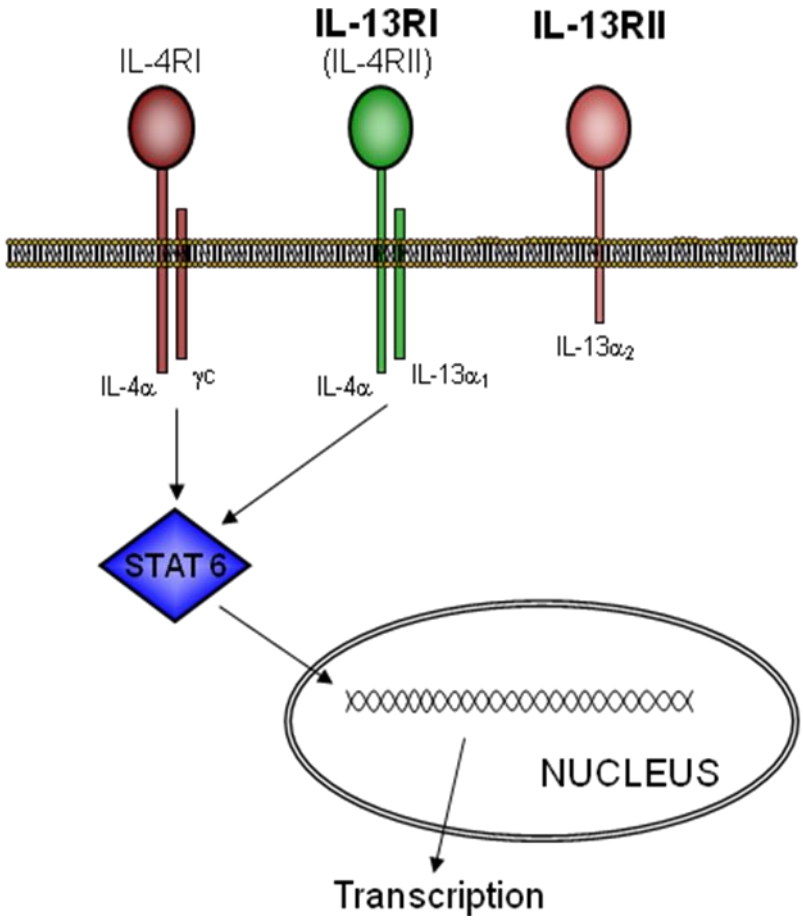


Figure 1.3 Schematic representation of IL-13 receptor

The functional components of the receptor are considered to be the IL-4Rα and the IL-13Rα1 subunits. Stimulation leads to activation of STAT 6 & STAT 1.

The role of IL-13Rα2 remains unclear.

selective and powerful inhibitor of IL-13 induced responses (Zheng et al. 2008). However, in the bleomycin model of lung fibrosis, a controversial role for the IL-13Rα2 subunit was proposed, which suggested that activation of this receptor led to induction of TGF-β and the development of lung fibrosis (Fichtner-Feigl et al. 2006).

1.3.3 Associations between airways disease and genetics variations in the IL-13 gene

A recent report re-examined the published asthma genetic studies (Vercelli 2008), including candidate gene studies and positional cloning, up to the end of December 2007. Two of the four genes with the highest number of positive association reports were IL4R and IL-13. Indeed polymorphisms within the IL-13 gene have been associated with various aspects of the asthma phenotype. Analysis of an adult Dutch population demonstrated the -1111 promoter region is strongly associated with asthma disease, airway hyper-responsiveness and atopy (Howard et al. 2001). In addition, polymorphisms within the IL-13 gene have been identified to predict asthma and higher serum IL-13 levels (van der Pouw Kraan TC et al. 1999) (Heinzmann et al. 2000). Recombinant IL-13 protein of one of these variants (Glutamine substitution for Arginine at position 110 on the mature protein-Arg110Gln) has demonstrated greater biological activity implying genetic variations in the IL-13 gene influence the asthma phenotype (Heinzmann et al 2000). The IL-13 gene locus is associated with atopy and allergy in the broader sense with 4 single nucleotide polymorphisms (SNPs) associated with a variant in the IL-13 gene (Arg130Gln polymorphism) resulting in elevated IgE in 3 separate populations of children (Graves et al. 2000).

In COPD compared with smokers and never smokers there was an association with the changes of cytosine to thymine at -1055 within a promoter region associated with IL-13 (van

der Pouw Kraan TC et al. 2002). Smoking has an influence upon specific SNPs with subjects with extensive smoking exposure possessing the C1112T allele developing worsening airflow obstruction (Sadeghnejad et al. 2007). Therefore, in COPD IL-13 polymorphisms have been associated with disease although the strength of this association is not as compelling as for asthma.

1.3.4 IL-13 expression within the lung

1.3.4.1 IL-13 expression in Bronchoalveolar Lavage Fluid (BAL)

Overexpression of IL-13 within the lung was first described in BAL from atopic asthmatic subjects undergoing bronchial segmental ragweed allergen challenge yielding elevated il-13 protein and mRNA primarily from mononuclear cells, in contrast to normal controls (Huang 1995). Further studies demonstrated elevated IL-13 specifically in the late phase (18 hours post allergen challenge) with positive correlation (rather than IL-4) with eosinophil numbers (Kroegel et al. 1996).

T-cells isolated from BAL specimens from both subjects with asthma and COPD have demonstrated IL-13.In asthma, IL-13 mRNA when assessed by RT-PCR was present in measurable quantities, with further upregulation with phytohaemagglutinin (PHA) stimulation (Bodey et al. 1999). In COPD when assessed using flow cytometry, a higher percentage of cells expressed IL-13 compared to smoker and never smoker controls. Statistically significant negative associations were seen with IL-13 expression and lung function (measured by FEV1%) in both CD4+ and CD8+ populations (Barcelo et al. 2006).

In addition, BAL derived macrophages are potentially rich source of IL-13 with significant amounts of IL-13 mRNA found within alveolar macrophages. Similar to previous findings

(Kroegel et al 1996), mRNA from BAL positively corrleated with BAL eosinophils (Prieto et al. 2000) implying cross-sectional evidence that IL-13 is chemotactic for eosinophil trafficking. In addition, IL-13 from alveolar macrophages in severe asthma may have a significant role with increased numbers of such macrophages containing IL-13 in severe asthma compared to healthy controls (Kim et al 2008).

The mechanism behind nocturnal asthma potentially may be attributable to IL-13 overexpression. In subjects with nocturnal asthma, the number of BAL cells expressing IL-13 mRNA was increased in with little attenuation under the influence of dexamethasone. BAL derived alveolar macrophages from these subjects have demonstrated overexpression of glucocorticoid receptor (GR)-β (a receptor complex that competes with the active GR-α receptor) but reduced expression when treated with IL-13 neutralising antibodies (Kraft et al. 2001a) which may explain the reduced glucorticoid receptor affinity seen when IL-13 is incubated with peripheral blood monocytes (Spahn et al. 1996).

In COPD, IL-13 protein quantification within BAL has not been determined but within BAL from asymptomatic smokers, IL-13 mRNA expression was lower compared to healthy never smokers ((Meuronen et al. 2008).

1.3.4.2 IL-13 expression in sputum

IL-13 protein as measured by ELISA was elevated in the sputum of steroid naïve asthmatics in a cross-sectional study when compared against healthy controls and subjects diagnosed with EB (Berry 2004). This observation was confirmed again in a later study with direct comparison of sputum IL-13 by ELISA from subjects with asthma and EB (Park et al. 2005).

IL-13 expression as assessed by mRNA has suggested overexpression in asthma is independent of disease severity. No difference in IL-13 mRNA expression was detected between disease severity subgroups though overexpression was demonstrated when all subjects with asthma were compared against controls. Similar to findings in BAL, IL-13 mRNA expression positively correlated with sputum eosinophilia (Truyen et al. 2006).

T-lymphocytes separated from induced sputum from asthmatics subjects, have exhibited a greater increase in intracellular Il-13 after stimulation with phorbol myristate acetate (PMA) and ionomycin compared to controls when measured by flow cytometry. Despite lymphocytes representing a substantial source of IL-13 mRNA (Minty et al 1993), there has been little correlation between sputum IL-13 and T-lymphocyte expression of IL-13. This may relate to the poor yield of lymphocytes within induced sputum samples.

Currently, there are no published studies detailing IL-13 expression in sputum from subjects with COPD. In addition virtually all published studies defining IL-13 expression in sputum have been cross-sectional in design. A single study has assessed IL-13 expression (in conjunction with other cytokines) post oral corticosteroid use for 2 weeks in a small group of corticosteroid naïve subjects (n=9) but IL-13 was below the limit of detection of the ELISA assay when measured pre and post steroid use (Scheicher et al. 2007).

1.3.4.3 IL-13 expression within the large airway

The initial studies which assessed IL-13 expression within bronchus, studied il-13 mRNA expression using radiolabelled in-situ hybridization. Increased IL-13 mRNA expression was first described within the submucosa in bronchial biopsies from a small population (n=9) of stable asthmatics compared to healthy control. IL-4 mRNA expression was also assessed and

the number of IL-13+ mRNA cells was significantly higher compared to IL-4+ mRNA cells. All cells that expressed IL-4+ mRNA, co-expressed IL-13 mRNA concurrently. In contrast only 60% of IL-13+ mRNA cells co-expressed IL-4+ mRNA. 90% of the cells expressing IL-13+ mRNA were characterized as CD3 T-lymphocytes (Kotsimbos, Ernst, & Hamid 1996).

One of the few studies to assess IL-13 in moderate-severe asthma (defined by progressive airflow obstruction and corticosteroid use) and the functional response to corticosteroid treatment, demonstrated increased numbers of IL-13+ mRNA cells compared to controls with a downregulation in subjects (n=6) after 7 days of treatment with oral prednisolone. Subjects with asthma who were predefined as being steroid resistant (n=5) did not demonstrate any change in IL-13+ mRNA expression (Naseer et al. 1997). This sample size was small but does suggest the actions of IL-13 are complex in the human airway asthma paradigm.

IL-13 expression within the airway, surprisingly is not related to atopy despite being a TH2 cytokine. IL-13 (mRNA) expression within the large airway has been assessed by RT-PCR from bronchial biopsies with statistically equivalent amounts measured regardless of atopy status (Humbert et al. 1997a). There was an indication (though not statistically significant) that IL-13 mRNA expression within atopic & non-atopic subjects with asthma, correlated with EG(2)+ eosinophils enumerated within bronchial biopsies as measured by immunohistochemistry, lending favour that IL-13 expression is linked with eosinophilia not only in BAL but the bronchial compartment as well.

IL-13 protein expression has been quantified using immunohistochemistry in large airway biopsy specimens from subjects with corticosteroid naïve asthma, EB and healthy controls. Increased inflammatory cells expressing IL-13 were found within the submucosa of the asthma group in comparison to the controls and the pro-TH2 cytokine condition EB. Over

80% of cells expressing IL-13 were eosinophils with 8% of cells being identified as mast cells (Berry et al 2004).

Mast cells are the only inflammatory cell to date to be localized within ASM in asthma and immunohistochemical techniques have shown IL-13 expression within the ASM was co-localised to mast cells (Brightling et al 2003c). In vitro action of IL-13 has demonstrated augmentation of contractility of airway smooth muscle (ASM) (Laporte et al 2001). This would suggest local secretion of this cytokine may contribute to the disordered airway physiological picture seen in asthma. Mast cells from lung tissue have been demonstrated to be a prime source of IL-13 with activated lung mast cells containing IL-13 protein comparable to amounts seen in lymphocytes (Jaffe et al. 1996).

Within the large airway, IL-13 has been suggested to have a role in patients suffering chronic bronchitis. In a cross-sectional study comparing chronic bronchitis sufferers with asymptomatic smokers, bronchial specimens taken post surgery were stained with polyclonal antibodies for IL-13 and IL-4. Of this target population of chronic bronchitis, 10/21 subjects with chronic bronchitis were diagnosed with COPD based on spirometry. Chronic bronchitis is a condition defined by sputum production. Increased expression of IL-13 and IL-4 was not detected within the epithelium or mucus glands but was seen over-expressed within a fixed distance beneath the epithelium (100μm) (Miotto et al 2003). Whether this finding of increased submucosal expression of IL-13 is a feature of COPD is unclear. Also with cytokine action only acting across short distances, the absence of IL-13 expression within glandular structures in a condition whose phenotype is defined by mucus production does not seem clear.

It remains unclear if there is overexpression of IL-13 protein in asthma and in COPD of increasing severity within the large airways. Appropriately designed studies examining the response of IL-13 with anti-inflammatory therapies are currently absent.

1.3.5 IL-13 expression within peripheral lung tissue

Examination of lung IL-13 expression in COPD has been directed towards mild disease and the more easily accessible proximal airways. Sampling peripheral airways and lung parenchyma with flexible bronchoscopy is potentially hazardous in subjects with COPD especially in sufferers with more severe disease. Consequently, specimens from surgery need to be studied. In parallel with murine models, over-expression of IL-13 might be expected in patients with emphysema. In contrast to this concept, one study demonstrated IL-13 as measured by mRNA and protein seemed specifically lower in tissue taken from patients with severe emphysema when compared to control groups (Boutten et al. 2004). This finding was later contradicted when studying IL-13 mRNA levels in tissue taken from patients with severe COPD undergoing lung transplant. Over expression of IL-13 was in parallel with MUC5AC mRNA expression, a marker of mucus cell metaplasia. IL-13 was consequently sourced to alveolar macrophages using immunofluorescent techniques. It has been suggested IL-13 laden alveolar macrophages are influenced by natutral killer T-cells also found in higher numbers in COPD peripheral tissue through an aberrant innate immunity response to previous viral infection as demonstrated in murine models (Kim et al 2008).

Currently there are no studies examining IL-13 expression in peripheral lung in asthma.

1.3.3 IL-13 expression within blood

Serum IL-13 protein assessed by ELISA was increased in asthma across a range of severity compared to healthy controls in a Chinese population (Wong et al. 2001). When this was further assessed between subgroups of asthma severity, no difference was found in IL-13 expression (Silvestri et al. 2006a) which reflects findings in sputum to date. In contrast, stable subjects with asthma showed further upregulation of serum IL-13 during exacerbations (Lee 2001). In COPD, serum IL-13 was not found to be raised in GOLD I-IV and smoker controls (Imaoka et al. 2008). This finding contrasts indirectly with measurement of IL-13 using multiarray platform assays in COPD subjects which identified an inverse relationship with airflow obstruction (as defined by FEV1%) and the lung gas transfer factor (as defined by %DLCO) (Lee et al. 2007).

Examination of the airways and lung parenchyma suggest eosinophil accumulation is intrinsic with IL-13 expression. This similar relationship is observed within the peripheral blood compartment. Peripheral blood eosinophils purified from eosinophilic disorders including eosinophilic oesophagitis and atopic eczema have demonstrated overexpression of IL-13, in addition to asthma. This finding is replicated when peripheral blood eosinophils from healthy normal controls are stimulated with GM-CSF and IL-5 (Schmid-Grendelmeier et al. 2002). In addition, peripheral blood eosinophils exhibit prolonged survival and activation (as determined by CD69 expression) under the influence of IL-13 (Luttmann et al. 1996; Luttmann et al. 1999).

Trafficking of eosinophils from the blood compartment to target tissue is mediated in part by IL-13. Increased adhesion of eosinophils to endothelium is recognized under the influence of IL-13 through the upregulation of p-selectin, suggesting IL-13 is implicated in the first stages

of transmigration of peripheral blood eosinophils to tissue which exhibit overexpression of IL-13 (Woltmann et al 2000).

Peripheral blood derived basophils can release IL-13 upon stimulation in particular by IgE receptor cross-linking (Ochensberger et al. 1996) (Gibbs et al. 1996). Peripheral blood neutrophils have not been able to show release of IL-13 protein (Schmid-Grendelmeier et al 2002). Peripheral blood T cells from atopic individuals upon stimulation with grass pollen and house dust mite showed elevated IL-5 & -13 production compared with non-atopic controls (Till et al. 1997; Hashimoto et al. 2004). Even with resolution of asthma symptoms, patients can retain a predisposition to expressing IL-13 cytokines by peripheral blood mononuclear cells (PBMC) (Smart, Tang, & Kemp 2002). In childhood, examination of PBMCs demonstrated IL-13 secretion is correlated with serum IgE.

1.3.6 IL-13 and Epithelium

Murine models have suggested the significance of the airway epithelial interface influencing the underlying structures to modifying the asthma phenotype. IL-13 action upon human epithelial cells lines can increase epithelial permeability as measured by mannitol influx and down regulate proteins associated with maintaining tight junction within these barriers (Ahdieh, Vandenbos, & Youakim 2001). The epithelial airway interface is normally absorptive in design but treatment with IL-13 yields a hypersecretory phase (as measured by the basal short circuit current and increased Ca2+ dependent anion conductance) which may contribute to increased airway inflammation (Danahay et al. 2002).

Airway epithelium can be a potent source of cytokines with IL-13 & IL-4 inducing the release of cytokines to influence the submucosal matrix and beyond. IL-13 can promote release of transforming growth factor beta (TGF-β) 2 from epithelial cells which in turn influences myofibroblasts into releasing cytokines, chemokines and actin smooth muscle (Holgate et al 2001). This specific paradigm is important as fibroblasts do not appear to secrete any TGF-β isoform post challenge with IL-13 or IL-4, highlighting the importance of IL-13 influence over airway epithelium. Both IL-13 & IL-4 induce granulocyte macrophage colony stimulating factor (GMCSF) and IL-8 release from ex-vivo epithelial cell lines, with greater potency displayed by IL-4. Concurrent addition of a common aeroallergen (Dermatophagoides pteronyssinus) did not foil this model with continued IL-8 and GMCSF secretion equal and if not greater to when the epithelial cell line was challenged by either IL-4 or IL-13 (Lordan et al 2002).

The role of epithelium within the asthma paradigm is multifaceted. Mucus hypersecretion is a recognized feature of asthma and as previously mentioned, transgenic IL-13 murine models have replicated this. The mechanisms behind this are multiple but IL-13 does have some role at the level of the epithelium, with microarray analysis indicating down-regulation of FOXA2, a known inhibitor of mucus production (Zhen et al. 2007).

1.3.7 IL-13 action upon fibroblasts

IL-13 and –4 exhibit substantial influence over fibroblast phenotype and function. Transformation of fibroblast to myofibroblasts can be induced as determined by over-expression of actin (Hashimoto et al. 2001). This process can be augmented with the synergistic effect of TGF-β with consequent additional expression of eotaxin (Richter et al

2001). Proliferation of fibroblasts appears to be a finding related to mild asthma compared to severe asthma (Kraft et al. 2001b). IL-13 action upon human lung fibroblasts alone can still promote a significant array of cytokines secretion e.g. beta 1 integrin, vascular adhesion molecule 1, monocyte chemoattractant protein 1and IL-6 which participate in airway inflammation and inflammatory cell recruitment (Doucet et al. 1998).

1.3.8 IL-13 action upon ASM

Human ASM demonstrates receptors for IL-13 & IL-4 (Laporte et al 2001; Kaur et al 2006b). IL-13 appears to have a dominant effect over IL-4 with consequent stimulation of such receptors leading to attenuation of the effects of beta agonists on ASM stiffness (Laporte et al 2001). ASM can be a potent source of cytokines; stimulation with IL-13 & IL-4 can lead to release of eotaxin which concurrently can be augmented by tumour necrosis factor (TNF) (Laporte et al 2001)and IL-9 (Baraldo et al. 2003). IL-13 induced release of eotaxin from ASM is increased with ASM taken from subjects with asthma compared to healthy controls.

IL-13 may be integral to the priming of ASM with microarray techniques demonstrating the IL-13 gene and its variant IL-13R130Q (associated with asthma) modulate genes of proteins associated with airway inflammation and remodeling – VCAM-1and Tenascin C (Syed et al. 2005).

Stimulation of ASM with IL-13 and other TH2 cytokines, mediates mast cell migration towards ASM. This is potentially a powerful mechanism with mast cell infiltration of the ASM being a key differentiating feature compared to healthy controls and EB, a fellow pro-TH2 condition.

1.3.9 In summary

IL-13 is a pleiotropic cytokine stored within a range of inflammatory cells including T-lymphocytes, eosinophils, mast cells and macrophages (figure 1.4). Upregulation is seen in allergen challenge studies. IL-13 expression is increased in sputum and the submucosa of the large airway in mild asthma. In addition, the ASM bundle is infiltrated with mast cells expressing IL-13. Studies with small numbers of subjects, have suggested IL-13 expression is persistent in severe steroid resistant asthma.

Murine models have suggested IL-13 is involved in key features of both asthma and emphysema. In humans, there is an absence of clarity with respect to IL-13 expression in COPD.

1.3.10 Unanswered questions

IL-13 has been assessed in the large airway in detail in mild steroid naïve asthma. In progressive disease, expression in the presence of steroids is unclear. In addition, IL-13 expression within the ASM in more severe asthma is unclear.

To date, IL-13 expression has been assessed in peripheral airways in COPD. Il-13 expression has been assessed in the large airway of subjects with chronic bronchitis with a varied range of airflow obstruction; this study was specifically flawed due to the absence of the use of appropriate positive controls. Il-13 expression in sputum and the large airway in a COPD population has yet to be definitively assessed.

In this thesis, I will assess the expression of IL-13 in sputum and large airway including the ASM bundle in moderate and severe asthma as well as COPD.

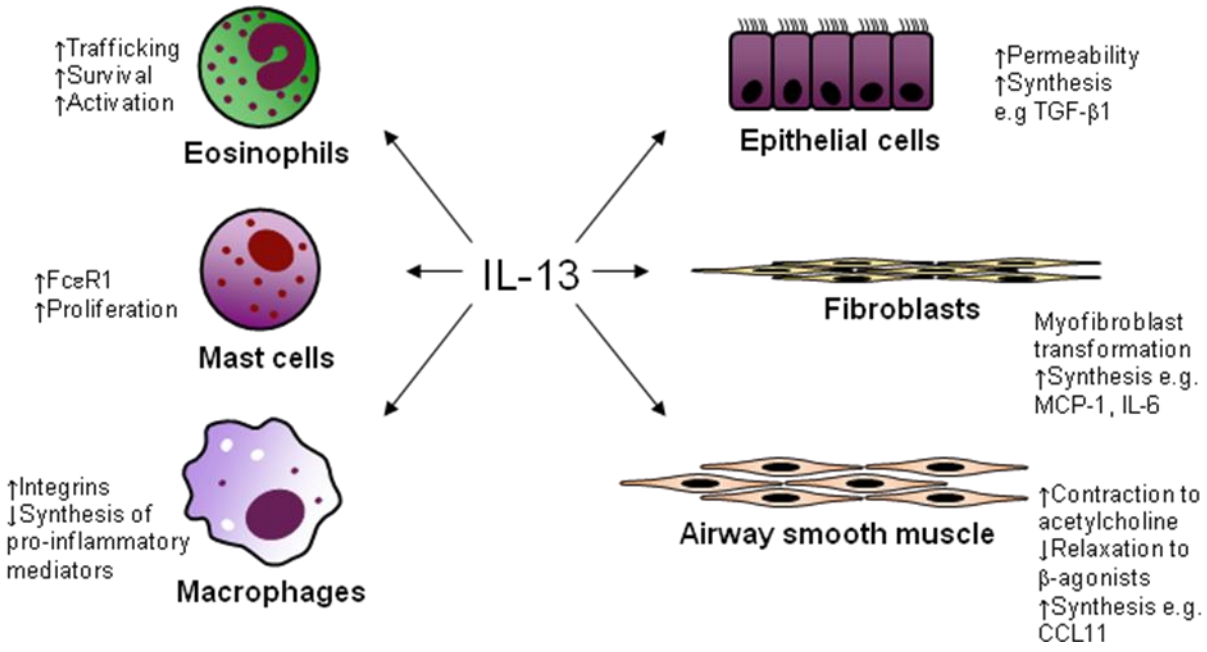


Figure 1.4 Actions of IL-13 upon inflammatory cells and large airway structures

1.4 Granulocyte Macrophage Colony Stimulating Factor (GMCSF) and the relationship with obstructive airways disease

Human granulocyte-macrophage colony stimulating factor is a 22kD glycoprotein that stimulates the growth of myeloid progenitor cells with a direct influence upon eosinophils, macrophages and neutrophils. Recombinant GM-CSF promotes eosinophil and neutrophil maturation and survival (Lopez et al. 1986). The GM-CSF gene is localized to the human chromosome region 5q21-5q32. Interstitial deletions are associated with acute myelogenous leukaemia (Huebner et al. 1985).

The influence of GMCSF over leucocytes has implications for both the asthma and COPD phenotype with respect to the action of the various leucocytes within the various compartments of the lung. In this introduction, the sequelae of GMCSF expression within lung and blood will be reviewed.

1.4.1 GMCSF role as examined by animal models

Animal models have demonstrated the importance of GMCSF in other mammalian systems. Transfection of rat lung with a murine GMCSF gene resulted in accumulation of eosinophils, macrophages and the development of fibrosis (Xing et al. 1996). Transgenic expression of GMCSF within airway epithelia in later murine models revealed increased airway eosinophilia with increases in IL-4 and -5 expression, though airway hyper-responsiveness was not specifically assessed (Lei et al. 1998). Specific neutralization of GMCSF in allergen challenged A/J mice considerably attenuated airway inflammation and airway hyper-responsivness (Yamashita et al. 2002). This model contrasts with a recent murine GMCSF

knockout model which displayed reduction in peribronchial eosinophilia in contrast to perivascular regions but persistent of airway hyper-responsivness. The absence of peribronchial eosinophilia but perivascular eosinophilia suggests an inability for eosinophils to migrate successfully to the target tissue (Su et al. 2008). Potentially murine models may demonstrate a dynamic role for GMCSF in COPD. LPS challenged mice can yield a COPD type phenotype. Administration of an anti-GMCSF antibody reduced lung neutrophil numbers and BAL levels of TNF-α and macrophage inflammatory protein-2 (Puljic et al. 2007).

1.4.2 GMCSF Receptors

The human GMCSF receptor (GMCSFr) is present on various cells including eosinophils, neutrophils and monocytes (Lopez et al. 1991) (Miyajima et al. 1993; Wognum et al. 1994). The receptor is a heterodimer constructed in part by α and β polypeptide chains, which both belong to the cytokine receptor superfamily (Bazan 1990, Ronco 1995). The α chain is specific for binding with GMCSF while the β chain is shared with receptors for IL-3 and -5 (Tavernier et al. 1991; Miyajima et al 1993; Okuda, Foster, & Griffin 1999) (figure 1.5).

In asthma, there is upregulation of GMCSFr mRNA within the large airway in nonatopic asthma which reflects preferential GMCSF expression in non-atopic asthma. Using in-situ hybridization, 80% of cells expressing GMCSFr mRNA were macrophages. Expression of GMCSFr mRNA reflects severity of airflow obstruction (Kotsimbos et al. 1997).

Prolonged activity after stimulation of the GMCSFr in eosinophils, occurs through interaction of intracellular adhesion molecule-1 (ICAM-1) and the β subunit of GMCSFr. Inhibition of this process attenuates the release of inflammatory cytokines (Pazdrak et al. 2008).

