THYMIC STROMAL LYMPHOPOIETIN AND ITS DOWNSTREAM NETWORKS IN SEVERE ASTHMA

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by

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Thymic Stromal Lymphopoietin and its downstream networks in Severe Asthma

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Abstract

TSLP is a cytokine implicated in the pathophysiology of asthma through a TSLP-OX40L-T cell axis and a TSLP-mast cell axis. Whether these pathways operate in human asthma is unknown. The objective was to investigate whether mucosal TSLP protein expression relates to asthma severity, and distinct immunological pathways. GMA-embedded bronchial biopsies from subjects and patients with mild, moderate and severe asthma were immunostained for TSLP, OX40/OX40L, CD83, IL-4, IL-13, and inflammatory cells. TSLP and IL-13 release were measured in supernatants from epithelial cells co-cultured with human lung mast cells (HLMC). There was considerable heterogeneity in TSLP, IL-13 and IL-4 immunostaining across the asthmatic subjects. TSLP protein expression was significantly increased in airway epithelium and lamina propria of asthmatic patients, in particular severe asthma which correlated with the severity of airflow obstruction. IL-13 staining was increased in non-epithelial cells within the epithelium in severe asthma of which lineage-negative CD45+ cells represented a substantial proportion. Asthmatic subjects with elevated IL-13 immunostaining in the lamina propria also had elevated levels of IL-4 and TSLP expression. Recombinant TSLP attenuated FcεRI-dependent HLMC degranulation and TSLP production, and HLMC rapidly degraded TSLP. In summary, TSLP expression is elevated in severe asthma despite high-dose corticosteroid therapy. Although the TSLP-OX40L-T cell pathway within asthmatic bronchial mucosa was not detected, it is possible that it operates in secondary lymphoid organs. The close approximation of airway stroma and mast cells suggests the TSLP-mast cell axis maybe active in asthmatic bronchial mucosa, but my in vitro data suggests that TSLP may inhibit HLMC activation. The interaction of TSLP with CD45+ lineage-negative innate lymphoid cells group 2 maybe the most important pathway contributing to increased IL-13 expression in subset of patients with asthma, including severe disease. Targeting TSLP may only be efficacious in the subset of asthma characterised by increased Th2 inflammation.

I dedicate this thesis to my bapa

દુર્ગમ કાજ જગત કે જેતે /

સુગમ અનુગ્રહ તુમ્હરે તેતે //

The burden of all difficult tasks of the world

become light with your kind grace

Jai Siya Ram

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First and foremost I offer my sincerest gratitude to my supervisor, Professor Peter Bradding for his knowledge and guidance, who has not only supported me throughout this study, but most importantly from the first day I started working at Glenfield Hospital. I thank him for convincing me to undertake a PhD in particular this study and for his patience every year I mentioned I was having a baby! He has encouraged and supported me throughout the study and has epitomised what good supervisors should be.

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Statement of Work Performed

The hypothesis and design of the study was designed by myself in conjunction with Professor Peter Bradding based on previous TSLP studies. Patient recruitment, clinical characterisation and bronchoscopies were carried out by Professor Peter Bradding, Dr Chandra Ohri and the research nursing team members Bev Hargadon and Maria Shelley.

Biopsy collection during the bronchoscopy processing into GMA resin was performed by myself. I undertook all of the bronchial tissue immunohistochemistry for inflammatory cells and cytokines expression. I also carried out the microscopy, assessment and quantification of immunohistochemical staining. I performed all the statistical data analysis.

Sputum processing was carried out in the Sputum Laboratory by the designated team. I conducted and analysed all sputum ELISAs. I provided bronchial brushings from the bronchoscopy for epithelial cell culture. Both epithelial cells and human lung mast cells were isolated by designated tissue culture staff. I designed and performed all cell culture experiments. I conducted all ELISAs, assays and subsequent analysis.

Finally I prepared all the abstracts and presented at internal and national conferences. The work arising from this thesis was published in peer-reviewed journal.

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Publications and Abstracts arising from this thesis

Publications

Shikotra, A., Choy, D.F., Ohri, C.M., Doran, E., Butler, C., Hargadon, B., Shelley, M., Abbas, A.R., Austin, C.D., Jackman, J., Wu, L.C., Heaney, L.G., Arron, J.R., Bradding, P., 2012.

Increased expression of immunoreactive thymic stromal lymphopoietin in patients with Severe Asthma.

Journal of Allergy and Clinical Immunology. 129, 104-111.e9.

Abstracts and Presentations

Shikotra, A., Hargadon, B., Shelley, M., Bennett, J., Brightling, C., Austin, C., Choy, DF., Wu, LC., Arron, JR., Bradding P.

Increased expression of immunoreactive thymic stromal lymphopoietin in Severe Asthma.

British Thoracic Society Winter Meeting, December 2010

Shikotra, A., Hargadon, B., Shelley, M., Arron, JR., Bradding P.

The Hunt for Airway Nuocytes (Innate lymphoid cells 2).

Belfast/Leicester/Hull Scientific Conference, 2012

Prizes

Commended Oral Presentation: University of Leicester: Department of Infection Immunity and Inflammation, 2009.