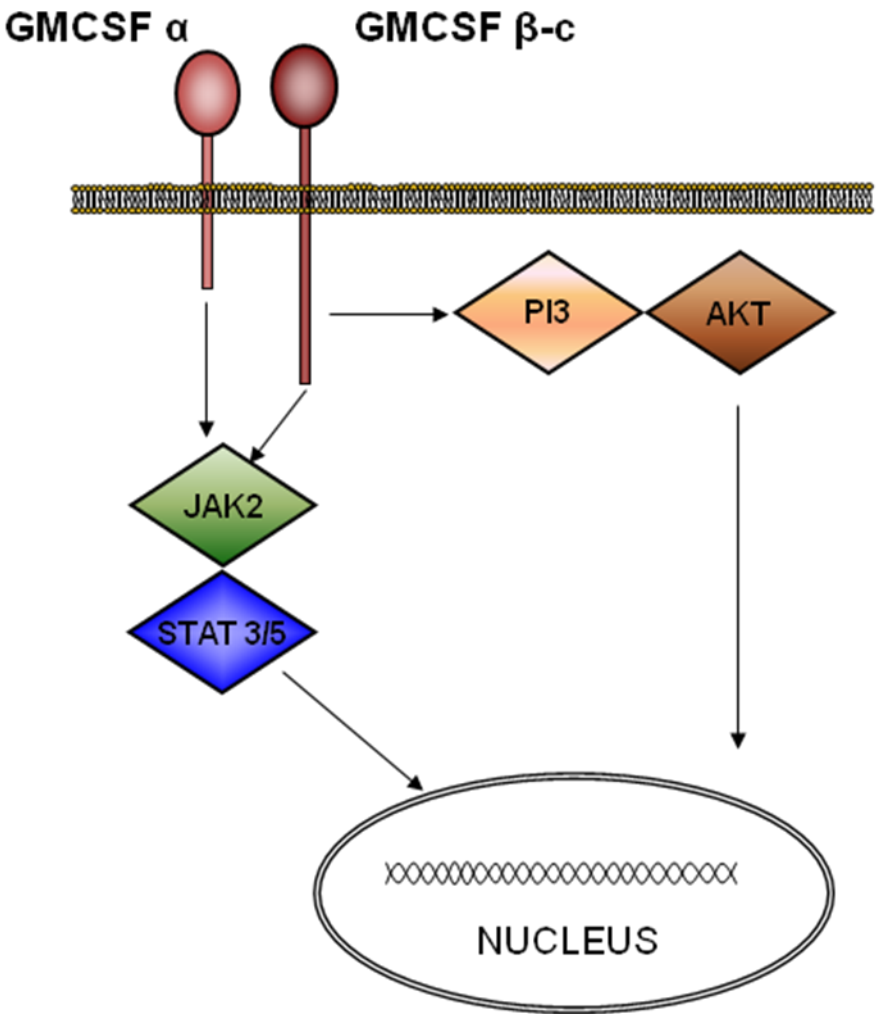


Figure 1.5 Schematic representation of GMCSF receptor

The α chain is specific for binding with GMCSF while the β chain is shared with receptors for IL-3 and -5.

GMCSF binds to the heterodimer to activate the JAK2 pathway; the β-c subunit is linked to phosphoinositide 3-kinase (PI3) system which activates AKT

1.4.3 GMCSF expression within the lung

1.4.3.1 GMCSF expression in Bronchoalveolar Lavage Fluid (BAL)

GMCSF expression within BAL has been extensively assessed over the past 17 years. In a small study of non-atopic subjects (n=6) with asthma exhibiting airway hyper-responsiveness, BAL exhibited elevated numbers of inflammatory cells but also increased levels of GMCSF protein compared to healthy controls. Dual staining of the cellular component of the lavage indicated GMCSF protein to be primarily stored within epithelia and monocytes (Mattoli et al. 1991). Increased levels of BAL GMCSF may be a reflection of active airways disease with a separate study demonstrating higher levels of BAL GMCSF when measured by ELISA, in subjects with symptomatic asthma compared with controlled disease (24 +/- 41 pg/ml versus > 8 pg/ml) (Broide et al. 1992).

Allergen challenge studies have demonstrated overexpression of BAL GMCSF post challenge, indirectly implicating GMCSF expression with active airway inflammation (< 4 pg/ml pre-allergen versus 180.5 +/- 46.9 pg/ml post-allergen) (Broide & Firestein 1991). A variety of cells have been identified as cellular sources of GMCSF with allergen challenging. In stable asthma, eosinophils within BAL express little GMCSF mRNA and protein, though with allergen challenge, BAL derived eosinophils express 45% and 59% GMCSF mRNA and protein respectively, suggesting an autocrine process may be present, driving the asthma airway inflammatory process (Broide, Paine, & Firestein 1992). Prior to allergen challenging, less than 1% of alveolar lymphocytes and macrophages expressed GMCSF mRNA; post challenge 92.6 +/- 3.4% of lymphocytes and 17.5 +/- 22.7% of alveolar macrophages exhibited GMCSF mRNA when assessed using in situ hybridization and immunoperoxidase staining (Broide & Firestein 1991). Further characterization of the lymphocyte population indicated CD4+ T-cells were activate post allergen challenge but not CD8+ T-cells with

expression of GMCSF along with interleukin(IL)-4 & -5. Statistical correlations have suggested the number of BAL cells expressing GMCSF are negatively correlated with airway hyper-responsiveness (Robinson et al. 1993). Allergen challenge studies additionally suggest a functional role for GMCSF in the asthma paradigm; BAL GMCSF concentration is increased 18hours post challenge in addition to increased CD69 expression upon BAL eosinophils, implying GMCSF overexpression influences the activation of eosinophils in the late phase of asthma (Julius et al. 1999). Further evidence to suggest the importance of GMCSF with late allergen response has been demonstrated with studies examining Cyclosporin which can attenuate the decrease in lung function seen specifically with the late allergen response. Subjects who have been defined as exhibiting this response also showed reduction in GMCSF expression post cyclosporin use (Khan et al. 2000).

BAL cellular expression of GMCSF may have important functional significance with respect to corticosteroid responsiveness. Lipopolysacchride (LPS) an endotoxin derived from the gram negative bacteria wall, can induce a strong innate immunity response and is an ubiquitous aeroallergen. Alveolar macrophages treated with LPS can exhibit 2 fold increases in GMCSF expression (Hallsworth et al. 1994) but modulation of GMCSF expression was possible with corticosteroid treatment with Budesonide (Linden 1994; John 1998). A double blind placebo study also demonstrated BAL cells production of GMCSF was markedly attenuated when LPS challenged cells were treated with a single bolus of intravenous prednisolone in subjects with asthma (Cotter et al. 1999).

GMCSF expression has been implicated with airway eosinophilia, and BAL cells expressing GMCSF correlate positively with activated eosinophils (defined by EG2 expression) with the bronchial tissue assessed by airway biopsy (Woolley et al. 1994). Further assessment post allergen challenge reflects a similar relationship, with increases in GMCSF expression and

eosinophilia (Sullivan & Broide 1996) with a positive correlation with BAL eosinophils (Woolley et al. 1995).

Certain physiological aspects of the asthma paradigm has been attributed to GMCSF expression within BAL. GMCSF gene expression in a small group of asthmatics (n=12) has been positively correlated with methacholine hyper-responsiveness testing. In addition, GMCSF mRNA may reflect susceptibility to the beneficial effects of ICS with both asthma and subjects with steroid responsive cough demonstrating elevated level in contrast to subjects with cough unresponsive to steroids (Gibson et al. 1998b).

BAL studies have been used infrequently to examine GMCSF expression in COPD. Alveolar macrophages from smokers & subjects with COPD show similar amounts of GMCSF release. GMCSF expression is inceased when exposed to cigarette smoke media but this process is unresponsiveness to the effects of dexamethasone (Culpitt et al. 2003) reflecting the clinical response of the majority of patients with COPD with corticosteroid therapy.

1.4.3.2 GMCSF expression in sputum

Attempts to quantify sputum GMCSF expression have been directed to assess differences between asthma, COPD and other conditions. GMCSF is persistently expressed in individuals with a history of mild asthma regardless of active symptoms compared to healthy controls. Two cohorts of adolescents with active asthma or childhood asthma (but persisting airway hyper-responsiveness) exhibited elevated sputum GMCSF. It cannot be commented upon if the persistence of GMCSF expression related to silent airway hyper-responsivness or a permanent predisposition to GMCSF expression regardless of symptoms (Obase et al. 2003).

Interestingly, this finding contrasts with previous results from the same group which in an earlier study, demonstrated GMCSF overexpression in sputum in subjects with mild asthma but not subjects with bronchial hyper-reactivity or atopy (Obase et al. 2001).

Assessment of sputum GMCSF expression across a severity of asthma disease suggests overexpression in severe disease (defined by higher inhaled corticosteroid use and substantially obstructive lung function values) but not moderate asthma when compared to healthy controls (Erin et al. 2008). This complements previous findings that sputum GMCSF is a steroid responsive target in mild-moderate asthma disease as a reduction of sputum GMCSF (as measured by ELISA) was shown in mild-moderate asthmatics using inhaled beclomethasone for 4 weeks (Inoue et al. 1999). The role of GMCSF overexpression in severe asthma is unclear, but sputum analysis in subjects with frequent exacerbations, has demonstrated higher levels of GMCSF (Dente et al. 2006).

GMCSF expression within BAL suggests an important role within the allergic asthma paradigm, with increases in GMCSF expression post challenge. This has not been reflected when using sputum to assess GMCSF expression post challenge in asthma (Keatings et al. 1997b). There is a paucity of published literature examining sputum GMCSF expression in relation to allergen challenging so it is unclear if this finding is consistent.

Preferential GMCSF expression between asthma and smoking related COPD phenotypes would be a significant difference. Small numbers of subjects with asthma have shown a greater number of sputum inflammatory cells to express GMCSF (n=12; 53.4 +/- 6.0%) compared to subjects with chronic bronchitis (n=12; 11.4 +/- 2.5%; $P < 0.001$). In asthma, the predominant cells expressing GMCSF were eosinophils and lymphocytes. Conversely in chronic bronchitis, the implicated cells were macrophages and neutrophils (Hoshi et al. 1995). Direct comparisons of sputum GMCSF between COPD, non-eosinophilic and eosinophilic

asthma have been complicated by the intercurrent use of inhaled corticosteroids potentially attenuating GMCSF expression and modifying the degree of eosinophilia. One study demonstrated detection of GMCSF within sputum supernatants from these 3 phenotypes with no preferential expression detected but uncertainty on interpreting this finding remains (Tsoumakidou et al. 2006).

Subjects with COPD do have greater levels of GMCSF released from cultured sputum cells compared to smokers and non-smoker controls. Consequently phosphodiesterase 4 inhibitors have been demonstrated to reduce GMCSF sputum levels (Profita et al. 2003). In a small study (n=12) similar to severe asthma, sputum GMCSF was shown to play a small role in COPD exacerbations, with a trend of elevated levels identified during exacerbations compared to stable disease (Tsoumakidou et al. 2005).

Sputum GMCSF overexpression in obstructive airways disease does not appear to be a non-specific marker of airways inflammation as low levels have been detected in cystic fibrosis compared to asthma (Koller et al. 1997).

1.4.3.3 GMCSF expression within the large airway

Examination of large airway GMCSF expression has supported BAL & sputum studies for increased expression of GMCSF in specifically asthma. Compared to normal controls and subjects with chronic bronchitis, GMCSF has been demonstrated to be preferentially localized to the epithelium and submuocsa compartments from bronchial biopsies using immunohistochemistry techniques. GMCSF was also localized to smooth muscle cells as well (Vignola et al. 1997). Additionally similar to BAL and sputum, large airway GMCSF expression is susceptible to the effects of beclomethasone, with down regulation of epithelial

GMCSF expression (Sousa et al. 1993; Trigg et al 1994; Wang et al. 1994). Specific down regulation of submucosal GMCSF expression as assessed by immunohistochemistry has also been seen with inhaled budesonide (Wilson et al. 2001).

Airway epithelium appears to be a rich source of GMCSF. GMCSF expression is persistently present in oral corticosteroid dependent patients with asthma with overexpression in large airway brushings compared to steroid naïve asthmatics. This may suggest a refactory state of GMCSF expression in certain patients with difficult to treat asthma (Vachier et al. 1998) 1998).

Bronchial tissue GMCSF expression has been correlated well with eosinophilia presented within the epithelium, submucosa and BAL (Woolley et al 1994; Wang et al 1994). Such studies have specifically assessed eosinophils in their activated form (expressing eosinophil cationic protein). This would imply GMCSF is intergral to the active airway inflammation that can define asthma.

The absence of atopy suggests preferential expression of GMCSF. In subjects with atopic and non-atopic asthma, GMCSF overexpression (as assessed by in-situ hybridization), was present when compared against their respective control groups, but also non-atopic asthmatics displayed increased number of cells expressing GMCSF mRNA compared to atopic asthmatics (Humbert et al. 1997b). This relationship was reflected in expression of GMCSF receptor mRNA in large airway biopsies from non-atopic asthmatics (Kotsimbos et al 1997). Again similar to previous analysis of sputum & BAL, airway hyper-responsiveness has negatively correlated with GMCSF expression (Wang, et al 1994; Woolley et al 1994).

Mast cells within the submucosa of the large airway in asthma contain GMCSF. Double staining of mast cells has highlighted GMCSF protein but this feature is more extensive in

aspirin sensitive asthmatics. Eosinophils are increased within the large airway in aspirin sensitive asthma and potentially this may be explained by a greater number of mast cells releasing GMCSF (Sousa et al. 1997).

Allergen studies using bronchial tissue have been somewhat contradictory with respect to GMCSF expression. In situ hybridization performed on bronchial tissue taken from subjects with asthma post allergen challenge, have demonstrated increases in GMCSF mRNA+ cells, mimicking allergen studies examining BAL (Bentley et al 1993). Conversely, immunostaining of bronchial tissue have shown reduction in GMCSF protein but increases within BAL samples (Woolley et al 1995). It is unclear if allergen challenging results in depletion of GMCSF protein from the large airway into BAL but overexpression of GMCSF mRNA is a reflection of active transcription in a bid to maintain large airway inflammation.

1.4.4 GMCSF expression within blood

Peripheral blood has been assessed for GMCSF concentration across a severity of asthma disease. Elevated GMCSF concentration has been seen in severe acute asthma compared to healthy controls but not mild asthma (Brown, Crompton, & Greening 1991).

Peripheral blood eosinophils express both GMCSF mRNA and protein (Moqbel et al. 1991). Secretion of GMCSF at sites of allergy has important implications. GMCSF has a multitude of effects over peripheral blood esoinophils so potentially autocirne and paracrine secretion of GMCSF is a significant mechanism. Stimulated eosinophils with calcium inophore, ionomycin leads to increased eosinophil survival. This activity can be attenuated with treatment of antibodies directed against GMCSF and IL-3 (Kita et al. 1991). Peripheral blood esoinophils from atopic asthmatics demonstrate the ability to migrate across in vitro models

of endothelial cells when exposed to GMCSF highlighting the ability for GMCSF to induce transmigration of eosinophils from the circulation into target tissue (Moser et al. 1992). GMCSF has the ability to prime peripheral blood esoinophils from healthy controls to undergo chemotaxis (Warringa et al. 1992). GMCSF can induce peripheral blood esoinophils into a pro-inflammatory state with expression of CD69, which upon cross-linking with platelets can lead to a release of inflammatory mediators (Hartnell et al. 1993). Potentially, GMCSF has a direct upon peripheral blood esoinophils with stimulated eosinophils releasing greater amounts of leukotriene C 4 (LTC4), a strong brochocnstrictor (Laviolette et al. 1995). GMCSF sitmulated release of LTC4 from stimulated eosinophils has been examined across subjects with severe asthma with differing responses to oral corticosteroid therapy. GMCSF enhanced release of LTC4 from steroid responding subjects showed marked reduction with oral corticosteroids. In contrast, GMCSF enhanced LTC4 release from steroid dependent asthma subjects was significantly greater than steroid sensitive and resistant subjects (Bosse et al. 2000).

Survival and viability of peripheral blood esoinophils has been promoted by GMCSF (Lopez, et al 1986; Lamas, Leon, & Schleimer 1991; Hallsworth, Litchfield, & Lee 1992). The effect of survival prolongation with GMCSF over eosinophils can be attenuated with corticosteroid therapy and also phosphodiesterase type 4 inhibitors (Hallsworth, Litchfield, & Lee 1992; Takeuchi et al. 2002). Eosinophils taken from healthy donors have displayed increasd cytotoxic and phagocytotic activity when stimulated by GMCSF (Lopez et al 1986).

The contribution of peripheral blood esoinophils derived GMCSF to the allergy model of asthma seems less prominent. Detection of both GMCSF protein and mRNA from peripheral blood esoinophils is absent in the early and late phase of allergen challenge studies in contrast to overexpression of GMCSF from BAL derived eosinophils (Sullivan & Broide 1996).

Survival of peripheral blood esoinophils seems less compared to BAL derived eosinophils. This mechanism may rest with an altered stabilization and release of GMCSF mRNA and protein respectively. Peripheral blood esoinophils stimulated with TNF and fibronectin, mediators found in increased concentrations in BAL have been found to increase peripheral blood esoinophils surivival, GMCSF mRNA stability and overexpression (Esnault & Malter 2001).

The influence of GMCSF over eosinophils is mediated through the p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-kappaB) enzyme systems. Stimulation of eosinophils with GMCSF leads to upregulation of gene expression of multiple adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), alpha6, beta2 integrin (CD18) and CD44 which can be involved in adhesion and migration of eosinophils from the systemic circulation to target sites (Ip et al. 2005).

CD4+ T lymphocytes express GMCSF mRNA in asthma, when assessed by RT-PCR. GMCSF mRNA is isolated in higher amounts during exacerbations with lower levels seen post treatment with oral corticosteroid therapy. Interestingly dissimilar to peripheral blood esoinophils and PBMC examinations, GMCSF mRNA expression was equivocal between stable asthma and healthy control groups (Lai et al. 1996). Theophylline in addition to corticosteroid treatment has also been demonstrated to reduce GMCSF gene expression (when assessed by RT-PCR) when CD4+ T cells have been previously stimulated (Choy et al. 1999).

Unstimulated peripheral blood mononuclear cells (PBMC) from asthma subjects release up to four times the amount of GMCSF compared to healthy controls (Hallsworth et al 1994). Stimulation with IL-2 and LPS leads to further increases in GMCSF release. This release of GMCSF is potentially very important and treatment of eosinophils with media from such

PBMC improves proliferation and stimulation of eosinophils. The value of GMCSF is confirmed with the addition of antibodies directed against GMCSF to this media with attenuating the original effect over eosinophils (Nakamura et al. 1993). Similar to lung findings, PBMC release of GMCSF can be reduced with treatment with corticosteroids (Nakamura et al 1993), (Linden & Brattsand 1994), (Cotter et al 1999).

GMCSF release from PBMC appears to be related severity of asthma disease. PBMC from severe asthma with chronic oral steroid use have continued to release increased amounts of GMCSF. Interestingly treatment with in vitro corticosteroids attenuated PBMC GMCSF release. Conversely, untreated uncontrolled asthmatics were able to demonstrate attenuated PBMC GMCSF release when given oral corticosteroids (Gagliardo et al. 2000). This specific subgroup of asthmatics with oral glucorticoid dependent asthma may have their phenotype explained through glucorticoid resistant expression of NF-κB. NF-κB has been demonstrated to be involved in the upregulation of many cytokines including GMCSF. In this complicated asthma subgroup, PBMC release of GMCSF was reduced using inhibitors of NF-κB (Gagliardo et al. 2003). Only one single study has suggested an absence of difference between basal PBMC GMCSF release between severe and non-severe asthmatics. This study used a luminex assay platform to assess multiple cytokines simultaneously which may reflect methodological differences when comparing cytokine release with previous studies. Histone Deacetylase (HDAC) affects histone and chromatin structure which in turn reduces transcription of genes of pro-inflammatory cytokines. NF-κB activity can be in part suppressed by HDAC action (Cosio et al. 2004). Interestingly, this study exhibited HDAC activity reduction correlated with the degree of steroid insensitivity of GM-CSF expression suggesting again the importance of NF-κB over GMCSF expression (Hew et al. 2006).

Supernatant from PBMC isolated from subjects with COPD have detectable amounts of GMCSF when measured by ELISA but these amounts are small (John et al. 2005).

Studies in children reveal PBMC release of GMCSF is increased in steroid naïve mild asthmatics and moderate asthmatics using inhaled corticosteroids. In this latter group, GMCSF was raised in subjects who suffered exacerbations (La Grutta et al. 2003).

Mast cells are an important source of GMCSF secretion within the allergy asthma paradigm. Originally GMCSF was identified from murine mast cells in response to IgE stimulation (Wodnar-Filipowicz, Heusser, & Moroni 1989). Mast cells derived from human lung tissue have also demonstrated release of GMCSF upon cross-linkage of the high-affinity Fc epsilon receptor. This mechanism of GMCSF release likely has significant influence over eosinophil activity with administration of anti-GMCSF antibodies to mast cells supernatant, considerably reducing the amount of eosinophil cationic protein (ECP) released from eosinophils. (Okayama et al. 1998).

Peripheral blood neutrophils (PBN) contain GMCSF mRNA and protein but in less quantity compared with peripheral blood esoinophils (Kita et al 1991). Traditionally the neutrophil has been considered one of the key effector cells in COPD. PBN taken from healthy donors have displayed increased cytotoxic and phagocytotic activity when stimulated with GMCSF. Increased degranulation was demonstrated as well as increase PBN survival (Lopez et al 1986). PBN taken from healthy donors and subjects with COPD reveal stimulation of neutrophils with GMCSF leads to increased survival, part mediated secretion of LTB4 and 5-lipoxygenase (Lee et al. 1999).

1.4.5 GMCSF and large airway epithelium

Large airway epithelium is a potent source of GMCSF that can be secreted into the underlying submucosa and associated structures. Expression of GMCSF mRNA and release of protein is increased from large airway epithelia in asthma compared to healthy control groups (Soloperto et al. 1991). Stimulation of the epithelium with certain cytokines (IL-1, IL-4 and –13, TNF-α, eotaxin) leads to significant release of GMCSF (Marini et al. 1991; Cromwell et al. 1992; Nakamura et al. 1996; Cui et al. 2002). The release of GMCSF from airway epithelia influences eosinophil activity. Supernatant taken from such stimulated cells prolonged eosinophil survival with attenuation induced with anti-GMCSF antibodies. Eosinophil survival was proportionate to the concentration of GMCSF of the supernatant (Nakamura et al 1996).

Epithelial release of GMCSF can be influenced in a paracrine fashion from eosinophil derived products. Eosinophil peroxidase treated against in vitro models of epithelial cell line has resulted in increased amounts of GMCSF release (Motojima et al. 1996). Epithelial GMCSF release is also influenced by additional exogenous factors. Rhinovirus is associated with the common cold and positively influencing airway eosinophilia in asthma. Treatment of epithelial cell line has led to increase in GMCSF expression suggesting the causative link between infection and asthma exacerbations. Again similar to GMCSF expression within peripheral blood esoinophils, GMCSF expression is associated with NF-κB dependent pathways (Sanders et al. 2001; Funkhouser et al. 2004).

Down regulation of GMCSF expression within epithelia from asthma groups is sensitive to down corticosteroid therapy and long acting beta agonists (LABA) (Marini et al. 1992; Adkins et al. 1998; Korn, Jerre, & Brattsand 2001; Silvestri et al. 2006b; Loven et al. 2007; Chiu et al. 2007). The mechanism by which steroid therapy and LABA therapy act are

significantly different. Transcriptional inhibitors reduce the effect of dexamethasone upon reducing GMCSF release from BEAS-2B epithelial cell line (Adkins et al 1998). LABA have been demonstrated to have an absent effect upon the glucocorticoid receptor (GR) reporter gene and small interfering RNA mediated depletion of the GR whilst still attenuating TNF stimulated epithelial GMCSF release (Loven et al 2007).

Examination of eotaxin stimulated epithelia cell release of GMCSF has demonstrated GMCSF secretion is dependent upon ERK 1/2 and p38 MAPK systems stimulated by eotaxins action upon receptor CCR3 (Cui 2002). The significance of the epithelium as a source of GMCSF in COPD seems less substantial. Epithelial brushings from subjects with COPD released little GMCSF compared to smokers and non-smoker controls (Profita et al 2003). Epithelial cell lines when exposed to cigarette smoke extract did not stimulate GMCSF release unless co-treated with LPS (Li 2007).

1.4.6 GMCSF action upon fibroblasts

Fibroblasts in close association with eosinophils can influence their activity within the submucosa layer. Peripheral blood esoinophils have demonstrated increased survival when exposed to culture medium from TNF stimulated and unstimulated bronchial myofibroblasts. GMCSF seceretion is increased and eosinophil survival can be abrogated with addition of anti-GMCSF antibody. GMCSF release from fibroblasts again seems to be steroid sensitive with reduction of GMCSF in media levels (and reduction in eosinophil survival) with treatment of prednisolone ((Zhang, Howarth, & Roche 1996) (Zhang et al. 1996). BAL eosinophils display an activated state characterized by upregulation of adhesion molecule CD11b and downregualtion of L-selectin. Lung fibroblasts can induce this state with

peripheral blood esoinophils but the administration of anti-GMCSF antibody, budesonide and/or fomoterol can reduce eosinophil activation to basal levels (Spoelstra et al. 1998; Spoelstra et al. 2000).

IL-13 and IL-4, cytokines implicated in the process of airway inflammation and hyper-responsiveness, have influenced stimulating human lung fibroblasts into secreting GMCSF along with other cytokines (Doucet et al 1998). Attenuation of GMCSF release (with other cytokines) from fibrobalsts has been possible in vitro, with macrolide use but the mechanism behind this remains unclear (Sato et al. 2001).

1.4.7 GMCSF action upon ASM

Airway smooth muscle (ASM) taken from subjects with asthma, reveal mRNA from multiple TH2 & TH1 cytokines including GMCSF and its receptor. Autocrine secretion of GMCSF may be an important mechanism as ASM treated with GMCSF displays icreased contractility to acetylcholine with reduced relaxation on exposure to beta agonist, isoproterenol (Hakonarson et al. 1999). In vitro studies have shown unstimulated ASM maintains a basal secretion of GMCSF. This release can be augmented by the actions of other solitary cytokines e.g. TNF-α and IL-1β, and mediators e.g. lipopolysaccharide, or simultaneous co-culture with multiple cytokines e.g. TNF-α, IL-1β and IFN-γ (Saunders et al. 1997). Further studies have identified GMCSF release from TNF-α and IL-1β stimulated ASM is mediated by the c-Jun NH2-terminal kinase (JNK) subgroup of the MAPK family (Oltmanns et al. 2003).

Collagen 1 and fibronectin are deposited within the submucosa and are involved with large airway remodelling associted with asthma. Treatment of ASM with GMCSF can induce

secretion of these proteins from the ASM and upregulation of TGF- β, implicating a role for ASM in airway remodeling (Chen et al. 2003).

Human mast cells infiltrate ASM from subjects with asthma in bronchial biopsy studies. Degranulation of mast cells releases histamine and tryptase. Histamine and tryptase alone did not induce GMCSF release from ASM. Pre-treatment with IL-β and TNF-α together promoted GMCSF release with stimulation with either tryptase or histamine. Histamine with IL-β alone, also induced ASM release from GMCSF. This suggests the infiltration of the ASM bundle with mast cells in asthma has functional significance for the asthma phenotype which is mediated in part by GMCSF (Chhabra et al. 2007).

In summary GMCSF is a pleiotropic cytokine stored within the epithelium and ASM in asthma. It can also be found within a range of inflammatory cells including eosinophils, macrophages and mast cells. Upregulation is seen in many allergen challenge studies. Studies suggest GMCSF is a steroid responsive target. Severe asthma can be divided between eosinophilic and neutrophilic phenotypes (Wenzel et al 1997; ten Brinke 2004). Hence the importance of GMCSF in severe obstructive airways disease. The functional properties of GMCSF in terms of survival and maturation of eosinophils and neutrophils suggests a significant role for this cytokine in severe obstructive airways disease (figure 1.6).

GMCSFr is distributed within the large airways with macrophages show high incidence of GMCSFr expression. Stimulation of GMCSFr in eosinophils leads to release of inflammatory cytokines.

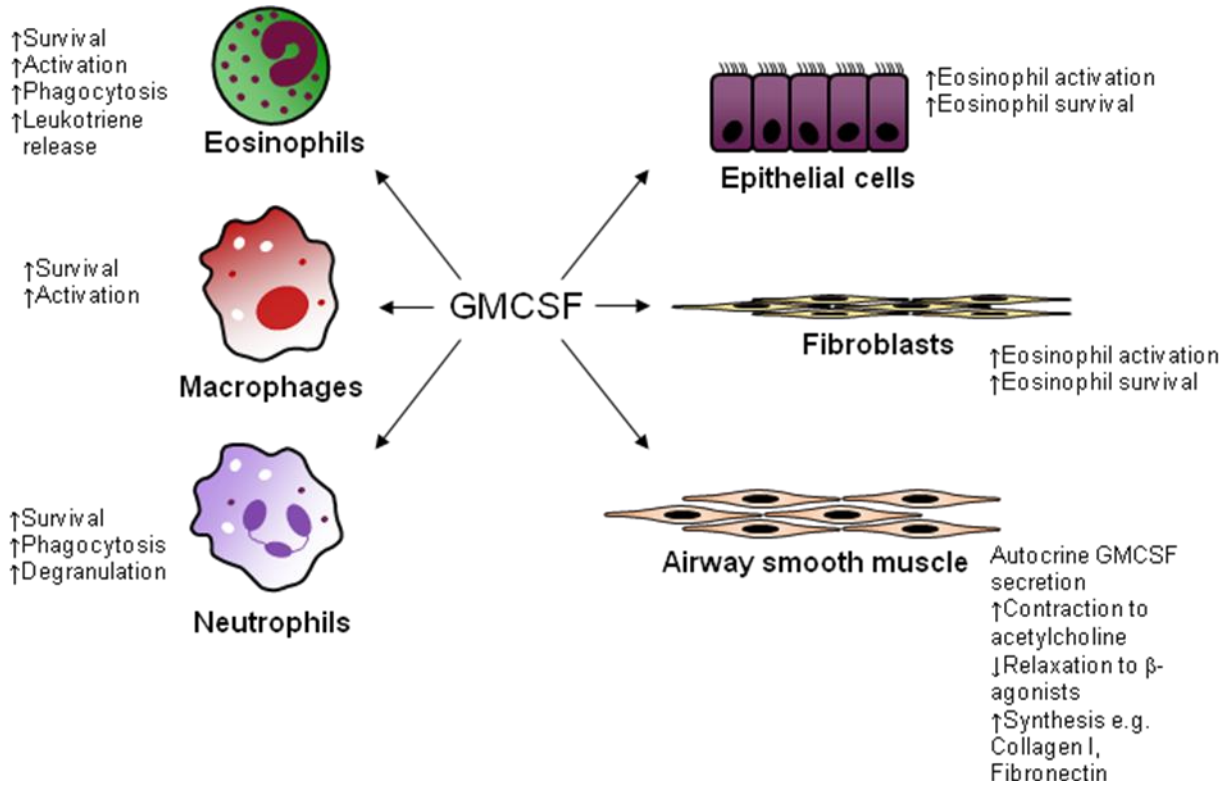


Figure 1.6 Actions of GMCSF upon inflammatory cells and large airway structures

GMCSF expression in COPD has been demonstrated in sputum but published data for GMSCF expression in other sources is lacking. Dissimilar to asthma, cultured epithelial cells from COPD do not release measurable amounts of GMCSF (Profita et al 2003).

1.4.8 Unanswered questions

Expression of GMCSF and upregulation of GMCSFr in asthma of progressive severity has not been definitely answered as of yet. Elevated GMCSF expression in sputum and blood in severe asthmatics has been identified (Brown, Crompton, & Greening 1991; Erin et al 2008). Epithelial brushings from subjects with severe asthma release significant amounts of GMCSF (Vachier et al 1998). Detailed quanitification of GMCSF and GMCSFr expression within the large airway in asthma of increasing severity is currently lacking.

Sputum examination of GMCSF expression in COPD to date has been in small numbers of subjects (n=10) and not related to severity of disease as measured by airflow obstruction (Profita et al 2003). In addition, large airway studies are in absence for the cross-sectional study of GMCSF and GMCSFr expression through the various comaparments of the airway. As part of this thesis, I will examine GMCSF expression in sputum and large airway in asthma over a range of severity and additionally, I will examine GMCSFr expression in the large airway. I will repeat this examination in COPD as well.

2. HYPOTHESIS & AIMS

I hypothesise preferential localisation of inflammatory cells and secretion of cytokines within the large airway, are evident in both asthma and COPD. Based upon murine models and human studies, 2 particular cytokines - IL-13 and GMCSF have diaplayed properties shared by both asthma and COPD phenotypes in terms of airway hyper-responsiveness, mucus hypersecretion, inflammatory cell migration and survival. It is on these merits, I hypothesise there will be upregulation of IL-13 & GMCSF within the large airway of subjects with asthma and COPD and in relation to severity.

Aims

1. To examine inflammatory cell localisation to submucosa and ASM in Asthma and its relation to disease severity
2. To examine inflammatory cell localisation to large airway structures in COPD
3. To examine large airway IL-13 and GMCSF expression in Asthma and its relation to severity and COPD
4. To examine IL-13 and GMCSF expression in Asthma and COPD and its relation to severity using sputum

3. METHODS

3.1 CLINICAL

3.1.1. Atopy skin testing

Atopy was assessed by skin prick tests to *Dermatophagoides pteronyssiunus*, cat and dog fur, grass and tree pollen, and *Aspergillus fumigatus* with normal saline and histamine controls (Alk-Abello, Berkshire, UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal >2mm more than the negative control along the volar aspect of the forearm.

3.1.2 Spirometry

Spirometry was measured with a Compact Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Bronchodilator reversibility was assessed by spirometry measured 15 minutes after of 200µg salbutamol administered via a volumatic spacer device. Forced expiratory value in 1 second (FEV_1) was recorded as the better of two consecutive readings within 100ml. Spirometers were calibrated daily.

3.1.3 Airway responsiveness

Using the Juniper tidal breathing method, the concentration of methacholine required to reduce the FEV_1 by 20% was recorded as the $PC_{20}FEV_1$ (Sterk et al. 1993). In brief following measurement of baseline FEV_1, subjects were instructed to inhale normal saline followed by doubling concentrations of methacholine 0.03-16mg/ml via a Wright's nebuliser (flow 0.13ml/min driven by dry compressed air). Subjects were instructed to inhale saline or

methacholine using tidal breathing for 2 minutes with a nose clip. FEV1 was measured at 30 and 90 seconds post nebulisation. The procedure was repeated with increasing concentrations of methacholine until a fall in FEV_1 by 20% was induced. If a 20% reduction in FEV1 was not observed with 16mg methacholine, the procedure was stopped. Methacholine $PC_{20}FEV_1$ concentration was calculated by linear interpolation of the log dose response curve. The output of the Wright's nebuliser was assessed at baseline by a qualified lung function technician and repeated at one monthly intervals by the same persons.

3.1.4 Sputum induction

Subjects were requested to inhale nebulised hypertonic saline (concentration between 3-5%) to aid generation of a sputum sample. The process of inhalation of nebulised hypertonic saline is explained in detail before the process begins.

Specific emphasis is given to the subject on the following:-

i. Deliberate spitting out saliva generated during inhalation of saline into a waste vessel.

ii. Blowing of their nose, rinsing of their mouth and swallowing water prior to trying to expectorate sputum.

iii. The method of effective expectoration is demonstrated. Subjects are shown to expectorate sputum forward from the back of the throat into a sterile container.

iv. A reminder not to swallow sputum as it comes up the bronchial tree.

v. Guidance on posture: sitting straight upright during nebulisation, and leaning forward during expectoration.

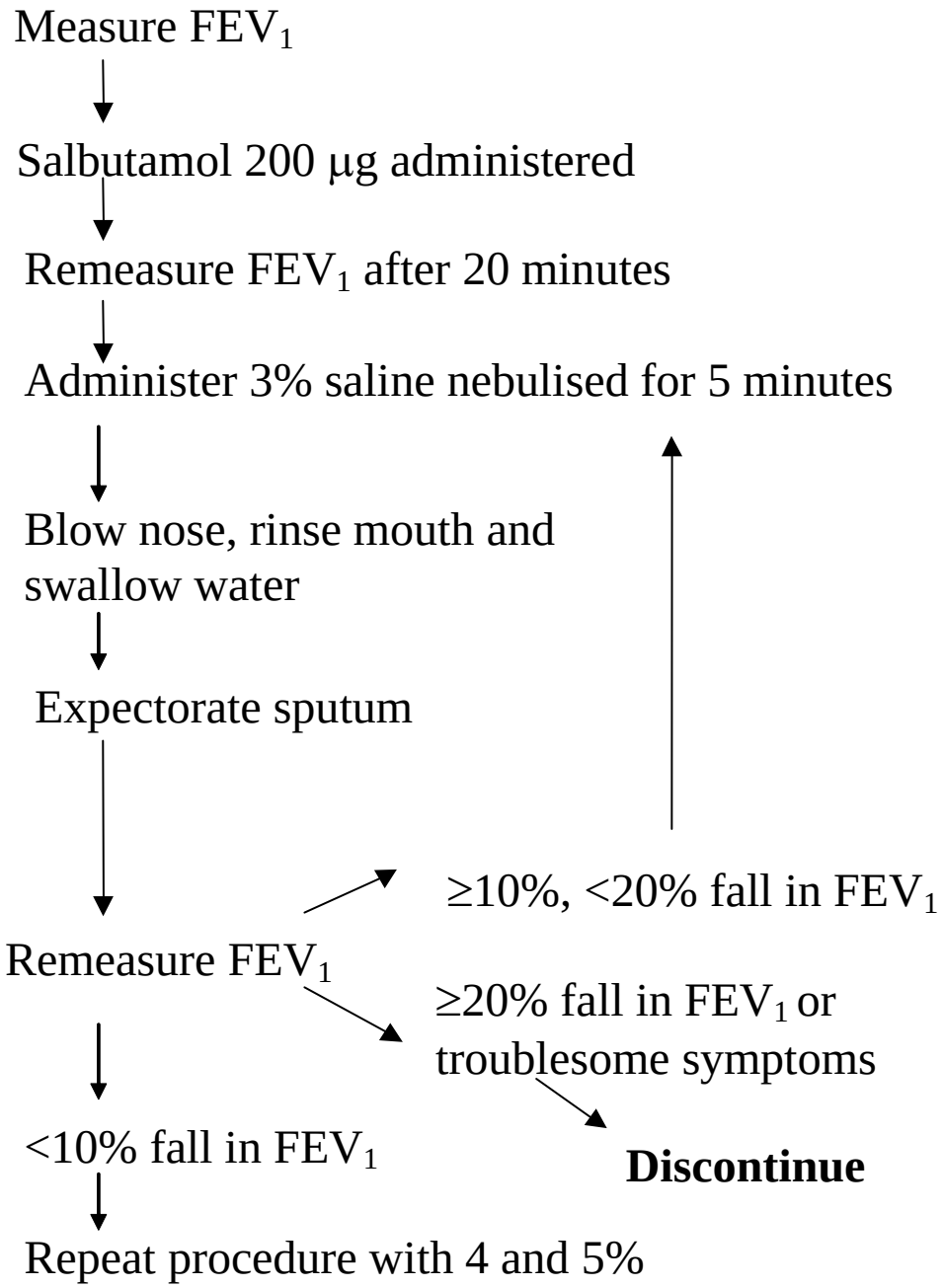
Subjects were requested to perform baseline FEV_1 on 3 occasions. To prevent bronchoconstriction during the induction, subjects were pre-treated with inhalation of 200µg of salbutamol by metered dose inhaler (MDI) and spacer. After 20 minutes, post bronchodilator FEV_1 was recorded 3 times. The highest post-bronchodilator FEV_1 value was used to calculate any subsequent fall in FEV_1 during the induction. The procedure was stopped if the post bronchodilator $FEV_1 < 1$ litre for safety reasons.

The nebuliser chamber was filled with 5ml of 3% pyrogen-free hypertonic saline. The subject was requested to breathe tidally, with a slightly deeper breath every minute.

After 5 minutes, subjects were instructed to rinse their mouth and throat with water and to blow their nose in order to reduce squamous cell contamination and post-nasal drip. Subjects were asked to cough any sputum into a plastic sputum pot using deep coughing until the cough becomes dry and unproductive.

Post sputum expectoration, the FEV_1 is measured again. The procedure is repeated with 4% and consequently 5% pyrogen-free hypertonic saline if the FEV1 does not fall by more than 10% or 200ml (which ever is greater) of the best postbronchodilator value (Pavord et al. 1997).

Fig 3.1 Sputum induction protocol



If the FEV_1 falls by more than 10% or 200ml (whichever is greater) but less than 20% or 400ml (whichever is greater), subjects were instructed repeat the steps with the same concentration of saline. Patients should not breathe saline for > 15 minutes in total.

If the FEV_1 fell by more than 20% or 400ml (whichever is greater) of the best post-bronchodilator value, or if significant symptoms occurred, nebulisation was stopped and repeat short acting β_2 agonist was administered. The nebuliser was calibrated by the same individual every month.

As inhaled hypertonic saline is a bronchoconstrictor the process of sputum induction was carried out in a careful safety first manner. Resuscitation equipment and nebulised salbutamol were available and a doctor nearby at all times.

3.1.5 Bronchoscopy

Bronchoscopy to procure bronchial biopsy specimens was performed as a day case procedure with adherence to the British Thoracic Society guidelines (BTS 2001). Written consent was obtained prior to procedure. Subjects were then premedicated with nebulised 2.5mg salbutamol. Topical lignocaine gel was applied to the nasal passages and topical lignocaine spray was applied to the oropharynx. Sedation was offered to the subjects and was given in boluses of 0.5mg midazolam to a maximum of 5mg. Supplemental oxygen at a rate of 2-4L min^{-1} was supplied and pulse oximetry monitored throughout the procedure. 2 aliquots of 2ml of 4% lignocaine were administered via the bronchoscope to the vocal cords and 3 aliquots of 2ml of 2% was applied to the trachea, right and left main bronchi. Four-six bronchial biopsies were taken from the segmental and subsegmental carina from the right lung using 20-cupped

biopsy forceps. A 2nd dose of nebulised salbutamol was administered to subjects with asthma post bronchoscopy.

3.1.6 Juniper asthma control questionnaire

The Juniper asthma control questionnaire (ACQ) was used to assess asthma control. It was a validated questionnaire designed in consultation with 100 international experts. Each symptom was scored for its importance in assessing asthma control. The questionnaire is responsive to change in asthma control ($p<0.0001$) (Juniper et al. 1999).

Juniper asthma control questionnaire

1. On average, during the past period, how often were you woken by your asthma during the night?

0 Never
1 Hardly ever
2 A few times
3 Several times
4 Many times
5 A great many times
6 Unable to sleep because of asthma

2. On average, during the past period, how bad were your asthma symptoms when you woke up in the morning?

0 No symptoms
1 Very mild symptoms
2 Mild symptoms
3 Moderate symptoms
4 Quite severe symptoms
5 Severe symptoms
6 Very severe symptoms

3. In general, during the past period, how limited were you in your activities because of your asthma?

0 Not limited at all
1 Very slightly limited
2 Slightly limited
3 Moderately limited
4 Very limited
5 Extremely limited
6 Totally limited

4. In general, during the past period, how much shortness of breath did you experience because of your asthma?

0 None
1 A very little
2 A little
3 A moderate amount
4 Quite a lot
5 A great deal
6 A very great deal

5. In general, during the past period, how much of the time did you wheeze?

0 Not at all
1 Hardly any of the time
2 A little of the time
3 A moderate amount of the time
4 A lot of the time
5 Most of the time
6 All the time

6. On average, during the past period, how many puffs of short acting bronchodilator (e.g., Ventolin) have you used each day?

0 None
1 1–2 puffs most days
2 3–4 puffs most days
3 5–8 puffs most days
4 9–12 puffs most days
5 13–16 puffs most days
6 More than 16 puffs most days

7. FEV_1 prebronchodilator: .....0 >95% predicted

1 95–90%

2 89–80%

3 79–70%

4 69–60%

5 59–50%

6 <50% predicted

3.2 LABORATORY

3.2.1 Sputum

3.2.1.1 Protocol for sputum processing

Induced sputum was collected on ice and processed at 4°C within 2 hours of expectoration. Sputum plugs were selected from saliva and transferred to a Petri dish. Sputum free from salivary contamination was transferred into an empty (pre-weighed) polypropylene centrifuge tube with screw top. The weight of the sputum plugs were derived from the weight of the empty centrifuge tube being subtracted from the weight of the tube with sputum plugs. Mucolytic dithiothrietol (DTT) freshly diluted to 0.1% (from a stock solution of 1%) using phosphate buffered saline was added to the sputum plugs using 4x weight/volume (e.g. 4 ml DTT per gram of selected sputum).To allow gentle dispersion, the sputum mixture underwent gentle aspiration into a plastic pipette followed by 15 seconds vortex and 15 minutes rocking on a bench rocker with ice.

An equal volume of Dulbecco's phosphate buffered saline (D-PBS) was added to the mixture and then vortexed for a further 15 seconds. The sputum suspension was filtered through a 48 mm nylon gauze pre-wet flat with D-PBS, any excess was shaken off and centrifuged at 2000 rpm (790 g) for 10 minutes. The consequent supernatant was taken in 0.5 ml aliquots into 2 ml microtubes, leaving behind a covering of fluid and the undisturbed pellet. Approximately 2-4 microtubes would be filled with supernatant and stored at -80°C until required for ELISA analysis.

The cell pellet was resuspended with 0.5 ml to 1 ml of D-PBS (depending on size of cell pellet) and mixed gently with a wide bore plastic pipette. The total cell count and cell viability was assessed using a Neubauer haemocytometer and the trypan blue exclusion method. This entailed flooding the haemocytometer with 10 µl of cell suspension mixed thoroughly with 10µl of 0.4% trypan blue. All cells within the centre square and in the four 1mm corner squares of chamber 1 of the haemocytometer were counted. Cells were divided into viable, non-viable and squamous. The mean number of cells per square and the portion of viable and squamous cells were calculated.

The total number of cells was calculated using the following:-

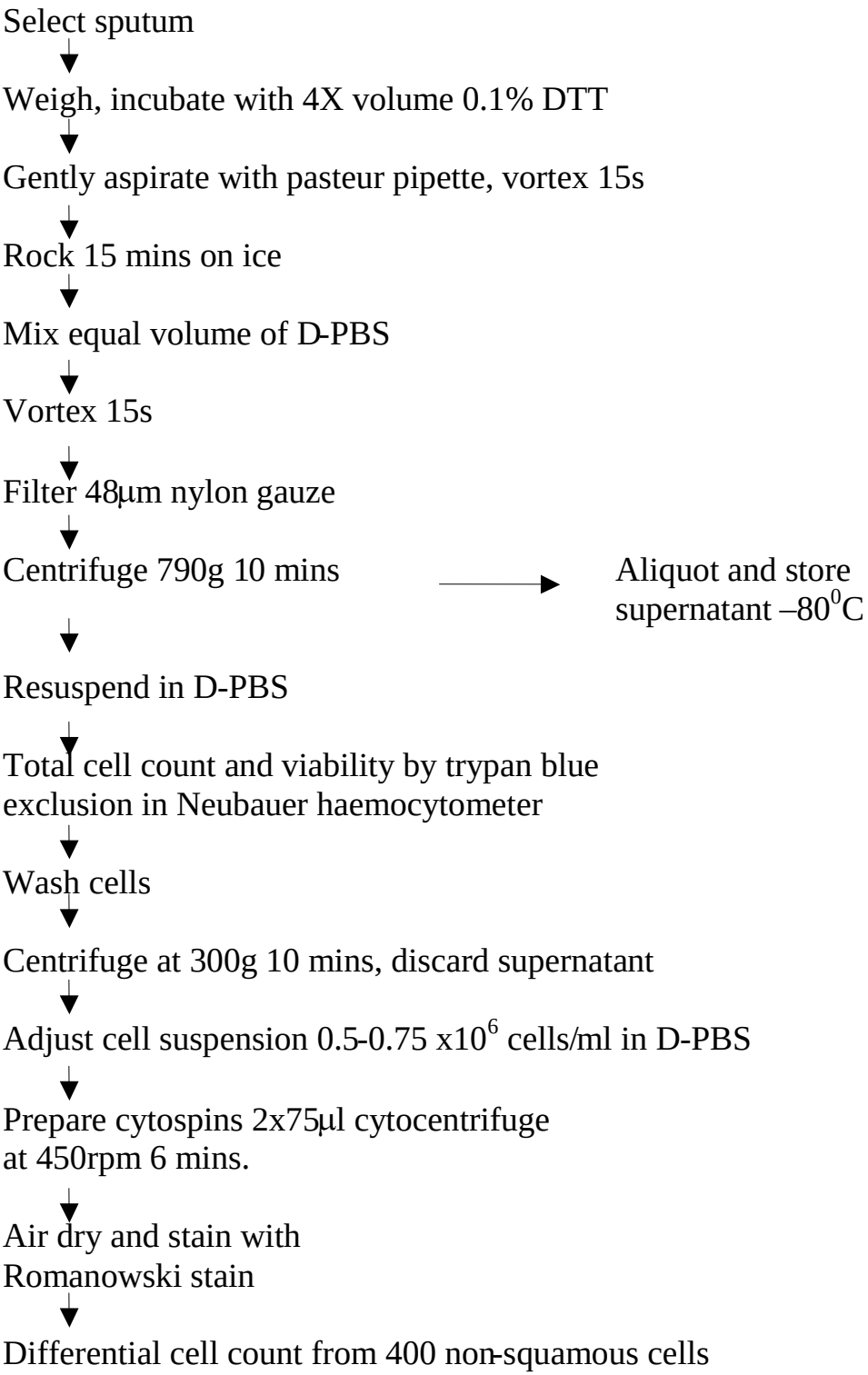
Total number of cells = mean number of cells/square x 2 x 10,000 x volume cells resuspended in (ml)

The total cell count (cells/g sputum) was calculated using the following:-

Total cell count (cells/g sputum)= mean number of cells/square x 2 x 10,000 x volume cells resuspended in (ml)/ weight of selected sputum (g).

The original cell pellet was diluted with D-PBS to make a cell suspension of 0.5-0.75 x 10^6 cells/ml . 50µl was taken off to prepare two cytospins, which were centrifuged at 450rpm (18.1 g) for 6 minutes using a Shandon III cytocentrifuge. Four slides were then air dried for at least 15 min at room temperature, Romanowski stain was added for 5 minutes. Slides were rinsed with distilled water and dilute Romanowski stain was added for a further 25 minutes. To differentiate between eosinophils, neutrophils, macrophages, epithelial cells and lymphocytes, 400 cells (non-squamous cells) were counted. Finally this information was used to calculate the total number of cells and the total cell count (cells/ml sputum) (International Committee for Standardization in Haematology 1984; Pavord, et al 1997).

Figure 3.2 Sputum processing protocol



Protocol for Romanowski stain preparation

1.5g Azure-B-thiocyanate in DMSO was dissolved at 37^{o}C and 0.5g Eosin was dissolved in 300ml methanol at room temperature. The Azure blue solution was slowly added to the Eosin and stored away from light.

Protocol for dilute Romanowski stain

62ml 10mM HEPES buffer pH 7.2

3.5ml DMSO

4.6ml Romanowski stain

3.2.1.2 ***IL-13 enzyme immunoassay in sputum supernatant***

The concentration of IL-13 was measured by a commercial ELISA kit (Caltag-Medsystems and R&D). The limit of detection was 10pg/g of sputum for IL-13. We found ELISA detection of sputum IL-13 was impaired by the mucolytic DTT. To improve recovery, we dialysed our samples in PBS (sigma) for a minimum of 12 hours prior to measurement by ELISA (see section 4.2.2.1 for results of validation) (Berry et al 2004). All samples were dialysed for 18 hours against PBS with a 10Kda dialyser (Sigma, Poole, Dorset, UK) and then measured by ELISA.

3.2.1.3 GMCSF enzyme immunoassay in sputum supernatant

Sputum GM-CSF was measured by a commercial ELISA kit (Caltag-Medsystems, Buckinghamshire, UK). The lower limit of detection was 10pg/g sputum. To assess the effect

of DTT upon the recovery of GMCSF from sputum, sputum from 3 subjects with asthma was spiked with exogenous GMCSF standard and processed as described previously. To assess the recovery of GMCSF was not affected by the freeze and thaw process with supernatant, an exogenous spike was added to sputum supernatant from 4 subjects (see section 4.3.2 for results of validation).

3.2.2 Large airway tissue

3.2.2.1 Collection of Large airway sample from surgical resection specimens

Bronchial tissue was dissected from lung tissue resected from operations from patients undergoing surgery for lung malignancy. A cross-sectional ring of bronchial tissue was dissected from the large airway and cut transversely into smaller pieces for further fixation, processing and embedding in glycolmethacrylate (see section 3.2.2.2 below). All patients provided written consent prior to their operation.

3.2.2.2 Large airway tissue fixation, proceessing and embedding in glycomethacrylate

Bronchial biopsy and large airway specimens were transferred immediately to ice-cooled acetone containing the protease inhibitors (20mM) (PMSF) (2mM) for fixation and then stored at -20°C for 24h. Samples were transferred into acetone followed by methyl benzoate at room temperature for 15 min each. Samples were infiltrated with 5% methyl benzoate in glycol methacrylate (GMA solution A, Polysciences, Northampton, UK) at 4°C, 3x2 hours and then embedded in a solution ofGMA A 10mls: GMA solution B 250μls: Benzoyl peroxide 45mg (the catalyst for the polymerisation). The blocks were polymerised at 4°C overnight and kept in dry airtight boxes at -20°C.

3.2.2.3 Immunohistochemistry

Two-micrometer sections were cut using a rotary microtome (Leica) (figure 3.3), floated on 0.2% ammonia solution in water for 1 min and dried at room temperature for 1-4h. The technique of immunostaining applied to GMA embedded tissue has been described previously (Britten, Howarth, & Roche 1993). In brief, tissue sections were coated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide to inhibit endogenous peroxide. Sections underwent 3 washes in TBS pH 7.6, blocking medium consisting of Dulbecco's MEM, 10% FCS and 1% BSA was applied for 30 min. Any excess was shaken off and sections were then incubated with the primary antibody for 16-20h overnight at room temperature at appropriate concentrations (Table 3.1). Bound antibodies were labelled with biotinylated rabbit anti-mouse Fab fragments (Dako Ltd., Ely, Cambridgeshire, UK) during a 2h incubation, and demonstrated using the streptavidin-biotin-peroxidase detection system (Dako Ltd). Biotinylated rabbit anti-rat Fab fragments were used against the monoclonal rat anti-GMCSF antibody and Rat IgG2a isotype control. Aminoethylcarbazole (AEC) was applied as the chromogen, which gives a red reaction product. Sections were counterstained with Mayers haematoxylin. Appropriate control sections were similarly treated either with the primary mAb omitted or in the presence of an unrelated antibody of the same isotype.