Abbreviations

AEC	3-Amino-9-ethylcarbazole
AHR	Airway hyper responsiveness
AP-1	Activator protein 1 transcription factor
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BEGM	Bronchial epithelial cell growth medium
BSA	Bovine serum albumin
BTS	British thoracic society
Ca^{2+}	Calcium
CD	Cluster of differentiation
DMEM	Dulbecco's modified eagle medium
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FcεRI	Fc epsilon Receptor I high-affinity IgE receptor
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GMA	Glycol Methacrylate
GM-CSF	Granulocyte macrophage-colony stimulating factor
GWAS	Genome wide association study
H & E	Haematoxylin and eosin
H	Hour

HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLMC	Human lung mast cells
HRP	Horse radish peroxide
ICOS	Inducible T-cell Co-Stimulator
ICS	Inhaled corticosteroids
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgG1	Immunoglobulin G
Ih2	Innate type 2 helper helper cells
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cells
JAK	Janus family kinases
kDa	Kilodaltons
L	Ligand
LABA	Long-acting β2 agonists
LTC_4	Leukotriene C4
MBP	Major basic protein
MC_C	Mast cell chymase
MC_T	Mast cell tryptase
MC_{TC}	Mast cell trytpase chymase
MHC	Major histocompatibility complex
MIN	Minutes
MPP^{type2}	Multi-potent progenitor type 2
mRNA	Messenger ribonucleic acid

NEAA	Non-essential amino acids
$NF_K\beta$	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHBE	Normal human bronchial epithelial cells
NHC	Natural helper cells
OVA	Ovalbumin
PBS	Phosphate buffer saline
PC20	Provocative concentration of inhaled methacholine required to reduce FEV1 by 20%
PGD_2	Prostaglandin D_2
poly I:C	Polyinosinic:polycytidylic acid
PVDF	Polyvinylidene fluoride
Rh.TSLP	Recombinant thymic stromal lymphopoietin
RPM	Revolutions per minute
RSV	Respiratory syncytial virus
SABA	Short-acting β2 agonists
SCF	Stem cell factor
Sec	Seconds
SIGN	Scottish Intercollegiate Guidelines Network
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TBS	Tris buffer saline
TGF	Tumour growth factor
Th	T helper cells
TLR	Toll like receptor

TNF	Tumour necrosis factor
TREG	T regulatory cells
TSLP	Thymic stromal lymphopoietin
TSLP-R	Thymic stromal lymphopoietin receptor
µg/mL	Micrograms per millilitre
µL	Microlitres
µm	Microns/micrometer

CHAPTER 1.

Introduction

1.1 Asthma Epidemiology

Asthma is an important cause of morbidity and mortality affecting 300 million people worldwide (Asher *et al.*, 2006; Masoli *et al.*, 2004). The prevalence of asthma in both Westernised and less-developed countries has risen in recent years making it a serious global health problem affecting individuals, families and communities (Pearce *et al.*, 2007) (Wilson *et al.*, 2006).

Approximately 10% patients with severe asthma are resistant to current therapies (Wenzel, 2005) (Proceedings of the ATS workshop on refractory asthma, 2000). As much as 50-60% of asthma healthcare costs are related to this group highlighting an unmet clinical need. The urgent requirement for better novel treatments has never been greater.

1.2 Asthma Aetiology

The exact cause of asthma is unknown and it is important to distinguish between asthma causes and asthma triggers. A cause is without which asthma would not occur. Potential aetiological factors involve the complex interaction of genetic factors (there is often a familial component) where there is a family history of asthma, atopy and genetic links and environmental stimuli such as allergens (house dust mite, moulds and pollen) (Sarafino & Goldfedder, 1995). In addition exposure to infection, exposure to second hand smoke, previous upper respiratory tract infections, low socio economic status and obesity are risk factors for the development of asthma.

A trigger induces the symptoms of asthma; however triggers vary between individuals. Triggers can include infections (colds, viruses, flu, sinus infections), exercise, changes in weather in particular cold air and/or humidity, allergens (dust mites, pollens, pets, mould spores, cockroaches), irritants (strong odours from cleaning products, perfume, wood smoke, air pollution) and extreme negative emotions such as anger or fear. Medications such as aspirin and other non-steroid anti-inflammatory drugs can also trigger symptoms.

1.3 Asthma Pathophysiology

Asthma is a chronic condition characterised by airway inflammation, often but not always with an eosinophilic component, airway hyper responsiveness (AHR) with variable airflow obstruction, and structural airway wall remodelling, Symptoms of asthma are recurrent episodes of breathlessness, chest tightness, coughing and wheezing often at night or early morning which can be relieved by bronchodilators. The key physiological abnormality is airway obstruction which occurs due to bronchoconstriction, mucous plugging, and airway wall oedema; this occurs as a result of structural and cellular changes seen microscopically within the airway wall. The appearance of symptoms varies in asthmatics; some are rarely symptomatic whilst others have persistent airflow obstruction or recurrent exacerbations.

1.4 Airway Inflammation and Airway Remodelling

Asthma is characterised by the presence of inflammation and structural changes within the airway wall often described as airway remodelling. It is often stated that remodelling occurs as a result of chronic inflammation, but there is

very little evidence to support this. The structural changes which occur include damage to the airway epithelium, sub-basement membrane thickening and increased extracellular matrix deposition, goblet cell hyperplasia and increase in smooth muscle mass which is accounted for by both hyperplasia and hypertrophy (Boxall *et al.*, 2006).

1.5 Assessment of Airflow Obstruction

Airflow obstruction is considered to be a consequence of airway inflammation in conjunction with airway remodelling and is measured by spirometry. Spirometry is a method of measuring the volume of air expelled from the lungs after maximal inspiration. The forced vital capacity (FVC) of the lung is the volume of air that can be expelled from the lung from the maximum inspiration to the maximum expiration. Forced expiratory volume in 1 second (FEV1) is the volume of air that can be expelled from maximum inspiration in the first second. The FEV1/FVC ratio is the FEV1 expressed as a percentage of the FVC. It distinguishes between reduced FEV1 due to restricted lung volume and that due to obstruction. If the FEV1/FVC ratio is <70% there is airflow obstruction.

1.6 Airway Hyper Responsiveness

Airway hyper responsiveness (AHR) is the overactive narrowing of the airway in response to an inhaled constrictor agonist leading to airflow obstruction. This can be termed direct or indirect AHR, whereby a direct constrictor directly stimulates airway smooth muscle contraction, whereas an indirect stimulus activates inflammatory cells such as mast cells which in turn release bronchoconstrictor mediators. Direct AHR is measured by a methacholine or

histamine inhalation challenge test which triggers bronchospasm in most symptomatic asthmatic individuals. Increasing concentrations of methacholine are administered to determine the PC20 (provocative concentration of inhaled methacholine required to reduce FEV1 by 20%). Those with AHR have a lower threshold, usually taken as a concentration of <8 mg/mL.