Midway through the staining of the large airway specimens, the intensity of staining with the IL-13 monoclonal antibody considerably reduced upon purchase of new monoclonal antibody. This was due to quality of the staining being related to a batch specific clone of the monoclonal antibody. Consequently, specific request for the original batch specific clone of antibody used in a previous study (Berry, Parker, Neale, Woodman, Morgan, Monk, Bradding, Wardlaw, Pavord, & Brightling 2004) was successfully made to allow completion of the study.

Protocol for Tris Buffered Saline pH 7.6

Sodium chloride	80g
Tris	6.05g
1M hydrochloric acid	38mls
Distilled water	10L

Mix buffer salts and acid in 1L of distilled water, adjust pH to 7.65 and add to remaining 9L of water to give a final pH of 7.6

Protocol for Blocking medium

Dulbecco's modified Eagles medium	80mls
Fetal calf serum	20mls
Bovine serum albumin	1g

Protocol for Tris HCl Buffer pH 7.6

0.2M Tris	12mls
0.1M hydrochloric acid	19mls
Distilled water	19mls

Mix all reagents together, adjust pH to 7.6

Figure 3.3 Leica microtome used to cut 2µm sections of GMA resin

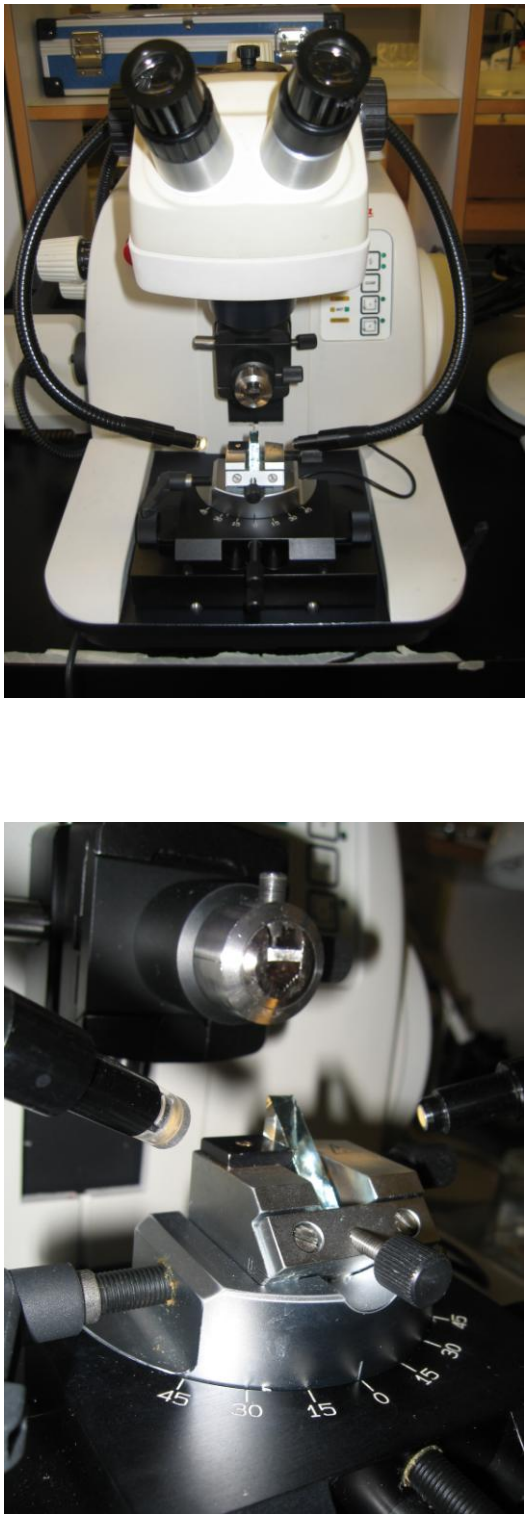


Table 3.1 Antibody source and concentration used for immunohistochemistry

Antibody	Clone	Epitope Stained	Source	Dilution
CD3	UCHT1	T lymphocytes	DAKO	1:1000
MBP	BMK-13	Major Basic Protein	Monosan	1:25
Tryptase	AA1	Mast cell tryptase	DAKO	1:1000
NE	NP57	Neutrophils	DAKO	1:1000
CD56	123C3	Macrophages	DAKO	1:50
IL-13	MAB 213	IL-13	R&D	1:100
GMCSF	BVD2-21C11	GMCSF	Cambridge Bioscience	1:100
GMCSF-R	2B7	GMCSF-R	Gift	1:200
IgG1		Mouse control Ig	DAKO	1:100
IgG2a		Rat control Ig	DAKO	1:100
Biot-rab anti Mouse		Second stage antibody	DAKO	1:300
Biot-rab anti Rat		Second stage antibody	DAKO	1:300
StABC-HRP		Third stage antibody	DAKO	1+1:200

4. STUDIES

4.1 INFLAMMATORY CELL INFILTRATION OF OBSTRUCTIVE AIRWAYS DISEASE

4.1.1 Inflammatory cell infiltration of large airway structures in asthma

Introduction

Asthma is characterised by the presence of variable airflow obstruction, airway hyper-responsiveness (AHR), and an airway inflammatory response often characterised by Th2-mediated eosinophilic airway inflammation with mast cell infiltration of the airway smooth muscle (ASM) bundle (Brightling et al 2002). Comparisons between asthma and non-asthmatic eosinophilic bronchitis (EB), a common cause of chronic cough, have been informative about the key immunopathological features of asthma. Importantly mast cell infiltration of the ASM-bundle are features of corticosteroid naïve asthma that are not shared by EB and have therefore been implicated in the pathogenesis of AHR (Brightling et al 2002).

Asthma is characterized by airway inflammation which is generally eosinophilic (Wardlaw et al 2002). This observation has not been reflected when examining ASM in mild corticosteroid naïve asthma. There is a paucity of inflammatory cells apart of mast cells infiltrating the ASM in mild asthma (Brightling et al 2002). Eosinophilic infiltration of the ASM in asthma of progressive severity is unknown.

Post-mortem studies have indicated have demonstrated an increase in numbers of mast cell degranulation within ASM in the bronchial wall in fatal asthma compared with non-fatal

asthma though (Carroll, Mutavdzic, & James 2002a). Though fatal asthma often reflects severe asthma, it remains unclear if significant mast cell infiltration of ASM is present in asthma of progressive severity. Post-mortem studies suggest there is an absence of difference with the density of mast cell infiltration in ASM in non-fatal asthma compared with fatal asthma (Carroll, Mutavdzic, & James 2002a). To date, there has not been a formal study of mast cell infiltration of the ASM in moderate-severe asthma.

We hypothesised that in addition to mild asthma, mast cell infiltration was present in asthma of progressive severity. We also assessed eosinophilic infiltration of the ASM in asthma of progressive severity.

To test our hypothesis we enumerated mast cells and eosinophils in the bronchial submucosa and ASM-bundle in a cross-sectional study that included mild, moderate and severe refractory asthmatics and healthy controls. To further define the possible role of ASM inflammatory cell infiltration, we assessed in addition, asthma control, AHR, spirometry and eosinophilic inflammation.

Methods

Subjects

Subjects were recruited from local primary health care, respiratory clinics, hospital staff and by local advertising. Asthma was defined and severity categorised by international (GINA) guidelines (Global Initiative for Asthma 2009) and ATS criteria for refractory asthma (ATS 2000). Normal subjects had no history of respiratory symptoms and normal spirometry. All

subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Clinical characterisation

Subjects underwent spirometry, allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat and grass pollen, a methacholine inhalation test using the tidal breathing method (Sterk, Fabbri, Quanjer, Cockcroft, O'Byrne, Anderson, Juniper, & Malo 1993) and sputum induction using incremental concentrations of nebulised hypertonic saline 3, 4 and 5% each for 5 minutes (Pavord et al 1997) (section 3.1.3&.4) . Subjects with a sputum eosinophil count>3% were defined as having eosinophilic asthma. In those subjects with moderate to severe disease symptom control was assessed by the Juniper Asthma Control Questionnaire (ACQ) (Juniper et al 1999) (section 3.1.6).

Mast cell & eosinophil measurement in endobronchial biopsies

Subjects with assessable ASM (>0.1mm2) in bronchial biopsies were recruited (Brightling et al 2002). Asthma was categorised as mild (GINA 1; n=14), moderate (GINA 2/3; n=7) or severe (GINA 4/5). All of the subjects in the severe asthma category had severe refractory asthma (ATS 2000). In order to examine IL-13 expression in non-eosinophilic asthma we included 7 GINA 1 asthmatics with an absence of sputum eosinophilia<1.9% on 2 separate occasions. In this cohort we chose to specifically compare corticosteroid naïve eosinophilic and non-eosinophilic asthmatics to exclude the possible confounder of treatment and applied a rigorous definition for non-eosinophilic asthma (Berry et al 2007).

After characterisation, subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines (BTS 2001). Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae; were fixed in acetone and embedded in glycomethacrylate as described previously (Britten, Howarth, & Roche 1993).

2µm sections were cut and stained using monoclonal antibodies against tryptase for mast cells (DAKO UK, Cambridgeshire, UK), major basic protein for eosinophils (Monosan, Uden, Netherlands) or appropriate isotype controls (DAKO). The number of positive nucleated cells was enumerated per mm2 of bronchial submucosa or ASM-bundle by a blinded observer (Dr Saha).

Statistical Analysis

Statistical analysis was performed using PRISM Version 4 and MINITAB13.31 (Minitab, Coventry, UK). Parametric data were expressed as mean (SEM), data that had a normal log distribution was log transformed and described as geometric mean (log SE) and non-parametric data were described as median (IQR). One-way analysis of variance and t-tests (Kruskal-Wallis and Mann-Whitney tests for non-parametric data) were used for across and between group comparisons respectively. Chi squared tests were used to compare categorical data. Correlations were assessed by Spearman rank correlation coefficients.

Results

Clinical and sputum characteristics for subjects shown in table 4.1 and the number of mast cells and eosinophils in the bronchial submucosa and ASM-bundle are shown in table 4.2.

Table 4.1 Clinical and sputum characteristics

	Normal	Mild	Moderate	Severe
Number	7	14	7	7
ICS use (%)	0	0	100	100
ICS dose (µg/day)#	0	0	942.9 (36.9)	1520.0 (224.5)*
Oral CS use (%)	0	0	0	71
Oral CS dose (mg/day)#	0	0	0	15.0 (5.4)
LABA use (%)	0	0	100	100
Age #	37.6 (7.3)	52.1 (3.8)	43.4 (4.7)	46.4 (2.7)
Male	7	8	3	3
Never smokers	7	14	7	6
Pack years#	0	0	0.83 (0.83)	3.4 (2.1)
Atopy (%)	14	50	86	71
$PC_{20}FEV_1$ (mg/ml)^	>16	0.79 (0.21)	0.66 (0.5)	0.47 (0.4)
FEV_1 % predicted#	106.6 (6.6)	95.3 (3.8)**	81.3 (8.4)**	74.3 (11.3)**
Bronchodilator reversibility(%)#	ND	4.4 (1.7)	6.3 (4.0)	15 (6.8)
FEV_1/FVC %#	81.7 (2.7)	71.2 (8.7)	76.3 (1.8)	70.9 (4.9)
Sputum Cell Counts				
Eosinophil %^	0.39 (0.15)	1.70 (0.2)	5.7 (0.29)∞	7.7 (0.21)∞
Neutrophil %#	38.7 (7.2)	60.6 (8.4)	41.4 (14.7)	34.5 (9.7)
Macrophage %#	53.6 (5.7)	28.2 (8.2)	35.9 (8.6)	46.2 (12.7)
Lymphocyte %#	4.2 (2.0)	0.59 (0.13)	1.6 (0.42)	1.1 (0.30)
Epithelial cells %#	6.0 (3.2)	3.2 (1.2)	7.4 (3.5)	2.84 (1.8)

#mean (SE), ^ geometric mean (log SE), CS- corticosteroid use, ND-not done

*$p<0.05$ Moderate vs Severe (Mann-Whitney)

**$p<0.001$ normal vs moderate/severe $p<0.01$ mild vs severe (Tukey's multiple comparison test)

∞$p<0.01$ (ANOVA)

Table 4.2 Median (IQR) inflammatory cell infiltration of submucosa and ASM

	Normal	Mild Asthma	Moderate Asthma	Severe Asthma
Submucosa cells/mm^2				
Tryptase+	16.4 (18.0)	21.8 (22.1)	24 (28.4)	21.5 (29.1)
MBP+	2.5 (7.4)	11.9 (25.9)*	8.1 (22.1)	21.8 (27.4)*
ASM cells/mm^2				
Tryptase+	0 (0)	9.1 (5.1)**	15.7 (13.5)**	16.2 (19.3)**
MBP+	0	0	0	0 (0.42)

*$p<0.05$ normal vs mild and severe groups (Mann-Whitney)

** $p<0.05$ normal vs mild and severe groups, $p<0.01$ normal vs moderate group (Mann-Whitney)

Representative photomicrographs mast cells in the ASM-bundle are as shown in figure 4.1. The number of mast cells within ASM-bundle in asthma was raised compared to the controls irrespective of disease severity (p=0.009; figure 4.2).

The number of mast cells in the ASM-bundle was increased in the eosinophilic mild asthmatics (11.3 [3.4]) compared to the non-eosinophilic mild asthmatics (7.5 [5.8]; p=0.018). There were no differences across disease severity. The number of mast cells within the ASM-bundle was related to asthma control (Rs=0.7 p=0.007).

There was an absence of eosinophils with the ASM bundle in asthma regardless of severity. In only 2/7 subjects with severe asthma demonstrated ASM eosinophil infiltration.

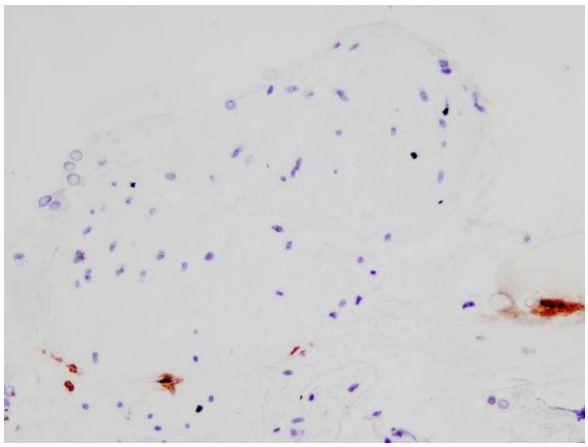
Discussion

We have confirmed our earlier observation that mast cell localisation to the ASM-bundle is a feature of mild asthma (Brightling et al 2002) and demonstrated for the first time that this is also characteristic of moderate and severe refractory disease In addition to previous findings of an absence of ASM eosinophilia in mild asthma, we did not demonstrate preferential eosinophilic infiltration of ASM in moderate and severe asthma. Mast cell microlocalisation to the ASM-bundle is a feature of asthma across severities(Amin et al 2005; Berger et al 2003a; Brightling et al 2002; Brightling, Ammit et al 2005a; Carroll, Mutavdzic, & James 2002a; El Shazly et al 2006;Koshino et al 1993).

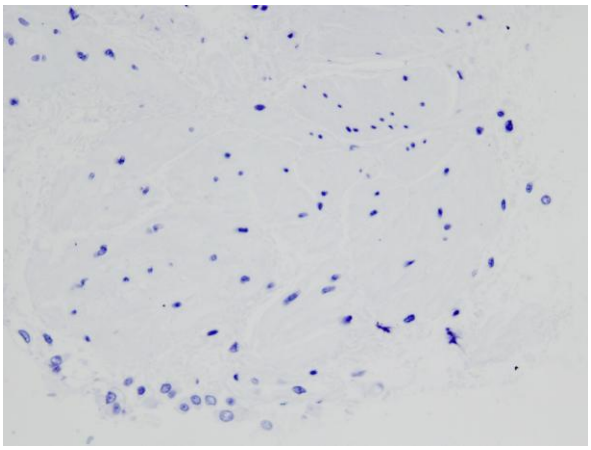
The immediate proximity of mast cells with ASM is likely to be functionally important through the action of locally secreted proteases and cytokines. Many cytokines exert their effects across distances of a few microns (Wardlaw et al 2002). In mild asthma mast cells in

Figure 4.1 Mast cells infiltrating ASM bundle in severe asthma

a) x200 magnification mast cell within ASM bundle



b) x 200 magnification isotype control



c) x 400 magnification mast cell within ASM bundle

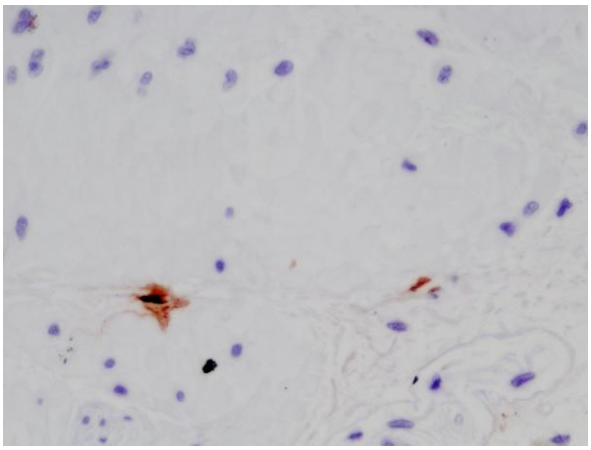
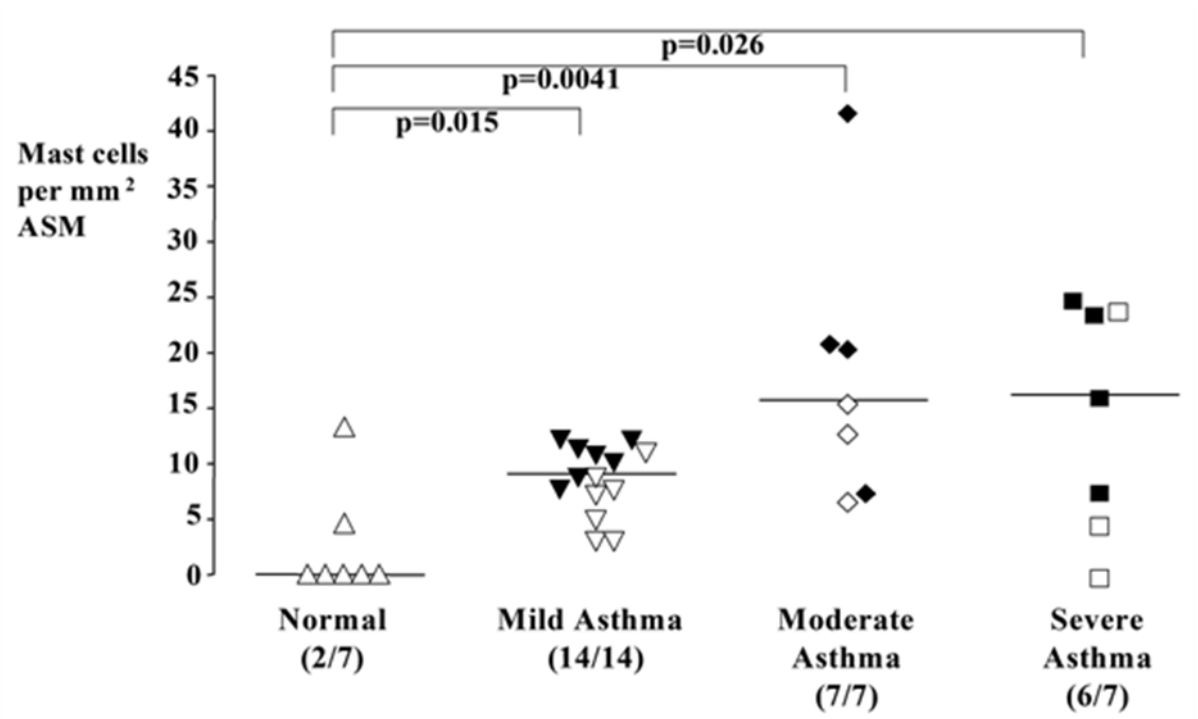


Figure 4.2 The number of mast cells in ASM in healthy controls and subjects with asthma.

Closed symbols represent subjects with sputum eosinophilia>3%. Horizontal bars represent median value. Values in parentheses represent subjects with mast cells in ASM/total subjects in group



the ASM-bundle have already been shown to express IL-4 and IL-13 (Brightling et al 2003c). ASM is an important source of pro-inflammatory mediators such as CCL11 and IL-13 induces the ASM-synthesis and release of this and other chemokines (Amin et al 2003;El Shazly et al 2006; Sutcliffe et al 2006). In section 4.2.1.1, expression of IL-13 by ASM mast cells in moderate and severe asthma is further assessed and discussed. Infiltration of the ASM bundle with mast cells has not been shown to be an exclusive feature of asthma (Gosman et al. 2008; Liesker et al 2007; Niimi et al. 2005; Zhu et al 2007). Potentially, in addition to the core properties of mast cells, it is likely the cytokine profile of mast cells will be important in influencing the phenotype of the patient.

The role of the mast cell myositis in asthma appears to be significant but the influence of mast cells within the ASM bundle is complex and is likely mediated through direct and indirect actions of locally secreted pleiotropic autacoid mediators, proteases and cytokines. Mast cell proteases, histamine and tryptase can modulate GMCSF and RANTES secretion via IL-1β and TNF-α but not by direct actions (Chhabra et al 2007). Ex-vivo mast cells within the ASM bundle can influence the phenotype of the ASM through release of tryptase. Increased contractility has been observed in ASM co-cultured with tryptase and mast cells via the release of TGF-beta. The significance of mast cells within the ASM is further supported with evidence of the degree of mast cell infiltration in the ASM having a direct correlation the degree of smooth muscle actin expression in asthma, which will ultimately have implications for lung function and airway hyper-responsiveness (Brightling et al 2002; Woodman et al 2008). The action of tryptase in ASM is partly via activation of the protease-activated receptor 2 (PAR-2) which causes beta-adrenoceptor densensitisation (Kobayashi et al. 2008).

Previously in steroid naïve asthma, mast cells within the ASM have correlated positively with airway hyper-responsiveness (Brightling et al 2002). This study did not support this when studying AHR across the asthma study groups. This is likely due to the concurrent use of steroids in the moderate and severe asthma groups but we did reflect siginificant correlation with asthma symptoms scores.

We saw an increased number of mast cells within the ASM in corticosteroid naïve subjects with eosinophilic asthma in comparison to corticosteroid naïve subjects with non-eosinophilic asthma. We did not see a correlation with mast cells in the ASM between eosinophilic infiltration of the submucosa or sputum eosinophilia across groups. Potentially masked by the confounding effect of concurrent steroid use in the moderate and severe group, we did not see increased numbers of mast cells in eosinophilic subjects with moderate and severe disease. We cannot comment on the basis of our data as to whether the inclusion of subjects with eosinophilic inflammation in the moderate and severe asthma led to an increase in ASM mast cells as reflected in our corticosteroid naïve subjects.

Criticisms of this study include the cross-sectional design,the inability to study the confounding effect of steroid therapy in the asthma groups and adequate number of subjects to see disease effect. In-vivo studies have shown modulation of human lung mast cells but to date, histological assessment of endobronchial mast cell myositis with corticosteroid modulation has not been studied. In our study groups, there was a trend of higher median number of ASM mast cells with disease severity. Though unlikely, it may be possible the presence of increasing corticosteroid therapy may promote recruitement of mast cells to the airways. This seems unlikely as previously mast cells have been shown to infiltrate the ASM in the absence of anti-inflammatory treatment, in mild disease.

The confounding factor of concurrent corticosteroid therapy may account for the absence of an ASM eosinophilia in severe disease. Previously steroid naïve mild asthma has shown an absence of eosinophils within the ASM bundle. This finding was replicated in the study groups, but the the eosinophil is often a steroid responsive target even in refractory disease (ten Brinke et al. 2004). It can only be speculated if eosinophils are present within the ASM in severe disease but the introduction of increased steroid therapy attenuates this. It would not be feasible to study these subjects in the absence of anti-inflammatory therapy. In the severe asthma group, 2/7 subjects demonstrated ASM eosinophils; whether this is a feature of corticosteroid restistant disease remains unclear.

Our study displayed a non-significant increase in mast cell infiltration through mild-severe disease. Each of our study groups including controls contained relatively small numbers of subjects. We were unable to derive a power calculation at the start of this study to decide upon minimum number of subjects needed per study group; it is possible that our study is underpowered. We cannot reliably comment based on our data if further subjects were recruited and study, would a significant increase in ASM mast cells be seen between mild-severe asthma.

In conclusion, mast cell infiltration of the ASM bundle is a feature of many patients with moderate and severe asthma. Eosinophil infiltration of the ASM bundle was not a feature of moderate and severe asthma. Dissimilar to mild asthma, mast cell infiltration of ASM did not correlate with disordered airway physoiology which may be due to the concurrent use of corticosteroid use. We suggest that mast cell infiltration of the ASM may have an important role in asthma and targeted therapies toward this feature may improve asthma control in severe disease.

4.1.2 Inflammatory cell infiltration of large airway structures in chronic obstructive pulmonary disease

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive condition characterised by fixed airflow obstruction with airway inflammation and remodelling of the large and small airways with peripheral alveolar destruction (Hogg et al 2004). Some of these inflammatory changes within the airways are mediated through cytokines and chemokines secreted from locally recruited inflammatory cells.

The localisation of inflammatory cells to structural components of the airway is likely to be a critical feature of disease as it facilitates cross-talk between cells (Siddiqui et al. 2007a). For examples, plasma cells and leucocytes within large airway submucosal glands have been identified in increased numbers in chronic bronchitis suggesting a role in mucus hypersecretion (Zhu et al 2007). In asthma, the location of mast cells within the airway smooth muscle (ASM)-bundle has been implicated in the development of disordered airway physiology in asthma (Brightling et al 2002; Siddiqui, et al 2007a). In the small airway, neutrophils and T-lymphoyctes have been implicated with airflow obstruction in COPD (Baraldo et al 2004).

To date the full range of inflammatory cells infiltrating large airway structures has not been examined in COPD; there is an absence of studies examining ASM and glandular structure infiltration by inflammatory cells which may explain the COPD phenotype. To investigate this further, we enumerated inflammatory cells within structural compartments of proximal airway specimens from subjects with COPD and smoking and non-smoking controls.

MATERIALS AND METHODS

Subjects

Subjects were recruited from respiratory clinics COPD was diagnosed and severity categorised by using Global initiative for chronic Obstructive Lung Disease (GOLD) criteria (Global Initiative for Chronic Obstructive Lung Disease 2009). All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Smoking history and spirometry was recorded in all subjects. , Large airway tissue from surgical specimens taken during resection for malignancy was obtained from 10 COPD subjects (GOLD stages 1-2), and 18 non-COPD controls. The controls consisted of 10 subjects with and 8 subjects without >10 pack year smoking history with normal spirometry.

Immunohistochemical assessment of proximal airway

Large airway samples were dissected transversely from surgical specimens either from a ring of bronchus proximal to the macroscopic tumour in non small cell lung cancer or from the proximal stump of the main bronchus with extrapleural pneumonectomy for mesothelioma.Samples were fixed in acetone and embedded in glycomethacrylate as described previously (Berry et al 2004; Brightling et al 2002)(section 3.2.2.2). 2µm sections were cut and stained using monoclonal antibodies against tryptase (Dako UK, Ely UK), major basic protein for eosinophils (Monosan, Uden Holland), CD68 (Dako), neutrophil elastase (Dako), CD3 (Dako), and appropriate isotype controls (Dako) (section 3.2.2.3). The number

of positive nucleated cells was enumerated per mm^2 of bronchial submucosa, ASM-bundle and mucosal glands.

Statistical Analysis

Statistical analysis was performed using PRISM Version 4. Parametric data were expressed as mean (SEM), data that had a log normal distribution was log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (IQR). One-way analysis of variance and T-tests (Kruskal-Wallis and Mann-Whitney tests for non-parametric data) were used for across and between group comparisons respectively. Chi squared tests were used to compare categorical data.

RESULTS

For clinical characteristics of the subjects see table 4.3.

Enumeration of inflammatory cell types within compartments in the proximal airway

There were no differences in cellular infiltration across groups in the submucosa or ASM-bundle (Table 4.4). Examples of inflammatory cell infiltration of the ASM are shown in figure 4.3. CD3+ cells were increased in the bronchial glands in subjects with COPD compared to healthy controls (p=0.04, Table 4.4 and figure 4.4). Collating all study groups, the pattern of inflammatory cells in the different compartments was distinct and the proportion of each cell type per compartment was shown figure 4.5.

Table 4.3 Clinical characteristics

	Non-smokers	**Smokers**	**COPD**
Number	8	10	10
Age	55.9 (3.1)	53.3 (7.5)	69.4 (8.0)
Male (n)	6	8	8
PYH	2.5 (2.5)	31.0 (6.6)	47.4 (8.8)
Current Smokers	0	0	2
ICS use	0	0	4
Beclomethasone (equivalent)	0	0	800mcg(0.0)
LABA use	0	0	4
FEV_1(L)	2.89 (0.28)	2.65 (0.20)	1.83 (0.20)*
FEV_1 % predicted	88.4 (4.5)	85.6 (3.6)	67.3 (4.0)*
FVC (L)	3.73 (0.33)	3.46 (0.28)	3.29 (0.37)
FEV_1/FVC(%)	76.8 (1.9)	76.5 (1.3)	56.0 (2.4)*
Peripheral blood eosinophils^	0.16 (0.07-0.36)	0.12 (0.08-0.18)	0.11 (0.07-0.2)

All data expressed as mean (SE) except ^geometric mean (lower-upper 95%CI)

*$p<0.05$

Table 4.4 Inflammatory cell infiltration of airway structures

	Never	Smoker	COPD
Cells/ mm^2 submucosa			
Mast cells	49.8 (42.8)	38.1 (37.2)	28.6 (42.3)
Eosinophils	0 (0)	0 (4.1)	0 (11.1)
Neutrophils	41.5 (25.1)	28.1(29.2)	22.4 (83.9)
Macrophages	47.0 (42.9)	87.9 (83.5)	25.7 (44.2)
T-cells	36.2 (72.2)	26 (51.7)	24.8 (55.7)
Cells/ mm^2 ASM-bundle			
Mast cells	7.9 (5.9)	7.3 (15.2)	5.0 (12.5)
Eosinophils	0 (0)	0 (0)	0 (0)
Neutrophils	0 (0.45)	0 (0.78)	0.25 (4.22)
Macrophages	10.6 (11.7)	6.2 (9.9)	3.9 (13.1)
T-cells	1.3 (4.8)	2.2 (7.3)	2.7 (4.0)
Cells/ mm^2 glands			
Mast cells	12.9 (12.5)	18.7 (18.5)	21.9 (27.0)
Eosinophils	6.2 (9.5)	2.9 (6.6)	7.8 (13.3)
Neutrophils	8.0 (14.3)	4.2 (9.5)	4.9 (8.63)
Macrophages	7.3 (28.3)	6.5 (11.2)	11.1 (36.1)
T-cells	2.8 (10.1)	2.8 (50.9)	36.4 (100.8)*

Median (IQR); ASM=airway smooth muscle; *$p<0.05$

Figure 4.3 Example photomicrographs of a COPD subject with inflammatory cell infiltration of the ASM

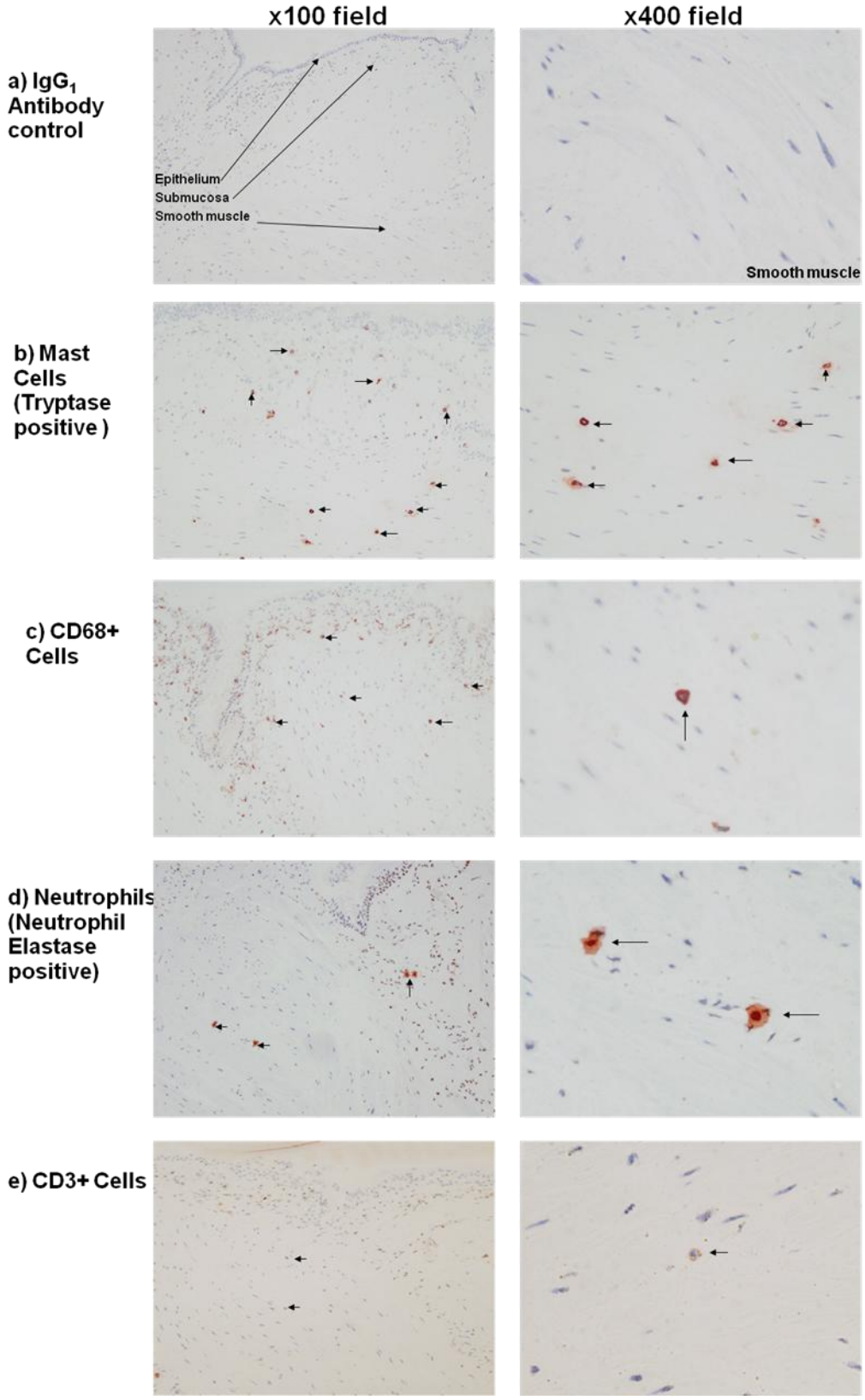


Figure 4.4 Example photomicrographs of a COPD subject with a) T-cells in glands and b) isotype control (x 400).

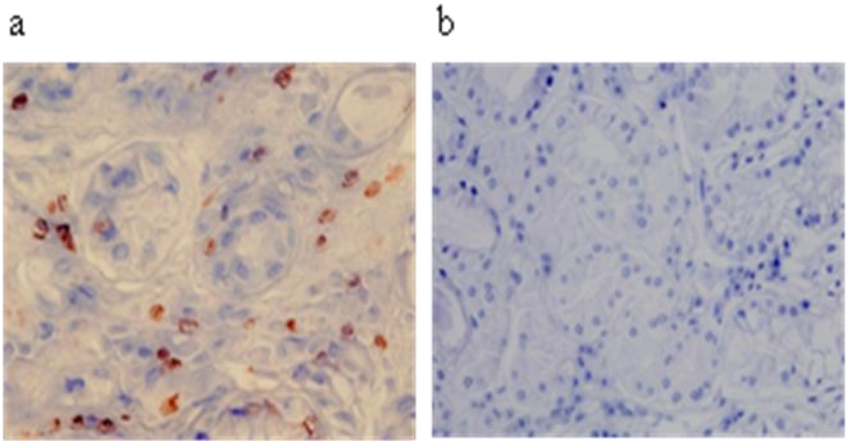
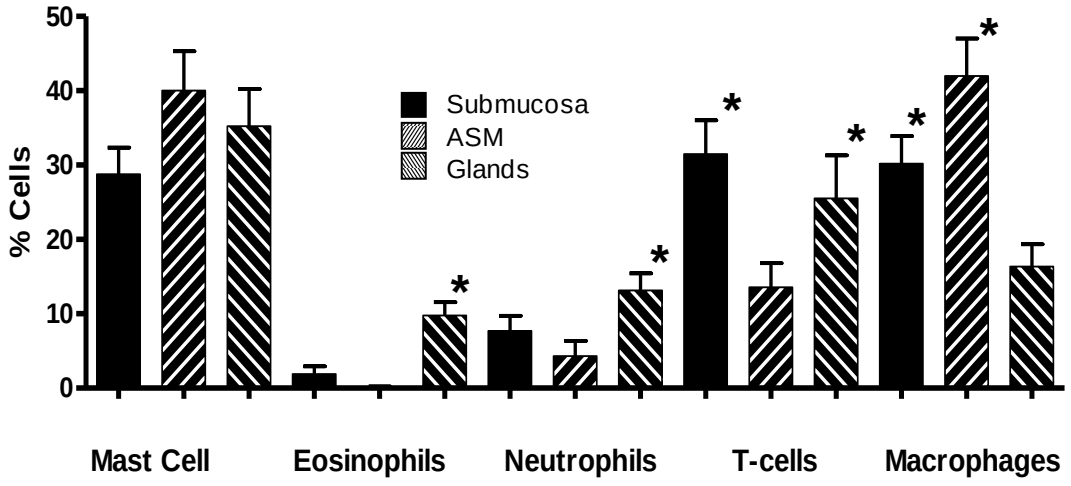


Figure 4.5 Mean (SEM) proportions of inflammatory cells in different airway compartments for all subjects. Proportions of inflammatory cells in different airway compartments for all subjects. *p<0.05



DISCUSSION

We identified that inflammatory cells were localised to all of the different airway compartments and the pattern of localisation was distinct for each inflammatory cell. These patterns were similar between those subjects with and without COPD with the exception of CD3+ cells in the bronchial glands which were increased in COPD.

Our study design enabled us to characterise for the first time the inflammatory cell localisation to different proximal airway compartments. The distribution of the inflammatory cells in the airway compartments was remarkably similar for subjects with and without COPD except for the number of T-cells in the bronchial glands. Collating cell counts from all study groups, the distribution of the inflammatory cells across large airway structures was distinct for each cell type (fig 4.5), suggesting that the recruitment and retention of inflammatory cells to different compartments of the airway wall is specific and under tight control (Siddiqui et al 2007a). We were unable to demonstrate an increase in the number of neutrophils or T-cells in the ASM- bundle as previously reported (Baraldo et al 2004) in the small airways in COPD or an increase in mast cells in the ASM-bundle as observed in asthma (Brightling et al 2002). Except for the preferential microlocalisation of CD3+ cells to glandular tissue in COPD, our findings of specific cell distribution across large airway compartments allows only speculation as to whether inflammatory cell microlocalisation and interaction between surrounding structures is different across COPD and control groups.

We report for the first time that CD3+ cells were increased in the bronchial glands in COPD. This is in contrast to previous reports in chronic bronchitis that have suggested increased

numbers of mast cells, neutrophils and macrophages, but not T-cells (Saetta et al 1997b; Siddiqui, et al 2007a). In fatal asthma the number of mast cells and neutrophils in mucosal glands was associated with mucus plugging (Carroll, Mutavdzic, & James 2002b). It is therefore likely that inflammatory cell-glandular interactions may be important in the development of glandular hyperplasia and mucus hypersecretion, which are important features of COPD.

Mast cell mysositis is a recognized feature of asthma (Brightling et al 2002). This study demonstrates an absence of preferential mast cell infiltration of the large ASM between COPD and control groups. This finding in surgical specimens, reflects endobronchial biopsy studies showing a similar degree of ASM infiltration in COPD and asthma (Slats et al 2007) and COPD and healthy controls (Liesker et al 2007). The influence of inflammatory cell infiltration upon ASM and consequent disease phenotype, may lie with the action of locally released cytokines. As discussed in section 4.1.1, ASM mast cells in asthma locally secrete IL-13 & -4 but not IL-5 which can influence the ASM contractility (Laporte et al 2001), (Brightling et al 2003c). Further study of the cytokine profiles of the inflammatory cells within the structures of large airway may further explain the disordered physiology seen in obstructive airways disease. There are a few methodological issues that require addressing with this study. One potential criticism of this approach is that the control subjects are undergoing surgery for malignancy and therefore are not healthy controls. The migration of inflammatory cells within the ASM may be a reflection of a loss of inhibitory forces associated with systemically secreted factors associated with carcinoma. In controls and COPD, we did not demonstrate any preferential distribution of inflammatory cells for the ASM bundle or submucosal glands except for CD3+ cells within glands. Our disease population reflected relatively mild COPD; potentially examination of the ASM bundle and

glandular structures in more severe COPD disease may demonstrate differences between disease and healthy controls. Unfortunately subjects with severe COPD are unlikely to be suitable for surgery, limiting tissue availability in severe disease.

In conclusion, inflammatory cell infiltration is evident in the ASM and glandular structures in COPD. Preferential localization of CD3+ lymphocytes is evident in COPD which may influence the phenotype. Further assessment of the role of T-cell infiltration of glandular structures is required.

4.2 EXPRESSION OF IL-13 IN OBSTRUCTIVE AIRWAYS DISEASE

4.2.1 LARGE AIRWAY

4.2.1.1 Increased bronchial biopsy IL-13 expression in severe asthma

Introduction

Asthma is characterised by the presence of variable airflow obstruction, airway hyper-responsiveness (AHR), and an airway inflammatory response often characterised by Th2-mediated eosinophilic airway inflammation (Wardlaw et al 2002) with mast cell infiltration of the airway smooth muscle (ASM) bundle (Brightling et al 2002). Comparisons between asthma and non-asthmatic eosinophilic bronchitis (EB), a common cause of chronic cough (Brightling et al. 1999a) have been informative about the key immunopathological features of asthma. Importantly overexpression of the Th2 cytokine interleukin (IL)-13 in sputum (Berry et al 2004; Komai-Koma et al. 2001), bronchial submucosa (Berry et al 2004), peripheral blood (Park et al 2005) and co-localisation to mast cells in the ASM-bundle (Brightling et al 2003c) are features of asthma that are not shared by EB and have therefore been implicated in the pathogenesis of AHR.

A role for IL-13 in the asthma paradigm is further supported by other human studies that have reported increased IL-13 mRNA expression in bronchial biopsies from subjects with moderate asthma (Humbert et al 1997a; Naseer et al 1997)and from sputum cells from corticosteroid naïve and inhaled corticosteroid treated asthmatics (Truyen et al 2006). In addition, following allergen challenge in mild asthmatics bronchoalveolar lavage IL-13 concentration was upregulated (Huang et al. 1995). This association between IL-13 and

asthma in humans is supported by animal models (Wills-Karp et al 1998). T-lymphocyte deficient mice have shown exogenous addition of IL-13 promotes AHR and airway inflammation, while neutralisation of IL-13 in murine models can resolve these features(Grunig et al 1998).

To date, human studies have focused their investigation on mild-moderate asthmatics (Humbert et al 1997a; Naseer et al 1997; Berry, et al 2004; Brightling et al 2003c; Komai-Koma et al 2001; Park et al 2005). Therefore whether IL-13 expression is associated with severe refractory disease (ATS 2000) is unclear. Refractory asthma accounts for a large proportion of the morbidity, mortality and health care costs associated with this disease. Thus, there is a pressing need to identify and test novel targets in this group of patients.

We hypothesised that, in addition to mild asthma, increased IL-13 expression is a feature of severe refractory asthma. To test our hypothesis we measured the sputum IL-13 concentration and the number of IL-13+ cells in the bronchial submucosa and ASM-bundle in a cross-sectional study that included mild, moderate and severe refractory asthmatics and healthy controls. To further define the possible role of IL-13 in asthma we investigated the relationship between IL-13 expression and disease severity, asthma control, AHR, spirometry and eosinophilic inflammation.

Methods

Subjects

Subjects were recruited from local primary health care, respiratory clinics, hospital staff and by local advertising. Asthma was defined and severity categorised by international (GINA)

guidelines (Global Initiative for Asthma 2009) and ATS criteria for refractory asthma (2000). Normal subjects had no history of respiratory symptoms and normal spirometry. All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Clinical characterisation

Subjects underwent spirometry, allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat and grass pollen, a methacholine inhalation test using the tidal breathing method (Sterk et al 1993) and sputum induction using incremental concentrations of nebulised hypertonic saline 3, 4 and 5% each for 5 minutes (Pavord, et al 1997)(section 3.1.3&.4). Subjects with a sputum eosinophil count>3% were defined as having eosinophilic asthma. In those subjects with moderate to severe disease, symptom control was assessed by the Juniper Asthma Control Questionnaire (ACQ; section 3.1.6) (Juniper et al 1999). 2/7 subjects with moderate asthma and 2/7 control subjects participated in sputum IL-13 study (see section 4.2.2.1).

IL-13 measurement in endobronchial biopsies

Subjects with assessable ASM (>0.1mm2) in bronchial biopsies were recruited (Brightling et al 2002). Asthma was categorised as mild (GINA 1; n=14), moderate (GINA 2/3; n=7) or severe (GINA 4/5). All of the subjects in the severe asthma category had severe refractory asthma(ATS 2000). In order to examine IL-13 expression in non-eosinophilic asthma we included 7 GINA 1 asthmatics with an absence of sputum eosinophilia<1.9% on 2 separate occasions. In this cohort we chose to specifically compare corticosteroid naïve eosinophilic

and non-eosinophilic asthmatics to exclude the possible confounder of treatment and applied a rigorous definition for non-eosinophilic asthma (Berry et al 2007).

After characterisation, subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines (BTS 2001). Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae; were fixed in acetone and embedded in glycomethacrylate as described previously (section 3.2.2.2)(Brightling et al 2002).

2µm sections were cut and stained using monoclonal antibodies against IL-13 (R&D systems, Oxfordshire, UK) in addition to staining for mast cells major basic protein for eosinophils appropriate isotype controls (DAKO) (see section 4.1.1). The number of positive nucleated cells was enumerated per mm2 of bronchial submucosa or ASM-bundle by a blinded observer. Sequential sections were stained for IL-13 and tryptase or MBP to assess colocalization as described previously (section 3.2.2.3)(Brightling et al 2003c).

Statistical Analysis-For statistical analysis refer to section 4.1.1.

Results

Clinical and sputum characteristics for subjects are shown in table 4.1 in section 4.1.1 and the number of IL-13+ cells in the bronchial submucosa and ASM-bundle are shown in table 4.5. Enumeration of large airway mast cells and eosinophils are presented in table 4.2 (section 4.1.1). Representative photomicrographs of IL-13+ cells in the submucosa and ASM-bundle are as shown in figure 4.6.

The number of IL-13+ cells in the bronchial submucosa was raised in all asthma severity groups in comparison to the normal controls (p=0.006; see figure 4.7a and table 4.5). The

Table 4.5 Median (IQR) inflammatory cell infiltration of submucosa and ASM

	Normal	Mild Asthma	Moderate Asthma	Severe Asthma
Submucosa cells/mm^2				
IL-13+*	0 (0)	3.7 (9.1)*	4.3 (12.0)*	12.7 (13.9)*
ASM cells/mm^2				
IL-13+**	0 (0)	1.0 (2.2)**	0 (0)	4.6 (5.2)**

*p<0.01 nomal vs other groups (Kruskal Wallis)

**p<0.05 mild vs severe group, p<0.01 moderate vs severe

Figure 4.6 Examples of IL-13+ cells in the submucosa and ASM bundle in subjects with asthma. Representative photomicrographs of bronchial biopsy sections from a subject with severe asthmatic illustrating isotype control (A; original magnification x200), IL-13+ cells present in the bronchial submucosa and ASM bundle (B; original magnification x 200), and IL-13+ cells within the ASM bundle (C; original magnification x400) are shown. IL-13+ cells are highlighted in the submucosa by arrows and in the ASM bundle by arrowheads.

A) B) C)

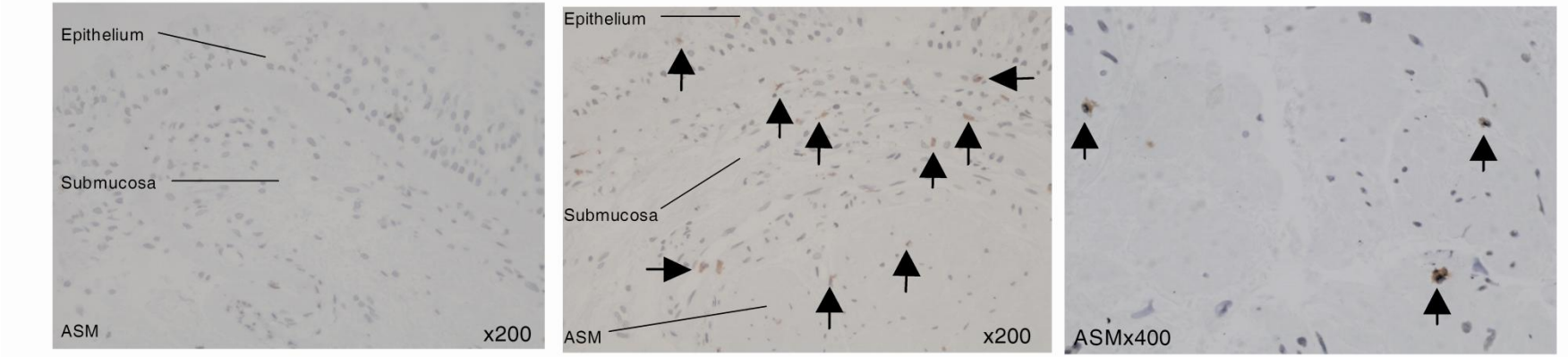


Figure 4.7a Number of IL-13+ cells in submucosa in healthy controls and subjects with asthma.

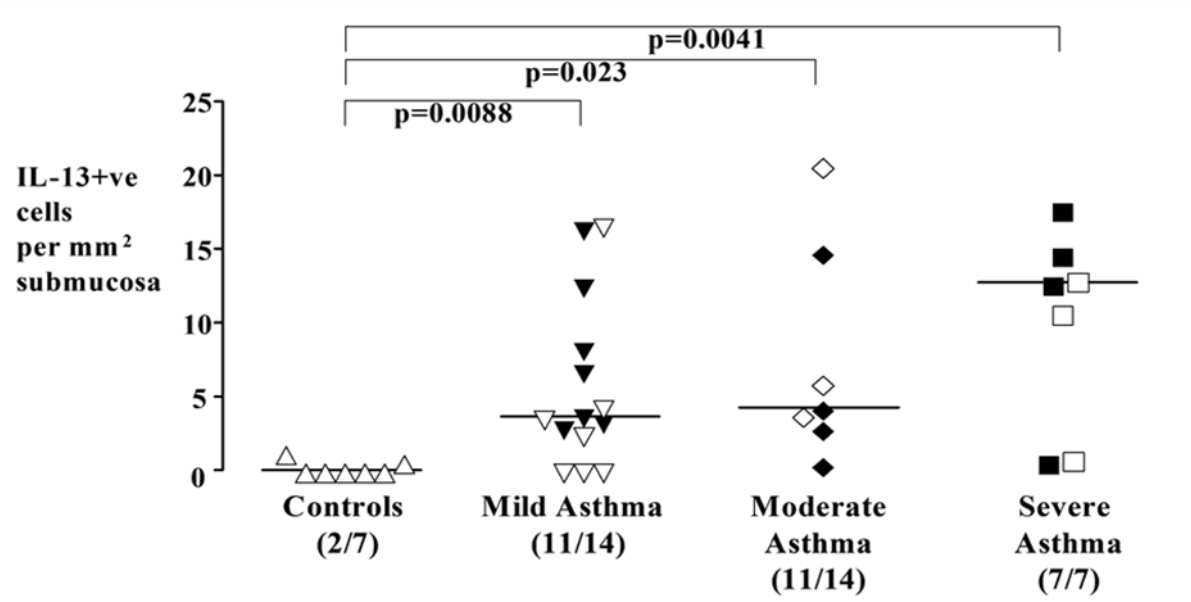
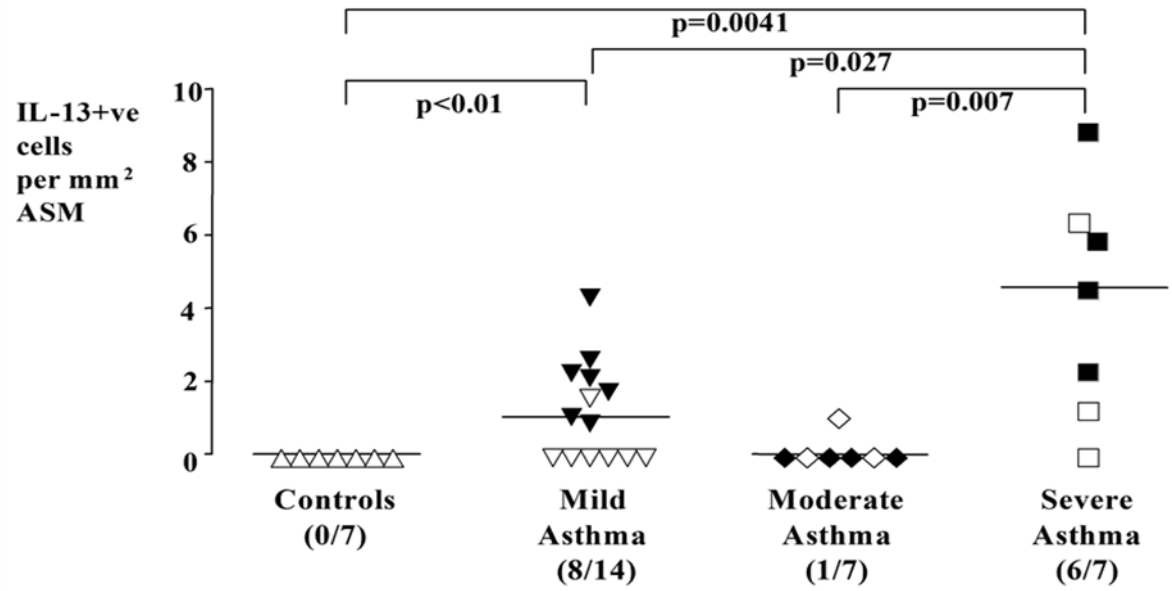


Figure 4.7b Number of IL-13+ cells in ASM in healthy controls and subjects with asthma



Closed symbols represent subjects with sputum eosinophilia>3%. Horizontal bars represent median value. Values in parentheses represent subjects with IL-13+ cells in submucosa or ASM/total subjects in group

mean (SEM) proportion of IL-13+ cells in the submucosa colocalized to mast cells was 22 (4)% and to eosinophils was 66 (6)%. There were no differences across disease severity. In the ASM-bundle the number of IL-13+ cells was increased in mild and severe asthmatics compared to the normal control group ($p<0.01$; table 4.5). The number of IL-13+ cells in the ASM-bundle was increased in the severe asthmatics in comparison to the mild asthma ($p=0.027$) and moderate asthma ($p=0.007$; figure 4.7b). The number of IL-13+ cells in the ASM-bundle was increased in the eosinophilic mild asthmatics (2.2 [1.57]) compared to the non-eosinophilic mild asthmatics (0 [0]; $p=0.002$) (figure 4.7b). The mean (SEM) proportion of IL-13+ cells in the ASM-bundle that were co-localized to mast cells was 99 (0.8)% and to eosinophils 0%. This was not different across disease severity. There was no significant correlation between the number of IL-13+ cells in either the ASM-bundle or submucosa and FEV1 % predicted or AHR.