1.7 Current Asthma Management

The goal of asthma treatment is to control the manifestations of the disease. The following treatments are commonly used depending on the severity of asthma.

- Short-acting β2 agonists (SABA) such as salbutamol,
- Inhaled corticosteroids (ICS) such as beclomethasone and budesonide,
- Long-acting β2 agonists (LABA) such as salmeterol and formoterol,
- Leukotriene receptor antagonists
- Theophylline
- Oral corticosteroids such as prednisolone

Figure 1.1 shows the BTS guidelines for the classification and management of asthma in adults.

Figure 1.1 Summary of stepwise asthma management in adults

Figure originally from the BTS/SIGN Asthma Guidelines January 2012 version. http://www.brit-horacic.org.uk/Portals/0/Guidelines/AsthmaGuidelines/sign101%20Jan%202012.pdf

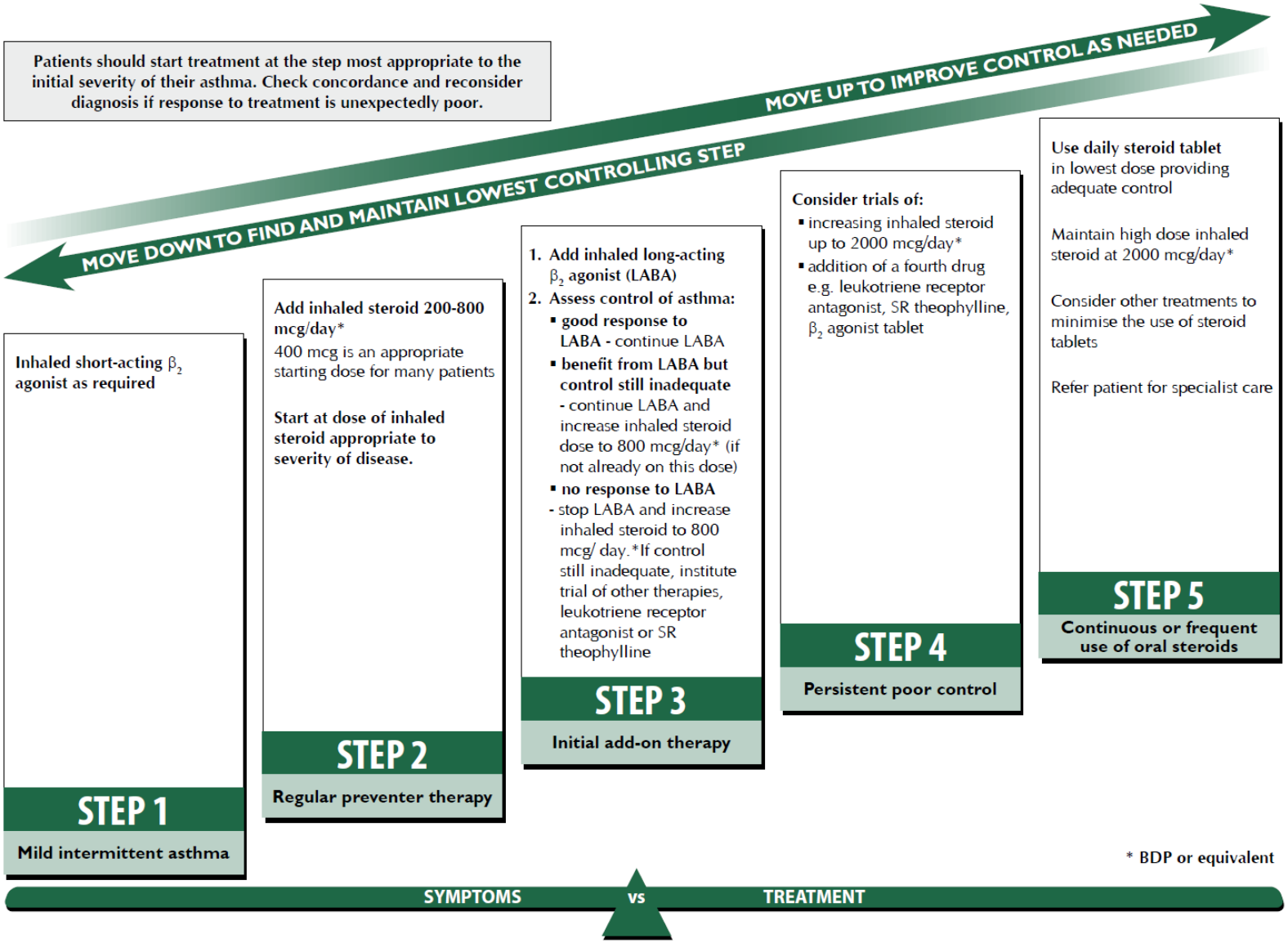


Figure 4: Summary of stepwise management in adults

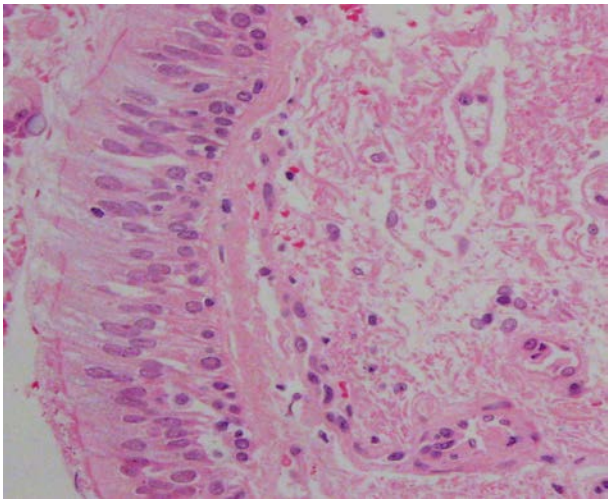
1.8 Cellular Dysfunction in Asthma

There are proponents of various cell types as key drivers of asthma pathophysiology. However, most if not all airway components are dysfunctional.

1.8.1 Airway Epithelial cells

The airway epithelium has historically been seen in a structural role as “just a physical barrier” separating the external settings and from the internal environment of the lung. However, recent advances have brought attention to the epithelium as a key player in airway disease in relation to epithelial damage, airway remodelling and airway inflammation.

A)



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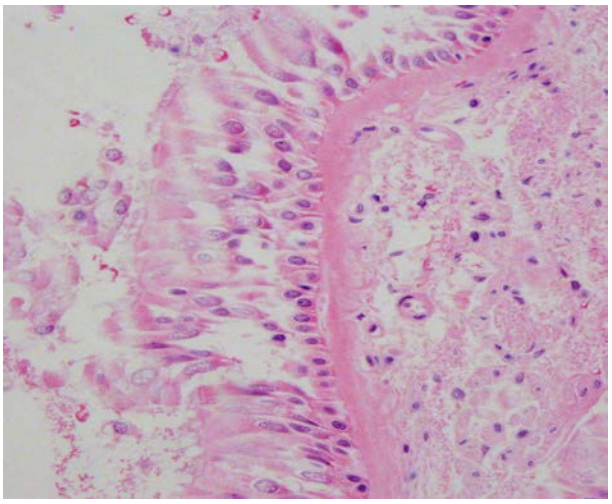


Figure 1.2 Photomicrograph of bronchial epithelium

Representative photomicrographs of bronchial epithelium in a bronchial biopsy obtained from A) healthy controls and B) asthmatic subjects and stained with haematoxylin and eosin. X400 magnification.

The epithelium is a pseudo stratified layer that essentially consists of basal, ciliated, mucus secreting goblet cells and Clara cells (Figure 1.2). Its role as a defence system against inhaled viruses, bacteria, allergens fungi, pollution and other environmental factors means epithelial cells undergoes rapid regeneration and repair whereby basal epithelial cells differentiate and proliferate to restore the damage (Runswick *et al.*, 2007) (Chen *et al.*, 2011; Olivera *et al.*, 2007; Rezaee *et al.*, 2011). The epithelial cells are connected together partly though tight junctions forming a highly impermeable barrier. Normally these tight junctions regulate intracellular transport, maintain cell-cell contact and most importantly protect the internal milieu from external stimuli. However in asthma the epithelium is vulnerable to stimuli as there is inefficient tight junction formation leading to epithelial dysregulation (Xiao *et al.*, 2011). Xiao *et al.* reported air liquid interface (ALI) cultures taken from asthmatic patients formed significantly less tight junctions compared with healthy controls which resulted in impaired epithelial cell function. In addition, immunohistochemical analysis revealed disintegration of tight junction formation in asthmatics compared to healthy controls which allowed inhaled substances to readily pass through the epithelium and interact with immune cells (Xiao *et al.*, 2011).

The cycle of frequent damage and inadequate repair of the epithelium is thought to facilitate epithelial damage and thickening of the basement membrane leading to subepithelial fibrosis (Roche *et al.*, 1989). Both are key features of airway remodelling. Subepithelial fibrosis is characterised by increased deposition of fibronectin, collagen, laminin, and tenascin (Altraja *et al.*, 1996) (Laitinen *et al.*, 1997) (Wilson & Li, 1997) (Hoshino *et al.*, 1998).

Transforming growth factor β (TGF-β) produced by eosinophils and myofibroblasts and epidermal growth factor (EGF) produced by epithelial cells promote the production of extracellular matrix and collagen. In response TGF-β airway epithelial cells can undergo epithelial–mesenchymal transition (EMT) which is characterised by a loss of epithelial cell markers and increased expression of mesenchymal cell markers in cell culture conditions and potentially contribute to subepithelial fibrosis (Hackett *et al.*, 2009). In addition periostin produced by asthmatic epithelial cells increases expression of collagen 1 by fibroblasts though the secretion of TGF-β (Sidhu *et al.*, 2010). Increased levels of TGF-β in the asthmatic airways induces epithelial cell apoptosis resulting in the detachment of epithelial cells, goblet cell proliferation and increased mucus secretion (Inman & Allday, 2000), (Chu *et al.*, 2004).

A second role of epithelial cells is to aid the immune response by releasing a range of chemokines and cytokines to recruit inflammatory cells. The chemokine CCL2 (monocyte chemotactic protein-1 (MCP-1)) and CCL20 (liver activation regulated chemokine (LARC)) attract monocytes and dendritic cells to the lung (Hammad *et al.*, 2009) (Nathan *et al.*, 2009). The cytokines granulocyte-macrophage colony stimulating factor (GM-CSF) and thymic stromal lymphopoitein (TSLP) initiate dendritic cell maturation with the latter also promoting growth and differentiation of IL-4 polarising basophils (Siracusa *et al.*, 2011). In addition, epithelial cell derived IL-25 and IL-33 stimulate innate lymphoid cell 2 (ILC2 previously named nuocyte) to produce Th2 cytokines, IL-5 and IL-13 (Neill *et al.*, 2010). More recently a number of groups have reported that bronchial epithelial cells are capable of producing IL-13 (Allahverdian *et al.*,