The ACQ scores in those subjects with IL-13+ cells in the ASM-bundle was not significantly higher than subjects without (2.4 [2.1] versus 1.9 [2.0]; $p=0.5$). There was no significant correlation between ACQ scores and IL-13 expression within the submucosa and ASM.

The number of IL-13+ cells within the submucosa positively correlated with the degree of sputum eosinophil count across all asthma disease groups ($Rs=0.42$; $p=0.042$). The number of IL-13+ cells within the ASM-bundle positively correlated with sputum eosinophil count ($Rs=0.40$; $p=0.05$) and the number of eosinophils in the submucosa ($Rs=0.39$; $p=0.038$).

Discussion

For the first time we have shown the number of IL-13+ cells in the bronchial submucosa and ASM-bundle were increased in severe asthma. Interestingly, in contrast to severe asthma IL-

13+ cells in the ASM-bundle were not observed in moderate disease. IL-13 expression within the submucosa and ASM-bundle positively correlated to the intensity of eosinophilic airway inflammation. Bronchial biopsy expression of IL-13 was not related to FEV_1 or AHR.

Consistent with the view that IL-13 is associated with eosinophilic airway inflammation we found that IL-13 expression in the bronchial biopsies was positively correlated with eosinophilic inflammation in sputum and biopsies. In our group of mild asthmatics we included a group of well-characterised non-eosinophilic asthmatics. These subjects had failed to demonstrate eosinophilic inflammation in their sputum on repeated occasions. The inclusion of this group gave us an opportunity to examine IL-13 expression in tissue in a group of non-eosinophilic asthmatics without the potential confounder of corticosteroid therapy. We found the number of IL-13+ cells in the ASM-bundle were markedly reduced in those subjects with non-eosinophilic asthma. The identification of differential expression of IL-13 in eosinophilic and non-eosinophilic asthma is important as it suggests there are fundamental differences in the underlying pathogenesis of these disease phenotypes and may be important in patient selection for the use of novel therapies in asthma.

In mild asthma mast cells in the ASM-bundle express IL-4 and IL-13 (Brightling et al 2003c). In this study we found that the number of IL-13+ cells in the ASM-bundle was increased in mild and severe asthma and in keeping with our earlier report (Brightling et al 2003c) the vast majority of these cells were mast cells. Many cytokines exert their effects across distances of a few microns (Wardlaw et al 2002). The immediate proximity of the IL-13+ cells and ASM is therefore likely to be functionally important. ASM is an important source of pro-

inflammatory mediators such as CCL11 and IL-13 induces the ASM-synthesis and release of this and other chemokines (Hirst et al 2002; Peng et al 2004; Sutcliffe et al 2006). In vitro IL-13, but not IL-4, has been shown to attenuate ASM relaxation to β-agonists (Laporte et al 2001) and augment contractility to acetylcholine (Grunstein et al 2002) suggesting that IL-13 may induce AHR by directly activating ASM. Mast cell derived IL-13 in the ASM-bundle has the potential to promote IgE-mediated mast cell activation and proliferation via an autocrine mechanism (Kaur et al 2006a). Hence the location of IL-13+ cells in the ASM-bundle and the consequent IL-13 ASM interactions may contribute to the pathogenesis of severe asthma.

We demonstrated a paucity of IL-13+ cells within the ASM bundle in subjects with moderate asthma compared to subjects with severe asthma. This may reflect successful downregulation of IL-13 expression by corticosteroid use in patients with potentially more corticosteroid responsive disease as compared to the severe asthma group. This is speculative especially as we did not see an inverse relationship between IL-13 expression and subject asthma control scores.

We showed positive correlation between eosinophilic inflammation (as measured by sputum eosinophilia) and IL-13 expression within the submucosa and ASM in asthma across disease severity. These observations may be a reflection of multiple testing but there is biological plausibility with the majority of IL-13+ cells being co-localised to eosinophils within the submucosa in our study.

One criticism of our study is the cross-sectional design. We have not assessed the response to corticosteroids within individuals and therefore we do not know whether the IL-13 expression in mild disease is corticosteroid responsive as suggested by the relative lack of IL-13 expression in moderate disease. However an earlier report found that in patients that were clinically corticosteroid responsive treatment with oral corticosteroid for 1-week led to a reduction in IL-13 mRNA expression in bronchial biopsies, whereas in those subjects that were clinically corticosteroid non-responsive IL-13 mRNA expression persisted after treatment (Naseer et al 1997). This is entirely consistent with our view that IL-13 expression is attenuated in those asthmatics with moderate disease adequately controlled by inhaled corticosteroids. This apparent shortcoming of our study design does not detract from our observation that severe disease was associated with IL-13 expression.

In conclusion, IL-13 over-expression is a feature of many patients with severe asthma with IL-13+ cells in the bronchial submucosa and ASM-bundle. IL-13 expression was related to asthma control and intensity of eosinophilic inflammation but not to severity of disordered airway physiology. We suggest that IL-13 may have an important role in the pathophysiology of severe asthma and future studies targeted at the IL-13 axis are eagerly awaited.

4.2.1.2 Bronchial mucosal expression of interleukin (IL)-13 is not increased in chronic obstructive pulmonary disease

Introduction

Airway inflammation and remodeling within the airways in COPD, are mediated through cytokines and chemokines secreted from locally recruited inflammatory cells (Siddiqui et al 2007a). Interleukin (IL)-13, a TH_2 cytokine, is found within T-lymphocytes, mast cells, eosinophils, basophils and macrophages and has been implicated in recruitment of inflammatory cells from the blood to lung tissue, regulation of matrix metalloproteinases and IgE production (Wills-Karp et al 1998). Murine models suggest a central role for IL-13 in the pathogenesis of COPD (Zheng et al 2000; Zhu et al. 1999). In contrast, data from human studies has been conflicting. IL-13 expression within macroscopic emphysematous lung tissue was low in severe emphysema (Boutten et al 2004), whereas IL-13 expression in the proximal airway from subjects with chronic bronchitis was increased within the submucosa (Miotto et al 2003). With this discordance in published literature, it is important to clarify if there is evidence to support large airway IL-13 expression in COPD. It may be the location of secreted. IL-13 that is more influential over nearby large airway structures with respect to COPD phenotype rather than the total amount of IL-13 measured within tissue. IL-13 has yet to be assessed in the structures of proximal airway in COPD.We hypothesised that in COPD inflammatory cells are increased in the submucosal glands and ASM-bundle with increased IL-13 expression. To test our hypothesis we examined the concentration of IL-13 in induced sputum samples, the number of inflammatory cells and IL-13 expression in the structural compartments of proximal airways specimens from subjects with COPD, and smoking and non-smoking controls.

Materials and Methods

Subjects

Subjects were recruited from respiratory clinics. COPD was diagnosed and severity categorised by using Global initiative for chronic Obstructive Lung Disease (GOLD) criteria (Global Initiative for Chronic Obstructive Lung Disease 2009). All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Smoking history and spirometry was recorded in all subjects. Large airway tissue from surgical specimens was obtained from 10 COPD subjects (GOLD stages 1-2), and 18 non-COPD controls. The controls consisted of 10 subjects with and 8 subjects without >10 pack year smoking history with normal spirometry.

Immunohistochemical assessment of proximal airway

Proximal airway samples were collected from surgical specimens and fixed in acetone and embedded in glycomethacrylate as described previously (section 3.2.2.2) (Berry et al 2004; Brightling et al 2002). 2µm sections were cut and stained using monoclonal antibodies against IL-13 (R&D, Abingdon UK) and appropriate isotype controls (Dako). The number of positive nucleated cells was enumerated per mm^2 of bronchial submucosa, ASM-bundle and mucosal glands (section 3.2.2.3).

Statistical Analysis For statistical analysis refer to section 4.1.2

RESULTS

IL-13 expression in large airway tissue specimens

Examples of IL-13+ cells in airway compartments are shown in figure 4.8. Clinical characteristics are shown in table 4.3 (section 4.1.2). There was a paucity of IL-13+ cells within the submucosa, ASM-bundle and glands with no significant differences across groups (Table 4.6).

DISCUSSION

IL-13 expression was not increased in the bronchial mucosa in COPD. The paucity of IL-13+ cells in the bronchial submucosa, glands and ASM-bundle compartments of the airway was striking. Our data is the first attempt to define IL-13 expression within the large airway in COPD. Large airway IL-13 expression in smokers has previously been characterized in sufferers with chronic bronchitis (Miotto et al 2003). This subject population displayed a range of airflow obstruction but further subdivision by airflow obstruction was not made. IL-13 was overexpressed in the submucosa but not bronchial glands in chronic bronchitis. Positive controls were not used with immunohistochemical staining in this study, so it is difficult to accept the validity of these findings.

The functional importance of large airway expression of IL-13 in COPD is unclear. The small airway and peripheral lung tissue in COPD are more influential upon measures of airflow

Figure 4.8 Example photomicrographs of IL-13+ cells in a COPD subject in a) submucosa, b) ASM-bundle, c) glands (x400) and d) isotype control (x200).

Arrows highlight IL-13+ cells in airway compartments.

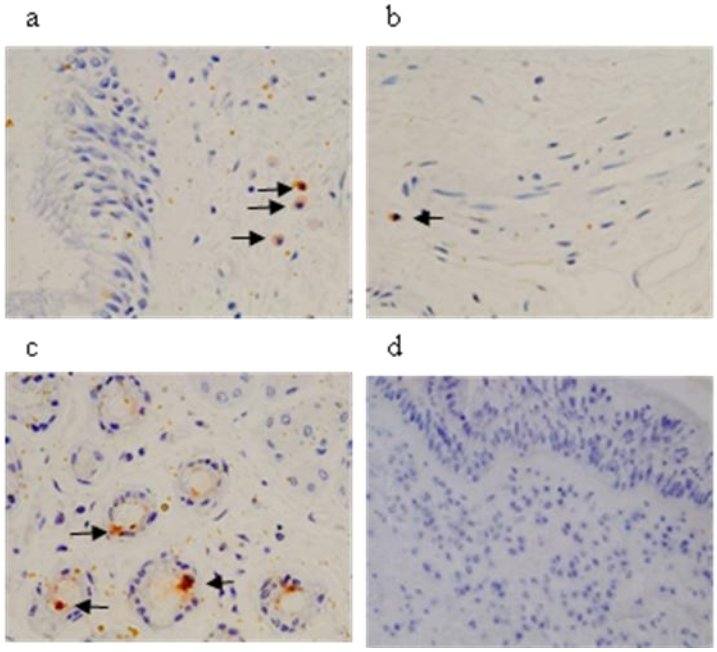


Table 4.6. **IL-13 cell infiltration of airway structures**

	Never	**Smoker**	**COPD**
Cells/ mm^2 submucosa			
IL-13 cells	1.3 (2.8)	1.0 (6.3)	0 (0.91)
Cells/ mm^2 ASM-bundle			
IL-13 cells	0 (0.02)	0 (0.13)	0 (0)
Cells/ mm^2 glands			
IL-13 cells	0 (0.35)	0.16 (0.97)	0 (0.35)

Median (IQR); ASM=airway smooth muscle;

obstruction (Hogg, Macklem, & Thurlbeck 1968). Potentially, IL-13 expression within the peripheral lung compartment may have more functional significance which this study did not address. Initial studies of IL-13 protein and mRNA content in peripheral lung tissue suggested an absence in emphysema (Boutten et al 2004). Recently IL-13 mRNA has been identified in ex-vivo lung from patients with COPD undergoing transplant. This has been correlated with mucus secretion and sourced to alveolar macrophages, suggesting IL-13 may be of functional importance in the peripheral lung.

One potential criticism of this approach is that the control subjects are undergoing surgery and therefore are not healthy controls. Although we concede this limitation this would only confound our findings if IL-13 expression was increased in all of the subject groups.

In conclusion, our findings do not support a role for IL-13 in the large airways in mild COPD. It is unclear if IL-13 expression is significant in COPD of greater disease severity. To attempt to address this issue, IL-13 expression in sputum across a range of severity in COPD is assessed in section 4.2.2.2.

4.2.2 SPUTUM

4.2.2.1 Increased sputum IL-13 expression in severe asthma

Introduction

Asthma is characterised by the presence of variable airflow obstruction, airway hyper-responsiveness (AHR), and an airway inflammatory response often characterised by Th2-mediated eosinophilic airway inflammation (Wardlaw et al 2002) with mast cell infiltration of the airway smooth muscle (ASM) bundle (Brightling et al 2002). Importantly overexpression of the Th2 cytokine interleukin (IL)-13 in sputum (Komai-Koma et al 2001) (Berry et al 2004), bronchial submucosa (Berry et al 2004), peripheral blood (Park et al 2005) and co-localisation to mast cells in the ASM-bundle (Brightling et al 2003c) are features of asthma that are not shared by EB and have therefore been implicated in the pathogenesis of AHR. To date, sputum IL-13 expression has been examined in mild asthma but expression in more severe disease is unknown.

We hypothesised that, in addition to mild asthma, increased sputum IL-13 expression is a feature of severe refractory asthma. We consequently measured the sputum IL-13 concentration in a cross-sectional study that included mild, moderate and severe refractory asthmatics and healthy controls. To further define the possible role of IL-13 in asthma we investigated the relationship between IL-13 expression and disease severity, asthma control, AHR, spirometry and eosinophilic inflammation.

Methods

Subjects

Subjects were recruited from local primary health care, respiratory clinics, hospital staff and by local advertising. Asthma was defined and severity categorised by international (GINA) guidelines (Global Initiative for Asthma 2009) and ATS criteria for refractory asthma (ATS 2000). Normal subjects had no history of respiratory symptoms and normal spirometry. All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Clinical characterisation

Subjects underwent spirometry, allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat and grass pollen, a methacholine inhalation test using the tidal breathing method (Sterk et al 1993) and sputum induction using incremental concentrations of nebulised hypertonic saline 3, 4 and 5% each for 5 minutes (Pavord et al 1997). Subjects with a sputum eosinophil count>3% were defined as having eosinophilic asthma. In those subjects with moderate to severe disease symptom control was assessed by the Juniper Asthma Control Questionnaire (ACQ) (Juniper et al 1999).

Sputum IL-13 measurement

Subjects with asthma were categorised as mild (GINA 1; n=34), moderate (GINA 2-4; n=21), or severe (GINA 5; n=26). All the subjects in the severe group also fulfilled the criteria for severe refractory asthma(ATS 2000). 11/26 of these severe asthmatics were treated with

intramuscular triamcinolone based on clinical grounds due to symptoms deemed unresponsive to oral corticosteroid therapy.

Sputum IL-13 was measured by ELISA (Caltag-Medsystems, Buckinghamshire, UK). The mucolytic dithiothreitol (DTT) affected recovery of IL-13. The percentage recovery (coefficient of variation) of standards spiked with DTT compared to standards without DTT was 38% (14%). Standards were consequently dialysed overnight in PBS (sigma) to remove DTT. Recovery of IL-13 standard post dialysis was 90% (13% coefficient of variation). Recovery of the exogenous IL-13 spike added to sputum samples was 95% (6.5% coefficient of variation) demonstrating validity of the assay. Consequently all samples were dialysed in PBS before assay. The lower limit of detection was 10pg/g sputum. An example of standard curve from IL-13 ELISA analysis is shown in figure 4.9

Statistical Analysis

For statistical analysis methodology refer to section 4.1.1

Results

Clinical and sputum characteristics are shown in table 4.7. The groups with asthma were well-matched for AHR and sputum eosinophilic inflammation. The sputum IL-13 concentration for each subject is shown in figure 4.10. The proportion of subjects with measurable IL-13 in their sputum supernatant was increased in those with severe asthma (10/26) and mild asthma (15/34) compared to normal controls (4/32) ($p<0.05$). In addition, the proportion of subjects with measurable IL-13 in mild asthma was raised compared to subjects with moderate asthma (3/21; $p=0.022$). In the 11 severe asthmatics requiring treatment with intramuscular triamcinolone, 6 subjects had measurable IL-13 in their sputum

figure 4.9 Example standard curve of IL-13 ELISA

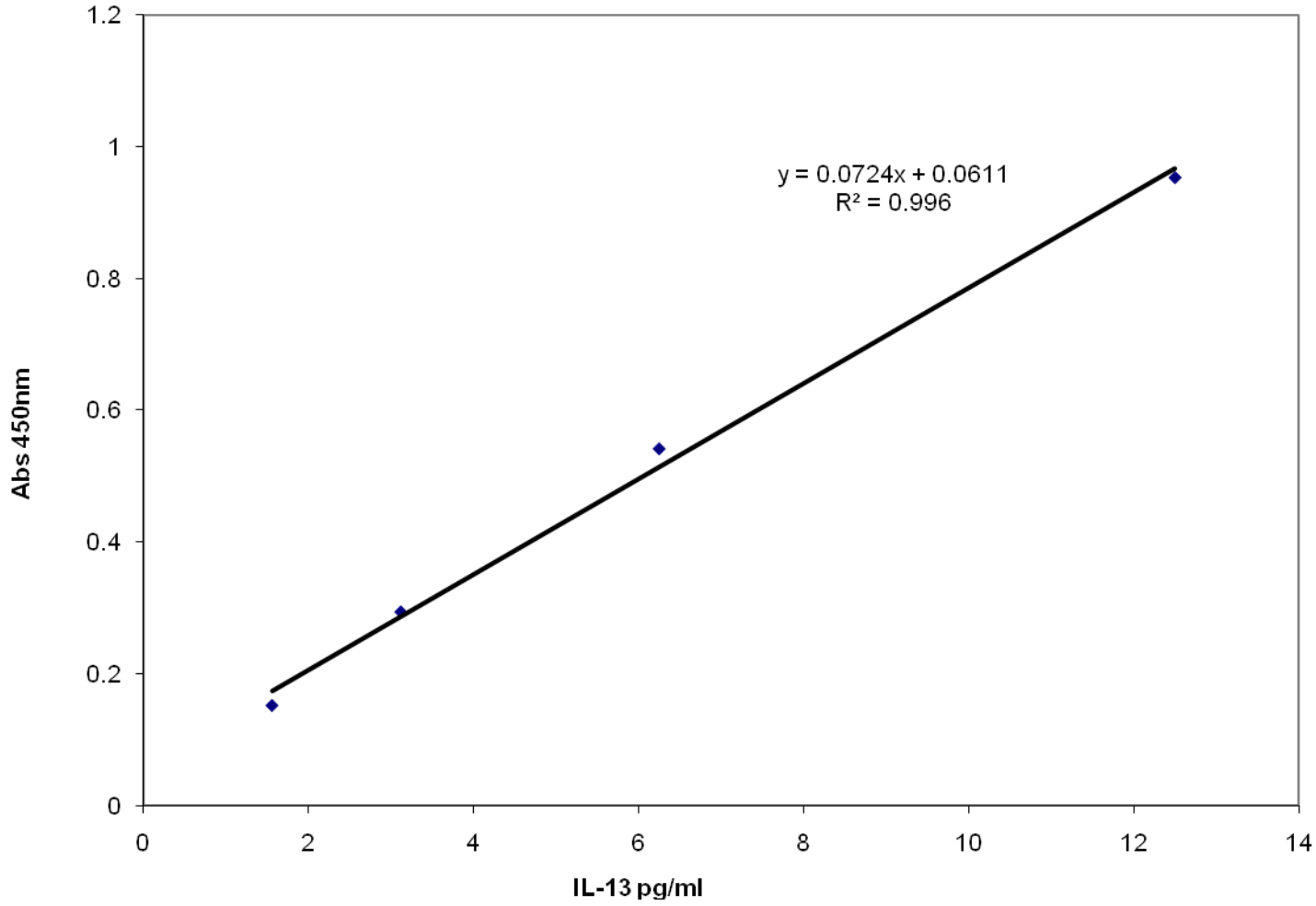


Table 4.7 Clinical and Sputum Characteristics

	Normal	Mild Asthma	Moderate Asthma	Severe Asthma
Number	32	34	21	26
ICS use (%)	0	0	100	100
ICS dose (µg/day)[#]	0	0	1575 (224.1)	1704 (95.7)
LABA use (%)	0	0	52	92
Systemic CS use (%)	0	0	0	100
Oral/Intramuscular CS dose (mg/day)[#]	0	0	0	10.3(2.2)/ 54.6(6.1)
Age [#]	47.6 (3.0)	48.5 (0.1)	49.5 (3.4)	48.3 (3.4)
Male	15	16	9	11
Never smokers	25	31	16	23
Pack years[#]	8.2 (2.9)	1.7 (0.8)	5.1 (2.3)	3.5 (2.2)
Atopy (%)	24	44	87	74
$PC_{20}FEV_1$ (mg/ml)[^]	>16	1.3 (0.12)	0.36 (0.2)	0.25 (0.4)
FEV_1% predicted[#]	96.4 (2.9)	82.4 (4.3)[*]	66.6 (6.7)[**]	59.6 (4.3)[***]
Bronchodilator reversibility (%)[#]	ND	5.8 (2.2)	6.5 (3.5)	8.6 (2.0)
FEV_1/FVC %[#]	78.7 (1.7)	72.0 (1.9)	68.6 (2.8)	66.9 (2.7)
Sputum Cell Counts				
Eosinophil %^[∞]	0.5 (0.1)	2.3 (0.1)[∞]	2.9 (0.2)[∞]	3.6 (0.1)[∞]
Neutrophil %[#]	46.6 (4.6)	61.6 (4.3)	59.7 (37.0)	64.5 (4.3)
Macrophage %[#∞]	48.3 (4.6)	28.0 (3.7)[∞]	26.5 (18.7)[∞]	17.7 (2.5)[∞]
Lymphocyte %[#]	1.8 (0.4)	1.1 (0.2)	0.4 (1.5)	0.4 (0.1)
Epithelial cells %[#]	3.5 (1.0)	3.1 (0.7)	2.3 (5.5)	5.6 (1.4)

[#]mean (SE), ^geometric mean (log SE), CS-corticosteroid, ND-not done

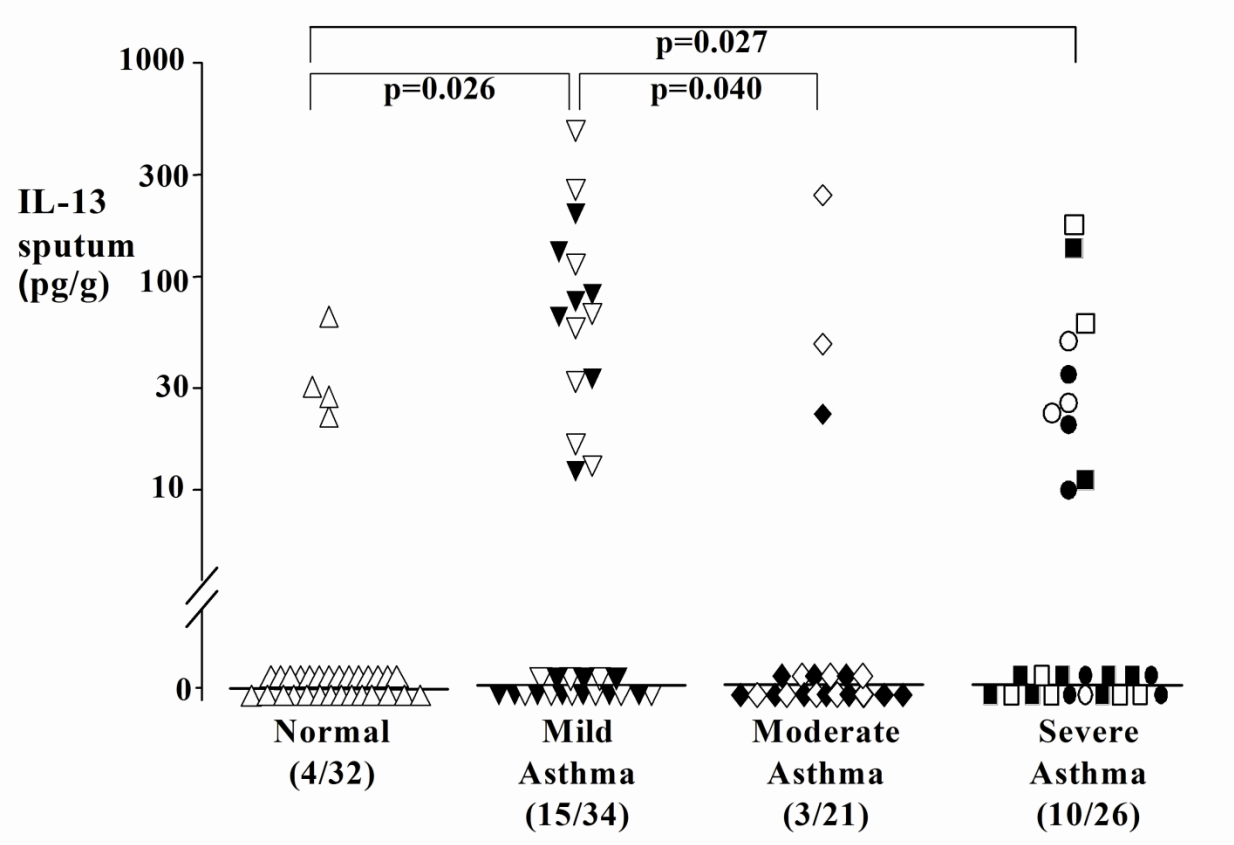
*p<0.01 mild vs severe, **p<0.001 normal vs moderate

***p<0.001 normal vs severe (Tukey's multiple comparison test)

[∞]p<0.0001 (ANOVA) normal vs mild/moderate/severe asthma

Figure 4.10 Sputum IL-13 concentration in controls and asthma.

Solid symbols indicate sputum eosinophilia greater than 3%. Horizontal bars represent median value. Values in parentheses represent subjects with measurable IL-13/total subjects in group**.** Circle symbols represent subjects in severe asthma group requiring parenteral triamcinolone (n=11/26)



(p=0.01; compared to healthy controls). The sputum IL-13 concentration was elevated in those with mild asthma compared to subjects with moderate disease (p=0.04) and controls (p<0.01). Sputum IL-13 concentration was raised in the severe asthma group when compared to controls (p=0.027) but was not significantly raised compared to moderate disease (p=0.059).

There was no significant correlation between sputum IL-13 concentration and any of the sputum differential cell counts, FEV_1 or AHR in the asthmatic subjects. Sputum IL-13 exhibited a significant positive correlation with ACQ scores (Rs=0.35; p=0.04) for subjects with moderate and severe asthma. In these 2 groups, subjects with detectable IL-13 had higher ACQ scores (3.2 [1.4]) compared to subjects with immeasurable IL-13 (2.1 [1.7]; p=0.05).

Discussion

For the first time we have shown that sputum IL-13 concentration was increased in severe asthma. Interestingly, in contrast to severe asthma increased sputum IL-13 concentration was not observed in moderate disease. This finding in sputum replicates IL-13 expression within the ASM in severe asthma (see section 4.2.1). Sputum IL-13 concentration was related to asthma control as determined by the ACQ. Both sputum and bronchial biopsy expression of IL-13 was not related to FEV_1 or AHR. We were unable to demonstrate a relationship between sputum IL-13 concentration and sputum eosinophil counts. However, this was perhaps not surprising as less than 50% of the subjects had measurable IL-13 in their sputum

There is compelling evidence, which implicates IL-13 as a central mediator in the pathogenesis of asthma from studies using animal models and in human disease (Wills-Karp & Chiaramonte 2003). A number of reports describe an association between polymorphisms in the IL-13 gene with aspects of the asthma phenotype (Howard et al 2001) (van der Pouw Kraan TC et al 1999). In mild to moderate asthma, but not in non-asthmatic eosinophilic bronchitis, IL-13 was elevated in BAL, bronchial biopsies and sputum (Berry et al 2004; Brightling et al 2003c; Naseer et al 1997; Park et al 2005). Similarly, IL-13 mRNA expression was increased in sputum cells from corticosteroid naïve and inhaled corticosteroid treated asthmatics (Truyen et al 2006). We now provide evidence to support a role for IL-13 in severe refractory disease. Sputum IL-13 concentration and the number of IL-13+ cells in bronchial biopsies were increased in severe disease. However, our data do suggest that the relationship between IL-13 expression in sputum and bronchial biopsies, disordered airway physiology and asthma control is complex. We were unable to demonstrate a correlation between IL-13 expression and AHR or FEV1. In contrast to severe disease IL-13 expression was not increased in moderate disease. The relative lack of IL-13 expression in this group of moderate asthmatics is likely to reflect a favorable response to corticosteroid therapy. However, these subjects had persistent AHR suggesting that AHR and IL-13 expression can be disassociated. Interestingly, sputum IL-13 concentration was related to asthma control. Severe refractory asthma is characterised by poor control, recurrent exacerbations and the development of persistent airflow obstruction. The rates of death and complications are high among patients with severe refractory asthma and these patients account for a disproportionate amount of the health care cost attributed to asthma (ATS 2000). There is therefore a significant unmet need in this group of asthmatics. Whether therapies targeted at IL-13 are effective in severe refractory asthma needs to be investigated.

One criticism of our study is the cross-sectional design. We have not assessed the response to corticosteroids within individuals and therefore we do not know whether the IL-13 expression in mild disease is corticosteroid responsive as suggested by the relative lack of IL-13 expression in moderate disease. This is entirely consistent with our view that IL-13 expression is attenuated in those asthmatics with moderate disease adequately controlled by inhaled corticosteroids. This apparent shortcoming of our study design does not detract from our observation that severe disease was associated with IL-13 expression. In addition, although the measurement of IL-13 in sputum is limited by the sensitivity of the assay with several subjects having undetectable sputum IL-13 we are confident that this observation is robust as it was confirmed in two cohorts in sputum and in bronchial biopsies. Importantly, sputum IL-13 concentration was increased even in subjects treated with intramuscular triamcinolone excluding the possibility of poor adherence to therapy.

In conclusion, IL-13 over-expression is a feature of many patients with severe asthma with increased sputum IL-13 concentration and IL-13+ cells in the bronchial submucosa and ASM-bundle. Sputum IL-13 expression was related to asthma control as measured by the ACQ. We suggest that IL-13 may have an important role in the pathophysiology of severe asthma and future studies targeted at the IL-13 axis are eagerly awaited.

4.2.2.2 Induced sputum Interleukin-13 is not increased in Chronic Obstructive Pulmonary Disease

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive condition characterised by fixed airflow obstruction with airway inflammation and remodelling of the large and small airways with peripheral alveolar destruction (Hogg, Macklem, & Thurlbeck 1968). Some of these inflammatory changes within the airways are mediated through cytokines and chemokines secreted from locally recruited inflammatory cells.

Murine models suggest a central role for IL-13 in the pathogenesis of COPD (Zhu et al 1999; Zheng et al 2000). In contrast, data from human studies has been conflicting. IL-13 expression within macroscopic emphysematous lung tissue was low in severe emphysema (Boutten et al 2004), whereas IL-13 expression in the proximal airway from subjects with chronic bronchitis was increased within the submucosa (Miotto et al 2003). Induced sputum can be an indirect method of assessing the bronchial tree. Currently it is unknown if large airway IL-13 is overexpressed in COPD and what role it plays in disease symptomology. Examination of induced sputum can give indirect evidence of large airway IL-13 expression.

We hypothesised that large airway expression of IL-13 in COPD would be detected in sputum and across disease severity. To test our hypothesis we examined the concentration of IL-13 in induced sputum samples from subjects with COPD, and smoking and non-smoking controls.

MATERIALS AND METHODS

Subjects

Subjects were recruited from local primary health care, respiratory clinics, hospital staff and by local advertising. COPD was diagnosed and severity categorised by using Global initiative for chronic Obstructive Lung Disease (GOLD) criteria (Global Initiative for Chronic Obstructive Lung Disease 2009). All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Smoking history and spirometry was recorded in all subjects. Subjects also underwent sputum induction (Pavord et al 1997), skin prick tests for common aeroallergens and total peripheral blood IgE. Healthy controls (n=14) were defined by an absence of respiratory symptoms with normal spirometry.

IL-13 measurement in sputum

Sputum IL-13 was measured by a validated ELISA (Bender-Med Caltag Systems) as described previously (see section 4.2.2.1). The lower limit of detection was 10pg/g sputum .

Statistical Analysis See section 4.2.1.2.

RESULTS

Sputum IL-13 concentration in COPD

Clinical and sputum characteristics are shown in table 4.8. Smoking pack year history was well matched for subjects with disease and controls. The sputum eosinophil count was increased in those subjects with COPD GOLD 3 (3.2 [1.7-12.4]%; $p<0.001$) and GOLD 4

(3.9 [1.3-11.5]; p<0.001) compared to controls (0.5 [0.3-0.8]). Sputum IL-13 was only measurable in 6/34 subjects with COPD and was not detected in any of the healthy controls (Table 9). There was no difference in sputum IL-13 concentration between COPD GOLD 2-4 or healthy controls (p=0.3).

DISCUSSION

IL-13 expression was not increased in induced sputum similar to bronchial mucosa in COPD (section 4.2.1.2). The very low concentrations of induced sputum IL-13 across disease severity reflect the paucity of IL-13+ cells in the bronchial submucosa, glands and ASM-bundle presented in section 4.2.1.2.

Several lines of evidence support a role for IL-13 in the pathogenesis of COPD. Murine models have proposed a central role for IL-13 in the pathogenesis of COPD with transgenic models exhibiting mucus hypersecretion and peripheral alveolar destruction indicative of emphysema (Zhu et al 1999; Zheng et al 2000). BAL lymphocytes from subjects with COPD have increased IL-13 intracellular expression (Barcelo et al 2006) and peripheral blood IL-13 concentration was related to FEV_1 % predicted (Lee et al 2007). In chronic bronchitis the number of IL-13+ cells in the central airways was increased compared to asymptomatic smokers (Miotto et al 2003). However, IL-13 mRNA and protein was decreased in emphysema (Boutten et al 2004). We report here for the first time in COPD the induced sputum IL-13 concentration and the number of IL-13+ cells in different airway compartments from proximal airway specimens. In contrast to our previous findings in asthma (Berry et al 2004), we found that IL-13 expression was not increased in COPD. Our data therefore challenges the role of IL-13 in COPD.

Table 4.8 Cohort 1 subject details, sputum characteristics and sputum IL-13 concentration

	Normal	GOLD 2	GOLD 3	GOLD 4
Number	14	10	14	10
Age	60 (3)	65 (3)	61 (2)	69 (3)
Male (n)	6	5	10	9
Never smokers (n)	7	0	0	0
Pack years	37 (4)	27 (3)	48 (5)	52 (7)
Atopy (n)	6	3	4	5
Total IgE	ND	47.5 (17.9)	94.9 (44.2)	204.8 (84.4)
FEV_1% predicted	96.2 (2.7)	60.8 (2.0)*	44.1 (6.3)*	23.4 (0.016)*
FEV_1/FVC %	76.1 (1.7)	60.4 (2.7)*	52.3 (8.7)*	48.3 (3.4)*
Eosinophil %^	0.5 (0.3-0.8)	1.2 (0.6-3.4)	3.2 (1.7-12.4)*	3.9 (1.3-11.5)*
Neutrophil %	56.7 (6.3)	58.9 (9.4)	61.6 (8.4)	68.8 (10.4)
Macrophage %	35.7 (6.0)	36.7 (8.9)	27.9 (6.9)	23.1 (9.5)*
Lymphocyte %	1.0 (0.23)	0.87 (0.17)	0.45 (0.14)	0.23 (0.091)
Epithelial cells %	4.3 (1.76)	1.63 (0.49)	2.99 (1.2)	0.90 (0.31)
Measurable IL-13 (n)	0/14	1/10	3/14	2/10
IL-13 (pg/g)[#]	0 (0)	0 (0-17.9)	0 (0-64.4)	0 (0-15.2)

Data expressed as Mean (SE); ^ Geometric mean (lower-upper 95%CI); [#]Median (range)

*$p<0.05$; ND=not done

We are confident that the sputum IL-13 measurements are robust as we have extensively validated this assay and meaningful differences were observed between subjects with asthma and healthy controls (Berry et al 2004). Furthermore the very low concentration of sputum IL-13 across severities reduces the likelihood that this cytokine is important in disease. We confirmed our sputum findings in surgical lung resection specimens (see section 4.2.1.2).

We have shown for the first time increased degree of sputum eosinophilia in subjects with COPD of worsening airflow obstruction as reflected by GOLD stage III-IV (FEV1<50%). Studies have demonstrated increased responsiveness to corticosteroid treatment in COPD subjects with greater degrees of sputum eosinophilia (Brightling et al 2000c, Brightling et al 2005c, Siva et al 2007). This finding may underlie the benefit seen with using high dose inhaled corticosteroid/long acting beta agonists in reducing exacerbations in subjects with COPD<50% (Calverley et al 2007). To know whether our finding is true, further studies are needed.

In conclusion, our sputum data supports our large airway data (see section 4.2.1.2) to suggest IL-13 expression does not have a role in COPD. Using sputum we have been able to study more severe COPD in contrast to our large airway study, and it appears unlikely IL-13 has a role in the COPD paradigm.

4.3 Granulocyte macrophage colony stimulating factor expression in asthma and COPD

4.3.1 Granulocyte macrophage colony stimulating factor expression in bronchial mucosa in asthma and COPD

Introduction

The airway diseases asthma and chronic obstructive pulmonary disease (COPD) are common and cause significant morbidity and mortality worldwide. Asthma affects 10% of children and 5% of adults, and its prevalence continues to rise (BTS 2008). Severe asthma accounts for about 10% of asthma, but is particularly important as it leads to debilitating chronic symptoms despite optimal standard asthma treatment and contributes to over half of the health care costs attributed to asthma (ATS 2000; 2008;Chanez et al. 2007). COPD is a major public health problem and will rank as the 3rd cause of death in 2030 (WHO 2009). Both conditions are characterised by airflow obstruction with airway inflammation, and remodelling. Although the inflammatory profiles of asthma and COPD have been described as overlapping (Saha & Brightling 2006), asthma is more commonly associated with Th_2 mediated eosinophilic inflammation (Wardlaw et al. 2000) whereas in COPD neutrophilic inflammation is more predominant (Saha & Brightling 2006). Several cytokines and chemokines have been implicated in driving the airway inflammatory response in asthma and COPD.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a major regulator of inflammatory cells of the myeloid lineage and has been implicated in asthma and COPD (Vlahos et al. 2006). It is released by a range of structural and inflammatory cells, including

airway epithelium, airway smooth muscle, fibroblasts, T-lymphocytes, mast cells, eosinophils, and macrophages. GM-CSF has recently been shown to signal via a ternary receptor complex (GMCSFR) composed a 2:2:2 hexamer consisting of two βc chains, two GMRa chains and two GM-CSF molecules (Hansen et al. 2008). GM-CSF is a pleiotrophic and proinflammatory cytokine that stimulates myelopoiesis, promotes leukocyte survival and activation. Its importance in airways disease is supported by evidence from mouse models of COPD (Vlahos et al 2006) and asthma (Yamashita et al 2002), whereby administration of anti-GM-CSF antibody attenuates the neutrophilic and eosinophilic inflammatory response respectively. Importantly, in human disease GM-CSF expression is increased in sputum, BAL and bronchial biopsies in asthma (Broide & Firestein 1991; Broide, Paine, & Firestein 1992;Woolley et al 1994; Obase, et al 2003; Dente, et al 2006; Erin, et al 2008). In contrast, in COPD there is a lack of direct evidence of increased GM-CSF expression in airway secretions or biopsy tissue. However, in culture GM-CSF secretion by ex vivo sputum cells is increased in COPD (Profita et al 2003). Similarly, whether GM-CSFR expression is increased in airways disease is contentious with one study suggesting that GMCSFR is increased in non-atopic, but not atopic asthma (Kotsimbos et al 1997). Therefore, GM-CSF and GM-CSFR expression in airways disease needs to be further defined.

We hypothesised that GM-CSF and GM-CSFR expression is increased in asthma and COPD and is related to disease severity. To test our hypothesis we enumerated in bronchial mucosa the number of GM-CSF+ and GM-CSFR+ cells in asthma and COPD.

Methods

Subjects

Subjects were recruited from local primary health care, respiratory clinics, hospital staff and by local advertising. Asthma was defined by one or more of the following objective criteria; significant bronchodilator reversibility of FEV_1 >200mls, a provocation concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) of less than 8mg/ml or a peak flow amplitude % mean over 2 weeks of more than 20%. Asthma severity was classified using the current global initiative for asthma (GINA) guidelines based upon the GINA treatment steps (Global Initiative for Asthma 2009). COPD was diagnosed and severity categorised by using Global initiative for chronic Obstructive Lung Disease (GOLD) criteria (Global Initiative for Chronic Obstructive Lung Disease 2009). Subjects were recruited as two independent cross-sectional cohorts, to assess GM-CSF and GM-CSFR expression in proximal airways in asthma (*Cohort 1*) and COPD (*Cohort 2*). Controls had normal spirometry and smokers with >10 pack year history were included to enable comparisons between smokers and COPD subjects. All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Clinical characterisation

For all subjects demographics and spirometry was recorded. Subjects with asthma and healthy controls in cohort 1 underwent methacholine inhalation test using the tidal breathing method (Sterk et al 1993)(section 3.1.3) and allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat and grass pollen. Sputum induction using incremental concentrations

of nebulised hypertonic saline 3, 4 and 5% each for 5 minutes was also performed in all subjects in cohorts 1 (Pavord et al 1997)(section 3.1.4).

GM-CSF and GM-CSFR expression in endobronchial biopsies

In cohort 1 subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines (BTS 2001), and biopsies were taken from the right middle and lower lobe carinae. In cohort 2 proximal airway samples were collected from surgical specimens.Specimens from 7/10 COPD subjects and 15/19 control subjects were previously examined in studies 4.1.2 & 4.2.1.2. Subjects with asthma had not participated in any previous sputum or biopsy study. All bronchial mucosal specimens were fixed in acetone and embedded in glycomethacrylate as described previously (Britten, Howarth, & Roche 1993)(section 3.2.2.2).

2µm sections were cut and stained using monoclonal antibodies against GM-CSF (clone: BVD2-21C11, Cambridge BioScience Ltd.**)**, GM-CSFR (clone: 2B7, gift from Dr Sleeman. MedImmune, Grant Park Cambridge), or appropriate isotype controls (Rat IgG2a [RαD systems Europe Ltd, Abingdon, UK] and mouse IgG1[Dako UK Ltd, Cambridge] respectively). The number of positive nucleated cells was enumerated per mm^2 of bronchial submucosa or ASM-bundle by a blinded observer (section 3.2.2.3).

Statistical Analysis

Statistical analysis was performed using PRISM Version 4. Parametric data were expressed as mean (SEM), data that had a normal log distribution was log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (interquartile range [IQR]). One-way analysis of variance (Kruskal-Wallis for non-parametric data) was used for across group comparisons with Tukey's and Dunn's post-hoc tests for between group comparisons respectively.

RESULTS

GM-CSF/R expression in large airway tissue specimens

Examples of GM-CSF and GM-CSFR+ cells in the bronchial submucosa in asthma are as shown in figure 4.11. Clinical characteristics of cohort 1 are as shown in table 4.9. The median (IQR) GM-CSF submucosa was increased in severe asthma (1.4 [3.0]) compared to mild-moderate asthmatics (0 [2.5]) and healthy controls (0[0.5]) ($p=0.004$ Kruskal-Wallis across groups; Figure 4.12a). The number of GM-CSFR+ cells/mm^2 submucosa and ASM was increased in severe asthma (2.1 [8.4]) and (2.4 [5.5]) compared to healthy controls (0 [1.6]) and (0 [0.8]), but not mild-moderate asthma (1.1 [5]) and (1.2 [2.2]) ($p=0.02$ and $p=0.049$ respectively Kruskal-Wallis; $p<0.05$ severe asthma versus control, Figure 4.12 b & c). The number of GM-CSF+ cells in the ASM-bundle was very low in subjects with asthma and healthy controls.

There were no differences in the number of GM-CSF or GM-CSFR+ cells within the submucosa, or ASM-bundle in lung resection tissue from subjects with COPD and controls with and without a significant smoking history (cohort 2; Table 4.10).

Figure 4.11. Examples of GM-CSF+ and GM-CSFR+ cells in the submucosa and ASM-bundlein asthma

Representative photomicrographs of bronchial biopsy sections from severe asthmatics illustrating isotype controls a) Rat IgG2a b) Mo IgG1, c) GM-CSF+ cells present in the bronchial submucosa and d) GM-CSFR+ cells in the submucosa (x400). GM-CSF/R+ cells highlighted by arrows.

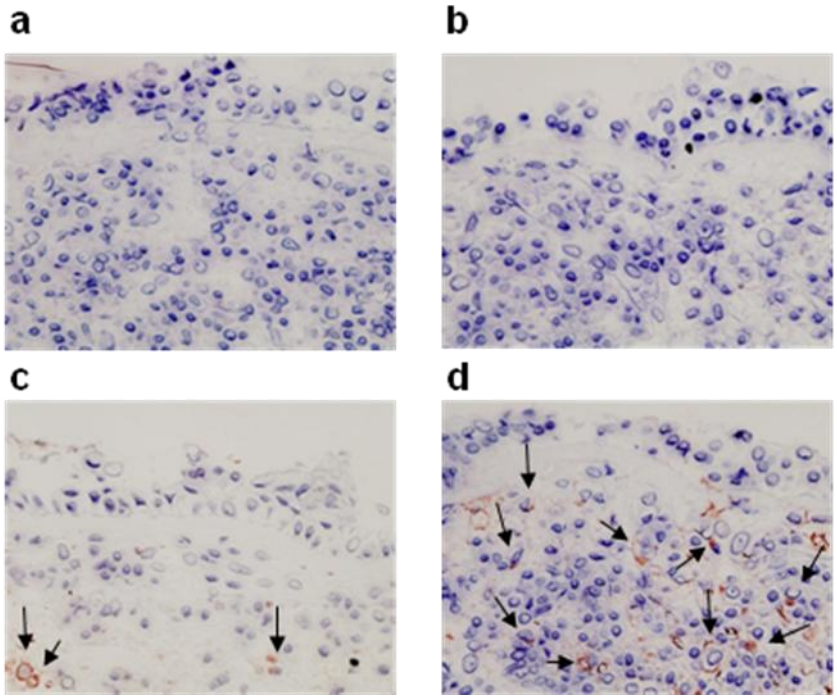


Table 4.9 Clinical and sputum characteristics of biopsy group asthma

	Normal	**Mild-moderate Asthma (GINA 1-3)**	**Severe Asthma (GINA 4-5)**
Number	10	15	12
Age [#]	38 (4)	48 (4)	50 (4)
Male/ Female	4/6	8/7	6/6
Never smokers	9	9	10
Pack years[#]	0 (0)	3 (1)	3 (2)
Atopy (%)	50	63	75
$PC_{20}FEV_1$ (mg/ml)[^]	>16	0.3 (0.1-0.9)	0.4 (0.1-1.4)
FEV_1% predicted[#]	98.4 (4.5)	89.7 (4.9)	80.2 (6.6)
BD response (%)[#]	1.1 (1.2)	8.8 (4.1)	12.5 (5.7)
FEV_1/FVC %[#]	77.9 (3.3)	74.4 (2.9)	74.5 (3.4)
Sputum Cell Counts			
TCC[#]	2.1 (0.6)	2.9 (0.7)	2.7 (0.6)
Eosinophil %^	0.4 (0.1-0.8)	0.9 (0.4-2.8)	2.9 (0.8-10.6)
Neutrophil %[#]	48.8 (17.7)	55.2 (7.0)	59 (9.7)
Macrophage %[#]	47.4 (11.7)	37.1 (6.3)	25.1 (5.8)
Lymphocyte %[#]	1.9 (1.2)	1.1 (0.2)	1.5 (0.7)
Epithelial cells %[#]	1.4 (1.2)	4.0 (1.6)	6.9 (3.3)
GM-CSF			
Submucosa[~]	0 (0.5)	0 (2.5)	1.4 (3.0)*
ASM[~]	0 (0)	0 (0)	0 (0)
GM-CSFR			
Submucosa[~]	0 (1.6)	1.1 (5)	2.1 (8.4)**
ASM[~]	0 (0.8)	1.2 (2.2)	2.4 (5.5)**

[#]mean (SE), ^ geometric mean (log SE), ~median (IQR)

*p<0.05 severe vs mild-moderate asthma & controls

**p<0.05 severe asthma vs controls

Figure 4.12

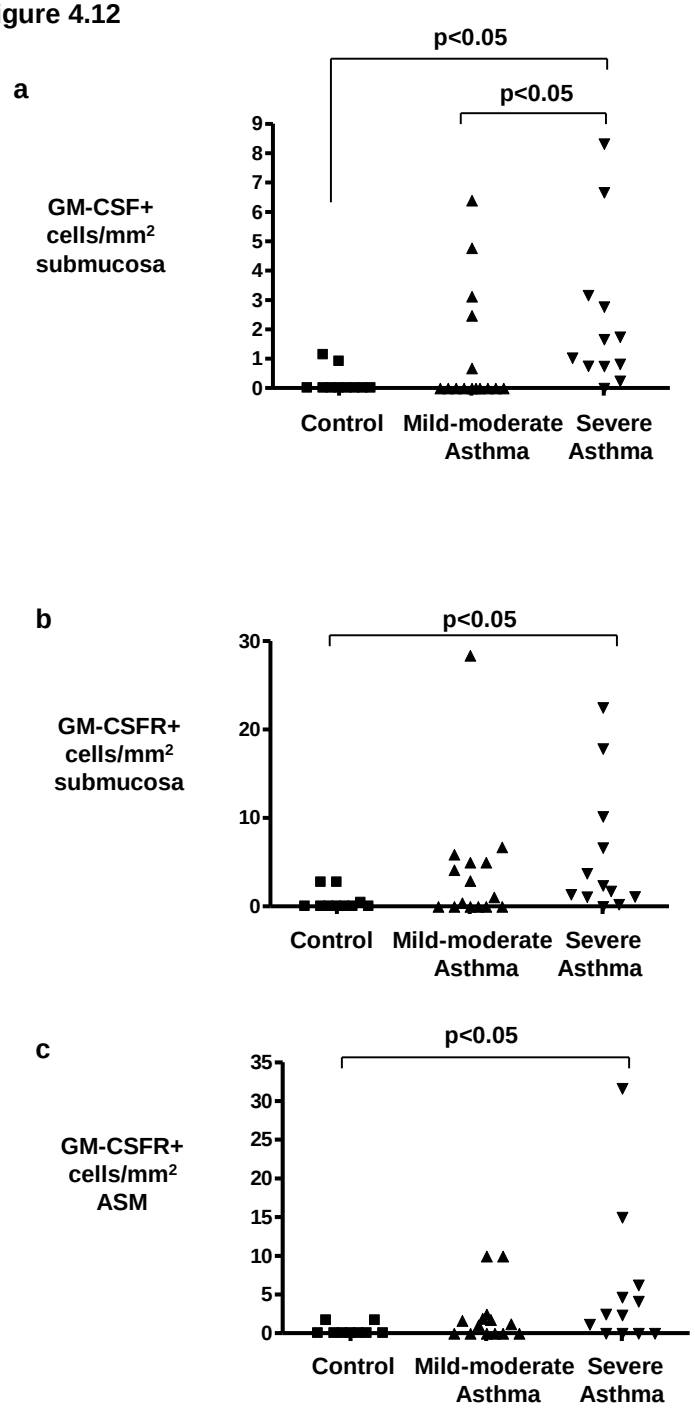

a
p<0.05
p<0.05
GM-CSF+ cells/mm² submucosa
9
8
7
6
5
4
3
2
1
0
Control
Mild-moderate Asthma
Severe Asthma
b
p<0.05
GM-CSFR+ cells/mm² submucosa
30
20
10
0
Control
Mild-moderate Asthma
Severe Asthma
c
p<0.05
GM-CSFR+ cells/mm² ASM
35
30
25
20
15
10
5
0
Control
Mild-moderate Asthma
Severe Asthma

Table 4.10 GMCSF/R COPD Tissue

	Normal	**Smoker**	**COPD**
Subjects	8	11	10
Male	7	8	7
Age	58 (3)	60 (3)	66 (3)
FEV_1[#]	2.8 (0.2)	2.6 (0.2)	1.8 (0.2)
FEV_1 % pred[#]	87 (3)	87 (4)	64 (4)
FEV_1/FVC[#]	79 (3)	81 (4)	55 (3)
Smoking history[#]	0 (0-10)	25 (10-60)	48 (15-56)
GM-CSF			
Submucosa~	0.5 (1)	0.8 (2.4)	0.2 (1.1)
ASM~	0 (0)	0 (0.1)	0 (0)
GM-CSFR			
Submucosa~	2.3 (6.1)	0.3 (1.3)	0.5 (4.4)
ASM~	0 (0.1)	0 (0)	0 (0)

[#]mean (SE), ~median (IQR)

We report for the first time increased GM-CSF and GM-CSFR expression in bronchial biopsies in severe asthma. We did not find increased GM-CSF or GM-CSFR expression in the COPD subjects that underwent surgical resection, although this aspect of our study was restricted to subjects with mild disease. Our study therefore supports our hypothesis that GM-CSF and GM-CSFR expression is increased in asthma and in asthma is related to disease severity.