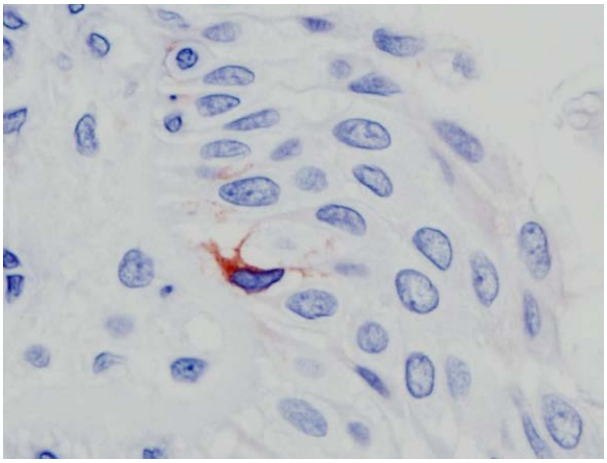
2008; Dougherty *et al.*, 2010; Semlali *et al.*, 2010). Gene expression microarrays in airway epithelial cells revealed 3 genes, periostin (POSTN), chloride channel regulator 1 (CLCA1), and serpin peptidase inhibitor, clade B, member 2 (SERPINB2) are specifically induced in asthma and are directly regulated by IL-13 (Woodruff *et al.*, 2009). Both TSLP and mechanical injury induce IL-13 mRNA and protein expression in confluent airway epithelial cell monolayers and air liquid interface (ALI) cultures (Allahverdian *et al.*, 2008) (Semlali *et al.*, 2010). A skewed Th2 response in asthma perpetuates inflammation as IL-5 and IL-13 induce epithelial expression of CCL11 (eotaxin 1) and CCL17 (Thymus and activation-regulated chemokine (TARC)) which are chemoattractants for eosinophils and Th2 cells (Matsukura *et al.*, 1999; Lordan *et al.*, 2002).

As a result the epithelium is infiltrated with mast cells, eosinophils, neutrophils and phagocytic cells (Brightling *et al.*, 2003b) (Dougherty *et al.*, 2010). These immune cells either reside in the epithelium or are in transition to the lumen to resolve or in the case of asthma maintain the immune response (Pesci *et al.*, 1993).

1.8.2 Dendritic cells

Dendritic cells are antigen presenting cells that bridge the innate and adaptive immune system. In the lung, gut and skin immature dendritic cells expressing $CD11c^+$ and $CD1a^+$ are located in the basement membrane where they are in direct contact with inhaled antigens in the epithelium (Figure 1.3) (Holt *et al.*, 1990).

A)



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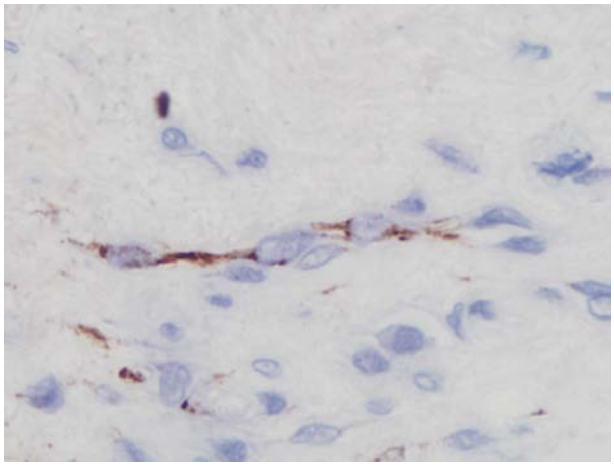


Figure 1.3 Photomicrographs of a dendritic cell

Representative photomicrographs of dendritic cell immunostaining in a bronchial biopsy obtained from a patient with asthma. A) CD1a+ immature dendritic cell, B) CD83+ dendritic cell. X400 magnification.

Immature dendritic cells express innate immune receptors however they also have the ability to take up antigen and present it to T cell receptors via major histocompatability complex (MHC) I and II (Banchereau & Steinman, 1998). On contact with antigen immature dendritic cells are recruited to the site and a dendritic cell network is established with the generation of long extensions connecting the epithelium and airway lumen (Figure 1.3B) (Huh *et al.*, 2003). These dendritic cells begin to mature over 24-48 hours and migrate to the draining lymph nodes which are rich in T cells. Once dendritic cell maturation begins, they modulate a range of regulatory chemokine and cytokine expression profiles which activate T cells enabling them to complete the immune response. For this reason dendritic cells have a unique ability to facilitate both the innate and adaptive immune system (Banchereau & Steinman, 1998).

There are several markers of dendritic cells; two of the most common markers are CD1a and CD83. CD1a belongs to a family of type1 CD1 membrane proteins and is known to be expressed in the interdigitating stratified squamous epithelium of the tonsil. CD1a is widely used as an immature dendritic cell marker in humans and upon maturation its expression is rapidly diminished (Brigl & Brenner, 2004) (Figure 1.3A). In contrast, CD83 is one of the best-known maturation markers for immunostaining mature dendritic cells (Figure 1.3B). Along with co-stimulatory molecules such as CD80 and CD86 it is strongly up regulated during dendritic cell maturation (Lechmann *et al.*, 2002).

The cell surface molecules, OX40 ligand (OX40L) and OX40 (CD134) are members of the tumour necrosis factor (TNF) receptor superfamily. Whilst

OX40 expression is located on naïve CD4+ T cells, OX40L is predominantly expressed by antigen presenting cells particularly dendritic cells (So *et al.*, 2008) (Ohshima *et al.*, 1997). The interaction of OX40L with its receptor OX40 is thought to be important in activating dendritic cell/T cell mediated Th2 inflammation (Liu *et al.*, 2007).

1.8.3 Mast cells

Mast cells were first discovered by Paul Ehrlich in his thesis in 1878 (Ehrlich, 1878) but it was only from 1957 that the mast cell gained attention in the study of allergy and asthma when they were reported to release histamine (RILEY & WEST, 1952). Mast cells originate from progenitor cells in the bone marrow and circulate in an immature form in the blood stream as CD34+ mononuclear cells. Mast cells only differentiate after recruitment into the target tissue and the precise characteristics are determined. Mast cells are commonly found in connective tissue and at mucosal surfaces placing them at the forefront of the human defence system. As a result mast cells have been implicated in the pathogenesis of many diverse diseases (Bradding & Holgate, 1999).

Despite mast cell populations exhibiting marked functional heterogeneity, their overall morphology is similar. They are elliptical cells up to 20 µm in diameter with an ovoid nucleus. Immature mast cells are multi-lobed whilst mature mast cells are mono-lobed and the cell membrane often show finger like projections. There is an abundance of cytoplasmic granules which contain preformed mediators (Figure 1.4).

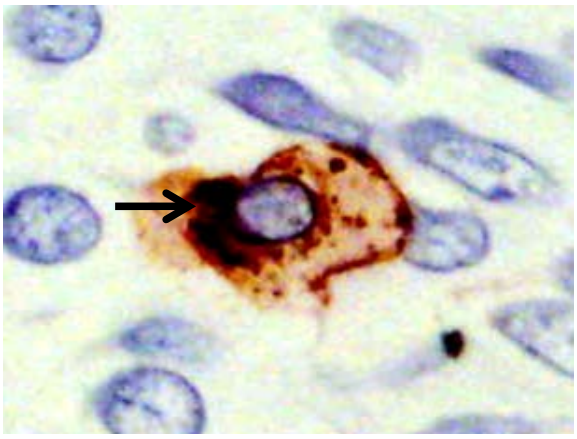


Figure 1.4 Photomicrograph of a mast cell

Representative photomicrograph of tryptase positive mast cell immunostaining in a bronchial biopsy obtained from a patient with asthma. This cell shows evidence of degranulation with loss of intracellular tryptase immunostaining in places (black arrow). X400 magnification.

Mast cells respond to a number of different stimuli resulting in the release of various mediators (Figure 1.5). Mast cell mediators can be divided into three subgroups, 1) preformed mediators including histamine, heparin, carboxypeptidase A, tryptase and chymase, TNF-α and IL-4 2) newly synthesised lipid mediators including the derivatives of arachidonic acid, prostaglandin D_2 (PGD_2) and leukotriene C4 (LTC_4) and 3) numerous cytokines and chemokines. The generation of these is not all-or-nothing, but highly dependent on the stimulus, allowing mast cells to achieve diverse biological effects. Table 1.1 and Table 1.2 summarises the principal biological properties of mast cell cytokines and mediators respectively.

Mast cells are divided into subtypes based on their protease content, tryptase positive mast cells (MC_T) and tryptase-chymase positive mast cells (MC_{TC}). Tryptase is expressed by all mast cells and is used to identify mast cells by immunostaining. In the lung MC_T is predominantly found in the lung parenchyma, bronchial epithelium and lamina propria. The MC_{TC} phenotype is found around blood vessels walls and in asthma, within the ASM bundles (Brightling *et al.*, 2002a) (Andersson *et al.*, 2009; Bradding *et al.*, 1995; Irani *et al.*, 1991). A third subtype chymase positive mast cell (MC_C) phenotype is rare and has been reported within the lung (Bradding *et al.*, 1995; Balzar *et al.*, 2005).

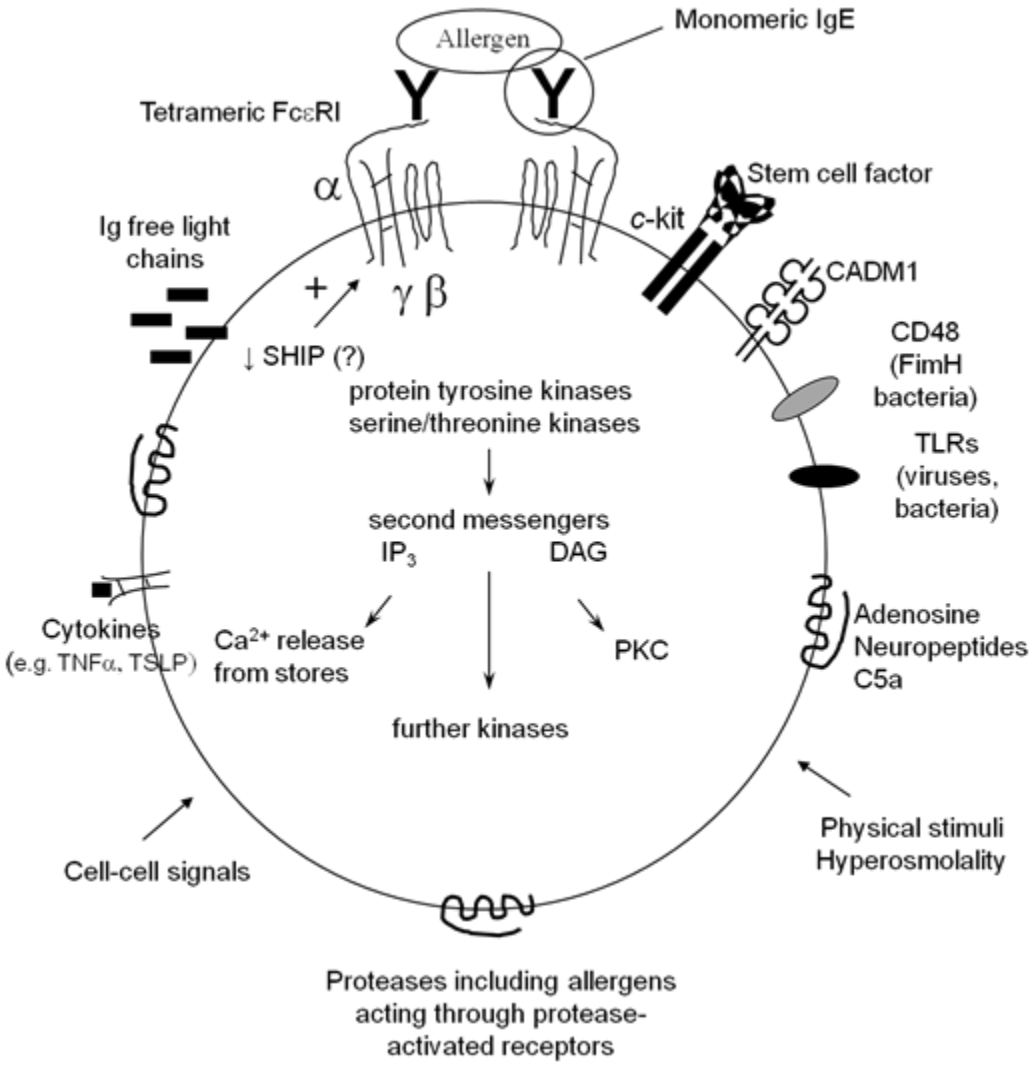


Figure 1.5 Mast cell activators.