In the resection samples we were unable to confirm that expression of GM-CSF or its receptor was increased. However, we only studied COPD subjects with milder disease and the control subjects had underlying lung cancer which may have masked differences between COPD and controls. Also the rapid turnover of GMCSF in vivo limits the detection of GMCSF expression by immunohistochemistry. Section 4.3.2 will examine the expression of sputum GMCSF in COPD further and its potential role within the COPD paradigm.

In asthma there is a wealth of data supporting a role for GM-CSF. In particular GM-CSF is pivotal in eosinophil maturation and survival (Lamas, Leon, & Schleimer 1991), a key effector cell in asthma. In animal models GM-CSF neutralisation attenuates airway inflammation and GM-CSF knockout mice (Su, Rolph, Hansbro, Mackay, & Sewell 2008) do not develop a bronchial eosinophilia in response to allergen challenge. In contrast to COPD, in asthma there are several reports of increased GM-CSF expression in airway secretions and tissue (Broide & Firestein 1991;Broide, Paine, & Firestein 1992; Woolley et al 1994; Obase et al 2003; Dente, et al 2006; Erin, et al 2008) . In particular increased sputum GM-CSF expression is associated with more severe disease (Dente et al 2006; Erin, et al 2008). We

report here for the first time that GM-CSF and GM-CSFR expression was also increased in the bronchial submucosa in more severe asthma. Therefore, in severe asthma there is a generalised upregulation in the GM-CSF/GM-CSFR axis suggesting that this mediator may play a prominent role in severe asthma.

Our study has a number of possible criticisms. This is a cross-sectional observational study and therefore whether GM-CSF expression is related to longitudinal clinical outcomes such as disease progression and exacerbations requires further examination. Similarly, we are unable to determine whether differences observed between mild and severe asthma reflect disease severity or are a consequence of differences in treatment. Therefore the effects of corticosteroids on GM-CSF need to be fully elucidated, although previous work suggests that GM-CSF expression in tissue is attenuated by corticosteroids (Wilson et al 2001). However, our ability to still observe differences between health and disease despite these technical limitations strengthens our observations.

In conclusion, we found increased bronchial submucosal expression of both GM-CSF and its receptor was a particular feature of severe asthma. Our findings therefore do support a role for GM-CSF in asthma and therapeutic strategies targeted at GM-CSF are eagerly awaited.

4.3.2 Granulocyte Macrophage Colony Stimulating Factor expression in sputum in asthma and COPD

Introduction

The inflammatory profiles of asthma and COPD have been described as overlapping (Saha & Brightling 2006), asthma is more commonly associated with Th2 mediated eosinophilic inflammation (Wardlaw et al 2000) whereas in COPD neutrophilic inflammation is more predominant (Saha & Brightling 2006). Several cytokines and chemokines have been implicated in driving the airway inflammatory response in asthma and COPD.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a major regulator of inflammatory cells of the myeloid lineage and has been implicated in asthma and COPD (Vlahos et al 2006). Its importance in airways disease is supported by evidence from mouse models of COPD (Vlahos et al 2006) and asthma (Yamashita et al 2002), whereby administration of anti-GM-CSF antibody attenuates the neutrophilic and eosinophilic inflammatory response respectively. Importantly, in human disease GM-CSF expression is increased in sputum in asthma (Dente et al 2006; Erin et al 2008). In contrast, in COPD there is a lack of direct evidence of increased GM-CSF expression in airway secretions. However, in culture GM-CSF secretion by ex vivo sputum cells is increased in COPD (Profita et al 2003). This pleiotropic cytokine has the potential to orchestrate the inflammatory milieu in both asthma and COPD.To date GMCSF expression in the large airway in asthma of increasing severity and in COPD has not been examined.We hypothesised that GM-CSF expression is increased in asthma and COPD and is related to disease severity. To test our hypothesis we have measured the sputum GM-CSF concentration in asthma and COPD as a measure of GMCSF expression within the bronchial tree.

Methods

Subjects

See Methods 4.3.1. Healthy controls had normal spirometry and some smokers with >10 pack year history were included to enable comparisons between healthy smokers and COPD subjects. 15/18 controls, 38/45 subjects with asthma and 18/47 subjects with COPD participated in previous sputum IL-13 studies (see section 4.2.2.1 & 4.2.2.2).

Clinical characterization See Methods section 4.3.1.

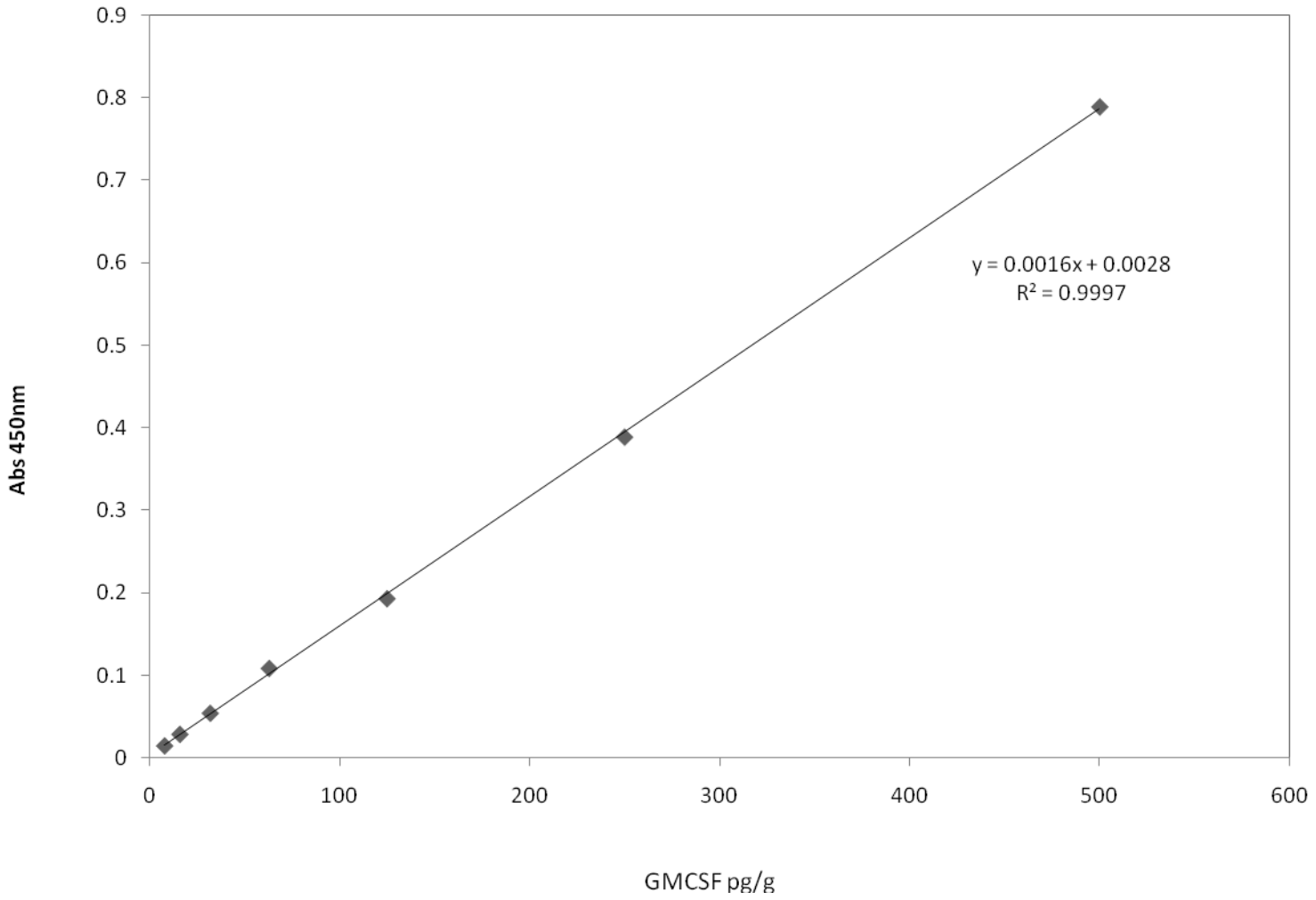
Sputum GM-CSF measuremen Sputum GM-CSF was measured by ELISA (Caltag-Medsystems, Buckinghamshire, UK). The lower limit of detection was 10pg/g sputum. We performed validation experiments to ensure adequate detection of sputum GMCSF:-

Spike of exogenous GMCSF was added to 50% of thawed supernatant volume from processed sputum (n=4 samples). Recovery of GMCSF spike was compared against the supernatant naïve to the spike. Recovery was 103% (15%).

Spike of exogenous GMCSF was added to selected sputum (n=3 samples) prior to processing with mucolytic DTT. Sputum was processed as per protocol (see 3.2.1.1). Samples underwent freeze (-80^{o}C) & thaw cycle. Recovery of GMCSF was 81% (9%) indicating DTT did not affect recovery.Example of standard curve for GMCSF is shown in figure 4.13.

Statistical Analysis For statistical analysis see section 4.3.1. Chi squared tests were used to compare categorical data. Correlations were assessed by Spearman rank correlation coefficients.

figure 4.13 Example of GM-CSF Standard Curve



RESULTS

Sputum GM-CSF concentration in asthma and COPD

Clinical and sputum characteristics are shown in table 4.11. The proportion of subjects with measurable GM-CSF in the sputum was raised in the moderate (7/14) and severe (11/18) asthmatics, and in those with COPD GOLD stage II (7/16), III (8/17) and IV (7/14) compared to controls (1/18) and mild asthmatics (0/13) ($p=0.001$). The sputum GM-CSF concentration was increased in subjects with COPD across severity compared to controls ($p=0.02$ Kruskal-Wallis; $p<0.05$ for COPD all severities compared to controls; Figure 4.14). Similarly, the sputum GM-CSF concentration was increased in severe asthma compared to mild asthma and controls and in moderate asthma compared to mild disease ($p<0.001$ Kruskal-Wallis; $p<0.05$ for between group comparisons; Figure 4.14). The sputum GM-CSF concentration was increased in the subjects with moderate-severe asthma combined compared to those with COPD GOLD II-IV combined ($p=0.004$). The sputum GM-CSF concentration was correlated with the sputum eosinophilia in subjects with disease as a whole group ($Rs=0.28$; $p=0.007$), all asthmatics ($Rs=0.3$; $p=0.04$) and moderate-severe disease ($Rs=0.41$; $p=0.018$), but not COPD. There was no association in subjects with asthma or COPD with sputum GM-CSF concentration and % predicted FEV1 ($R=-0.26$, $p=0.09$; $r=-0.07$, $p=0.7$) or FEV1/FVC ratio ($R=0.06$, $p=0.7$; $r=0.1$, $p=0.5$) respectively.

	Normal	Mild Asthma (GINA 1)	Moderate Asthma (GINA 2-4)	Severe Asthma (GINA 5)	GOLD 2	GOLD 3	GOLD 4
Number	18	13	14	18	16	17	14
Age [#]	54 (3)	53 (4)	51 (4)	49 (5)	71 (2)	68 (2)	72 (11)
Male/ Female	4/14	8/5	4/10	7/11	9/7	12/5	13/1
Never smokers	9	11	10	16	2	0	0
Pack years[#]	17 (5)	2 (1)	6 (3)	4 (3)	43 (8)	52 (4)	56 (9)
Atopy (%)	33	54	79	72	44	41	36
$PC_{20}FEV_1$ (mg/ml)[^]	>16	1 (0.3-4.2)	0.4 (0.1-1.5)	0.1 (0-1.6)	X	X	X
FEV_1% predicted[#]	98.7 (3.0)	80.4 (5.1)	66.4 (4.4)	56.4 (6.8)	60.1 (1.5)	39.9 (1.3)	24.1 (1.3)
BD response (%)[#]	1.5 (0.7)	6.0 (3.6)	5.0 (4.6)	9.6 (2.0)	4.3 (2.2)	6.0 (1.8)	4.1 (3.2)
FEV_1/FVC %[#]	77.6 (1.7)	72.1 (3.6)	67.5 (3.0)	69.7 (2.8)	59.4 (2.2)	50.9 (2.1)	40.3 (2.0)
Sputum Cell Counts							
TCC[#]	3.8 (0.9)	2.34 (0.82)	3.31 (1.8)	6.57 (3.3)	3.3 (0.6)	4.3 (0.9)	11.2 (3.2)
Eosinophil %^	0.5 (0.3-0.8)	2.3 (0.6-7.9)	2.7 (1.0-7.3)	3.8 (1.8-8.0)	2.3 (1.7-6.3)	2.6 (1.4-4.7)	1.0 (0.4-2.3)
Neutrophil %[#]	55.4 (39.6)	67.3 (6.9)	57.8 (5.9)	64.3 (6.0)	72.2 (5.0)	71.0 (4.3)	85.6 (2.9)
Macrophage %[#]	36.9 (32.2)	20.6 (4.6)	21.4 (4.7)	19.2 (4.9)	29.2 (4.6)	21.3 (3.3)	8.7 (1.7)
Lymphocyte %[#]	1.0 (1.4)	0.5 (0.2)	0.4 (0.1)	1.9 (1.5)	1.6 (1.1)	0.6 (0.2)	1.0 (0.3)
Epithelial cells %[#]	0.7 (4.6)	3.7 (1.8)	3.6 (1.7)	3.3 (1.6)	3.3 (0.6)	4.3 (0.9)	11.2 (3.2)

Table 4.11 Clinical and Sputum characterisation [#]mean (SE), ^ geometric mean (log SE), ~median (IQR)

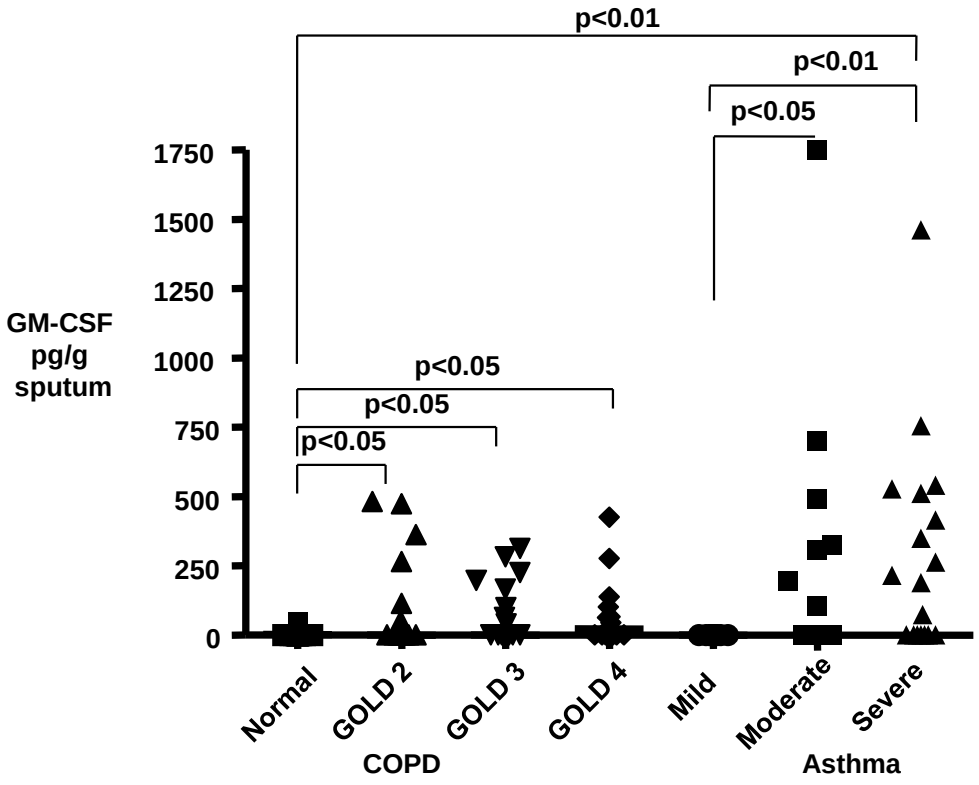


Fig 4.14 Sputum GMCSF concentration in asthma, COPD and controls

DISCUSSION

We report here for the first time that the sputum GM-CSF concentration was increased in COPD, independent of disease severity, and confirm that in asthma the sputum GM-CSF concentration is associated with more severe disease. In asthma our sputum findings were supported by increased GM-CSF and GM-CSFR expression in bronchial biopsies in severe disease (see Results section 4.3.1). Our study therefore supports our hypothesis that GM-CSF expression is increased in asthma and COPD and in asthma is related to disease severity.

We have confirmed these earlier reports and found that sputum GM-CSF concentration was increased in moderate-severe asthma, but not in mild disease. In addition the intensity of the sputum GM-CSF expression was related to the sputum eosinophilia. In conjunction with our previous novel findings reflecting increased GMCSF & GMCSFR in bronchial tissue in severe asthma, there is an upregulation of the GMCSFR/GMCSF axis in severe asthma (see section 4.3.1 for further discussion)

Several lines of evidence support a role for GM-CSF in COPD. GM-CSF is induced by the presence of airway pathogens (Berclaz et al. 2002; Bozinovski et al. 2002) and is known to be an important regulator of the activation and survival of key effector cells in COPD namely the neutrophil and macrophage (Akagawa et al 1988; Stankova, Rola-Pleszczynski, & Dubois 1995). Critically, neutralisation of GM-CSF in animal models attenuates airway inflammation in response to cigarette smoking (Vlahos et al 2006). However, to date there has been a paucity of direct evidence of increased GM-CSF expression in airway secretions. Indeed sputum GM-CSF concentration was not increased in subjects at exacerbations compared to their stable state at recovery (Tsoumakidou et al 2005), although in contrast GM-CSF release by sputum cells in culture was increased (Profita et al 2003). *In vivo* and *in vitro* GM-CSF is rapidly internalised following receptor binding and therefore it is likely that the measurement

of sputum GM-CSF is under-estimated by ELISA (Metcalf et al. 1999; Vlahos et al 2006). In spite of this we have here validated the measurement of GM-CSF in sputum and found that it was increased in COPD subjects across all disease severities compared to smoking and non-smoking controls.

Our study design allowed for direct comparison of the sputum GM-CSF concentration in asthma and COPD. Comparisons in sputum GM-CSF concentrations were undermined by the number of subjects that had concentrations below the level of detection of our assay. This is likely to reflect the rapid internalisation of GM-CSF. In spite of this limitation we found that sputum GM-CSF was increased in both COPD and moderate-severe asthma, and importantly it was greater in moderate-severe asthma than COPD. Whether GM-CSF plays a more important role in severe asthma than COPD requires further investigation.

Our study has a number of possible criticisms. See section 4.3.1 The rapid turnover of GM-CSF in vivo limits the interpretation of protein expression by ELISA. However, our ability to still observe differences between health and disease despite these technical limitations strengthens our observations.

In conclusion, we found that sputum GM-CSF concentration was increased in COPD across disease severity and in moderate to severe asthma. Our findings therefore do support a role for GM-CSF in asthma and COPD and therapeutic strategies targeted at GM-CSF are eagerly awaited.

5. Conclusions

5.1 Summary of findings

This aim of this thesis was to establish common immunobiology between asthma and COPD, including assessment of disease severity.

We demonstrated mast cell infiltration of the large ASM in asthma was not a feature of mild asthma alone but a feature present with moderate-severe disease in comparison to healthy controls. In corticosteroid naive disease, eosinophilic asthma displays higher number of mast cells within the ASM than in non-eosinophilic asthma. The degree of infiltration of the ASM correlated with symptom control but no physiological parameters.

We discovered the novel finding of localisation of CD3+ lymphocytes to bronchial glands in COPD. Our study was not designed to examine this feature against disease phenotype. We found a wide range of inflammatory cells present in the ASM and in glandular structures within the proximal airway in subjects with COPD and the control groups. The physiological significance of this is unclear. We accept we cannot compare these findings in surgical specimens to biopsy samples in our mild-severe asthma airway study (above).

We identified for the first time increased numbers of IL-13+ cells within the large airway submucosa in moderate-severe asthma in comparison to healthy controls. The majority of these cells were identified as eosinophils. In addition, we found an increase in IL-13+ cells in the ASM of subjects with severe asthma compared to controls and mild-moderate asthma. Theses IL-13+ cells were predominantly identified as mast cells. IL-13 expression within bronchial tissue in asthma correlates with eosinophilic inflammation in sputum. We saw a paucity of IL-13 expression within the submucosa, bronchial glands and ASM bundle in the

proximal airway of surgical specimens from mild COPD with no difference in expression compared against non-smoker and smoker controls.

In severe asthma, there is overexpression in the sputum supernatants from subjects with severe asthma compared to healthy controls. Sputum IL-13 was increased in mild asthma compared to moderate asthma as well as healthy controls. This pattern of IL-13 expression is similar to ASM IL-13 expression in mild-severe asthma. Sputum IL-13 correlated with disease control in subjects with moderate-severe asthma In a subset of subjects with severe asthma requiring parental corticosteroid therapy, sputum IL-13 was overexpressed compared to healthy controls. In mild-severe COPD we found little expression of IL-13 with no difference compared against appropriate control groups.

For the first time we have demonstrated GMCSF overexpression within the submucosa of the large airway in severe asthma compared to mild-moderate asthma and healthy controls. GMCSF receptor+ cells were preferentially expressed in the submucosa and ASM bundle in severe asthma compared to controls. There was a paucity of GMCSF and GMCSF receptor expression within the proximal airway in COPD. In mild-severe asthma and mild-severe COPD, there were increased levels of sputum GMCSF compared to controls. We saw correlations between sputum GMCSF and sputum eosinophilic inflammation across the whole group of subjects with asthma and COPD but also with all subjects with asthma and subjects with moderate-severe asthma.

In summary, inflammatory cell microlocalisation to important structures is present in moderate-severe asthma and COPD. IL-13 expression appears to have a different pattern of expression between asthma and COPD but there is some overlap in GMCSF expression reflected in sputum only. This broadly supports our original hypothesis that similar features of airway inflammation are shared between asthma and COPD.

5.2 Criticisms

Some criticisms of the studies presented in this thesis, have already been addressed in the discussion of each chapter. Some consistent issues over study design have recurred though each chapter, and require comment.

Each sputum and large airway study in asthma and COPD has been cross-sectional design. The longitudinal measurement of cytokine expression in stable asthma and COPD with GMCSF and IL-13 remains unknown. The repeatability of GMCSF and IL-13 overexpression in individuals within our studies is not accurately known. Within our asthma study groups, the cross-sectional design did not allow correction for the use of inhaled and oral corticosteroid therapy. With GMCSF, in vitro evidence suggests GMCSF is a steroid responsive target suggesting our findings of increased expression of GMCSF in progressive more severe asthma, is not a result of increased corticosteroid therapy. With IL-13, we demonstrated increased expression in mild steroid naive asthma, with further overexpression seen in severe disease in both sputum and biopsy tissue. The paucity of expression seen in moderate, more controlled disease would suggest corticosteroid therapy does not have a stepwise influence upon IL-13 expression. Also we still do not know if mast cell infiltration and consequent cytokine expression is modifiable with corticosteroid therapy.

We were not able to demonstrate increased cytokine expression or a preferential relationship of inflammatory cell microlocalisation within the large airway in COPD, except for CD3+ T-cells infiltration of mucus glands. Potentially we may be missing a significant relationship as our study population possessed mild COPD predominantly but also was atypical in their recruitment. We used large airways dissected from lung tissue resected in surgery for malignancy. Hence by screening for fitness for surgery, our study population would have had reasonable absence of significant disability from COPD. The systemic influence of

malignancy upon cell trafficking is unclear but with a general paucity in cytokine expression in mild COPD, we can only speculate malignancy does not generate a pro-inflammatory response within the large airway. Study of the large airway in more severe COPD is more technically difficult as such patients do not undergo surgery and submission of COPD volunteers for bronchoscopy for research purposes, raises ethical issues. In addition, an absence of differential features in terms of cytokine GMCSF and IL-13 expression and inflammatory cell microlocalisation in the large airway in COPD, may not confirm the absence of meaningful role within the COPD paradigm. The small airway obstruction is a defining feature of COPD but technically difficult to sample; potentially examination of the small airways may suggest a significant role for IL-13 and GMCSF expression in COPD.

5.3 Future laboratory studies

As previously mentioned, this thesis has primarily studied the infiltration of large airway structures by inflammatory cells and in addition, the cross-sectional expression of cytokines GMCSF & IL-13 in stable volunteers. Further studies need to be formulated thereafter to assess the effect of systemic corticosteroid therapy. A double blind oral corticosteroid placebo study would allow assessment of cell microlocalisation and cytokine expression within the structures of the large airway and sputum in asthma and COPD, in response to systemic anti-inflammatory treatment. The placebo arm would allow determination of repeatability of sputum and large airway expression of GMCSF and IL-13 as well as ASM and glandular structure infiltration by inflammatory cells.

Our study (section 4.1.1) determined a relatively narrow range of inflammatory cells (tryptase positive mast cells and MBP positive eosinophils) in moderate and severe asthma. Future

studies should be designed to assess the significance of other inflammatory cells and their role with infiltration of significant structures in the large airway in moderate and severe asthma. This question remains pertinent and may unravel further the severe asthma paradigm in view of an absence of inflammatory cells within the ASM in mild asthma (Brightling et al 2002).

With sputum analysis, we have limited ourselves to study only 2 cytokines by separate ELISA kits. New techniques have arisen using multi-platform array methods to assess multiple cytokines in single specimens. While validation of these techniques raise their own difficulties, we and others have begun to answer these important questions, which I anticipate will further inform our understanding of the basic mechanisms of airway diseases and have significant impact on our clinical practice.

5.4 Future clinical studies

Our studies suggest IL-13 & GMCSF to be potential targets in the treatment of severe asthma. To date, studies targeting GMCSF in asthma have not happened. Trials targeting IL-13 have already happened. Early studies with a soluble recombinant human interleukin-4 receptor (altrakincept, Immunex [Amgen], USA) in patients with mild-to-moderate asthma showed some efficacy in maintaining asthma control when inhaled corticosteroids were being withdrawn (Borish et al. 2001), but this effect was not subsequently confirmed and development was stopped. Two recent placebo-controlled allergen challenge studies showed that an interleukin-4 variant (pitrakinra) administered subcutaneously or nebulised can inhibit the binding of interleukins 4 and 13 to the IL-4Rα subunit. Pitrakinra reduced the allergen-induced late-phase response and the need for rescue medication in asthmatic patients (Wenzel

et al. 2007). Trials are now underway using an inhaled preparation (Getz et al 2008). Similarly, a humanized monoclonal antibody IMA-638 inhibited both the early and late allergen challenge response, but did not affect allergen-induced hyperresponsiveness to methacholine (Gauvreau et al 2008). Intriguingly, although preclinical data supports the view that IL-13 is critical in the development of airway hyperresponsiveness to date studies that have included allergen induced airway hyperresponsiveness have failed to show an effect on this outcome. Whether anti-IL-13 strategies have an impact on 'wild-type' airway hyperrresponsiveness needs to be addressed. Several other monoclonal antibodies against interleukin 13 have completed early safety trials in humans, including CAT-354 (Bhowmick et al 2008) and AMG 317 (Banfield et al 2008), and are undergoing clinical trials for asthma. To date there are no studies of anti-IL-13 or –GMCSF therapies in COPD.

'This thesis has made a substantial contribution to our understanding of the potential role of IL-13 and GM-CSF in asthma and COPD and importantly has underscored the importance of recognising the clinical and immunopathological heterogeneity of airways disease. Whether specific therapies directed towards IL-13 and GM-CSF are valuable in the managment of airways disease is uncertain and may require careful individualised therapy. Interesting times are ahead as we have the opportunity to test the role of these cytokines in human disease. We are therefore likely to have in the very near future new treatments, a better understanding of disease and possibly both.

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