Mast cells in lung inflammation Mast cell Biology: Contemporary and Emerging Topics, E.P Moiseeva and Peter Bradding edited by Alasdair M. Gilfillan and Dean D. Metcalfe. 2010 Landes Bioscience and Springer Science+Business Media

Cytokine	Function
IL-4	IgE, CD40, CD25, IL-6, MHC class II production, B cell, and fibroblast proliferation, T cells proliferate to a Th2 cell phenotype, transendothelial migration of eosinophils and increased FcεRI and ICAM-1 expression on mast cells
IL-3, IL-5, GM-CSF	Growth, adhesion, transendothelial activation and migration of eosinophils. Prolonged survival of eosinophils
IL-6	Mast cell survival increased IgE synthesis by B cells and T cell activation and differentiation
IL-13	IgE synthesis by B cells, decreased cytokines production by macrophages, activation and prolonged survival of eosinophils
TNF-α	Histamine and tryptase secretion by mast cells, enhanced cytotoxicity of eosinophils, T cells and macrophages, mucous gland hyper secretion
SCF	Growth differentiation, survival chemotaxis of mast cells
TGF-β	Smooth muscle differentiation and activation, epithelial cell proliferation inhibited
IFN-α	Increased cytotoxicity of natural killer cells, maturation of macrophages and dendritic cell activation, promotes survival of activated T cells
TSLP	Induction of Th2 immunity by dendritic cells and increased IL-13 expression

Table 1.1 Biological effects of human mast cell derived cytokines

Adapted from Mast cells in lung inflammation Mast cell Biology: Contemporary and Emerging Topics, E.P Moiseeva and Peter Bradding edited by Alasdair M. Gilfillan and Dean D. Metcalfe. 2010 Landes Bioscience and Springer Science+Business Media

	Mediator	Effects
Preformed	Tryptase	Increases bronchial hyperresponsiveness, epithelial cell activation, enhances mast cell histamine release, fibroblast proliferation, collagen synthesis, indirect collagenase activator, generates C3a and bradykinin
	Histamine	Mucus secretion, bronchoconstriction, dendritic cell activation, fibroblast proliferation, endothelial cell proliferation
	Heparin	Anticoagulant, fibroblast activation, endothelial cell migration, mast cell mediator storage matrix
	Chymase	Mucus secretion, SCF release, IL-4 degradation, activated IL-1β, degrades extracellular matrix, inhibits airway smooth muscle T cell adhesion
Newly synthesised	PGD_2	Mucus secretion, bronchoconstriction, dendritic cell activation, eosinophil, Th2 T cells and basophil chemotaxis, tissue oedema
	LTC_4/D_4	Mucus secretion, bronchoconstriction, dendritic cell maturation, tissue oedema, mast cell IL-5, IL-8, and TNF-α release, smooth muscle proliferation via IL-13, IL-4 secretion by eosinophils, tissue fibrosis

Table 1.2 Biological effects of human mast cell mediators

Adapted from Mast cells in lung inflammation Mast cell Biology: Contemporary and Emerging Topics, E.P Moiseeva and Peter Bradding edited by Alasdair M. Gilfillan and Dean D. Metcalfe. 2010 Landes Bioscience and Springer Science+Business Media

Mast cells express a range of receptors. Two of the most important are Kit (CD117), the receptor for stem cell factor necessary for growth and differentiation of mast cells and FcεRI which is the high affinity IgE receptor. FcεRI is a tetrameric structure consisting of a single α-subunit, a four transmembrane spanning β-subunit and two disulphide bonded γ-subunits (Blank *et al.*, 1989).

Contact with allergen initiates cross-linking of bound IgE which promotes FcεRI aggregation at the cell surface, eventually leading to the secretion of preformed mediators (degranulation). Monomeric IgE from B cells induces up-regulation of FcεRI surface expression, prolonged survival and cytokine release in mast cells (Knol, 2006; Blank & Rivera, 2004). In human lung mast cells, in the presence of stem cell factor (SCF), IgE induces histamine, LTC_4 and IL-8 release (Cruse *et al.*, 2005).

In healthy lung, mast cells are found beside blood vessels in the lamina propria however in asthma they migrate into three key structures; airway mucous glands, airway epithelium, and airway smooth muscle (Bradding *et al.*, 1994) (Brightling *et al.*, 2002a) (Carroll *et al.*, 2002).

Mast cell numbers are increased in submucosal glands from non-fatal asthmatic patients compared to controls. In both fatal and nonfatal asthma there are a significantly higher number of degranulated mast cells present. This infiltration of both granulated and degranulated mast cells in mucous glands correlates with the degree of mucous-related airway obstruction suggesting a role for mast

cells in mucous gland hyperplasia and mucous gland hypersecretion (Carroll *et al.*, 2002).

Activated mast cells are found within and in close proximity to the bronchial epithelium in asthmatic patients compared to controls (Bradding *et al.*, 1994) (Dougherty *et al.*, 2010). This places them at the point of entry for stimuli such as aeroallergens and can aid the inflammatory response. Through a carbohydrate dependent mechanism mast cells strongly adhere to the epithelial cells where their survival is partly promoted through epithelial cell derived stem cell factor (SCF) (Sanmugalingam *et al.*, 2000) (Miyata *et al.*, 2008). In addition epithelial cells suppress mast cell chymase expression (Allakhverdi *et al.*, 2007) and inhibit constitutive and IgE-dependent HLMC degranulation though a G0/Gi receptor coupled mechanism (Yang *et al.*, 2006b). (Martin *et al.*, 2012).

The microlocalisation of mast cells in the airway smooth muscle is a unique feature of asthma. The absence of other inflammatory cells (T cells and eosinophils) suggests that mast cells are selectively recruited to the ASM bundle (Brightling *et al.*, 2002a). Mast cell microlocalisation to the airway smooth muscle is mediated through the activation of CXCR3 expressed on mast cells by the ASM-derived chemoattractant CXCL10 (Brightling *et al.*, 2005). Once mast cells are recruited they avidly adhere to smooth muscle cells partly via a cell adhesion molecule 1 (CADM1) (formally known as tumour supressing lung cancer-1 (TSLC-1)) and partly through a Ca^{2+} independent pathway (Yang *et al.*, 2006a) (Yang *et al.*, 2006b)

In turn, this direct CADM1 dependent cell-cell adhesion drives HLMC proliferation and maintains their survival through the expression of SCF and IL-6 by ASM. Furthermore the cell-cell interaction results in mast cell degranulation (Hollins *et al.*, 2008). As a result many mast cell derived mediators are able to induce ASM contraction and/or induce AHR (see Table 1.2). For example, through the autocrine upregulation of TGF-β mast cells drive ASM to a more contractile phenotype (Woodman *et al.*, 2008).

The majority of these ASM-infiltrating mast cells are of the MC_{TC} phenotype and also express Th2 cytokines IL-4 and IL-13 (Brightling *et al.*, 2003b). IL-13 can attenuate ASM relaxation to β-actin and facilitate ASM contractility to acetylcholine therefore potentially inducing AHR (Grunstein *et al.*, 2002) (Laporte *et al.*, 2001). The number of mast cells in the ASM correlates with the degree of AHR and to the reduced bronchodilator response to deep inspiration (Slats *et al.*, 2007) suggesting that the presence of mast cells in smooth muscle is central to asthma physiology (Brightling *et al.*, 2002a) (Siddiqui *et al.*, 2008). This is strengthened by the absence of mast cells in the ASM of patients with eosinophilic bronchitis (EB). EB presents with chronic cough, is associated with sputum eosinophilia, and has a Th2 cytokine driven airway inflammation that is similar to asthma. AHR and variable airflow obstruction, which are the defining features of asthma, are not present in eosinophilic bronchitis suggesting that the presence of mast cells in ASM is specific to AHR seen in asthma. (Brightling, 2011) (Brightling *et al.*, 2002b).

1.8.4 Eosinophils

Eosinophils are derived from the bone marrow and develop from pluripotential stem cells in response to IL-3, IL-5, and granulocyte macrophage-colony stimulating factor (GM-CSF) (Metcalf *et al.*, 1987; Metcalf *et al.*, 1986; Yamaguchi *et al.*, 1988). In healthy controls eosinophils mature and migrate to the lamina propria of the gastrointestinal tract but in inflammatory disease like asthma eosinophils are recruited to the lungs.

Eosinophils have a bi-lobed nucleus connected by a nuclear material band and their size ranges from 12-17 µm in diameter. With an eosin dye these cells stain pink due to the acidophilic content and in immunohistochemistry antibodies to major basic protein are used as a specific eosinophil marker (Figure 1.6).

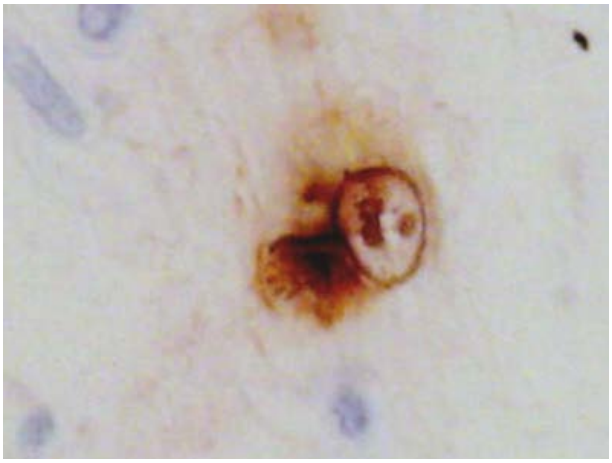


Figure 1.6 Photomicrograph of an eosinophil

Representative photomicrographs of MBP positive eosinophil immunostaining in bronchial biopsy obtained from a patient with asthma. X400 magnification

Eosinophils contain preformed stores of cytokines (Ponath *et al.*, 1996). They also contain the acidophilic granule proteins major basic protein (MBP), eosinophil peroxidases and eosinophilic cation protein. Elevated MBP levels have been reported in sputum of asthmatic patients and in high concentrations MBP is reported to be toxic to the bronchial epithelial cells (Frigas *et al.*, 1981), but the clinical significance of this is uncertain. In addition the release of these proteins causes mast cell degranulation and ASM hyperresponsiveness (Gundel *et al.*, 1991; Piliponsky *et al.*, 2001). Airway eosinophila is a key feature of asthma. Bronchial biopsies, induced sputum and bronchiolar lavage (BAL) fluids demonstrate elevated eosinophil numbers in steroid-naïve asthmatics (Frigas *et al.*, 1981) (Broide *et al.*, 1991) (Bradding *et al.*, 1994). Sputum eosinophil counts serve as valuable diagnostic marker and in this subset of patients a reduction in sputum eosinophils is associated with a reduction in asthma exacerbations (Green *et al.*, 2002a). However in a clinical study of an α-IL-5 antibody that inhibits eosinophil activation (mepolizumab) there was no improvement in day-to-day symptoms or AHR despite significant improvements in exacerbations (Haldar *et al.*, 2009).

1.8.5 Neutrophils

Neutrophils are polymorphonuclear cells whose name derives from the characteristic neutral pink staining seen on a haematoxylin and eosin (H & E) slide**.** Mature neutrophils have a segmented nucleus with 2-5 lobes interconnected by a nuclear material band and are 8-9 µm in diameter (Figure 1.7). They contain neutrophil elastase, cytokines, lipid mediators and reactive oxygen compounds which lead to tissue damage and possible mucous hyper-

secretion. Similar to various cells including mast cells, neutrophils express CXCR1 and CXCR2. These chemokine receptors are involved in neutrophil chemotaxis and activation (White *et al.*, 1998; Sabroe *et al.*, 1997).

Studies have shown a correlation between airway neutrophilia and the severity of asthma (Fahy *et al.*, 1995). Non-atopic asthmatics that have predominantly airway neutrophilia and an absence of sputum or tissue eosinophils do not respond to steroid treatment suggesting neutrophilic asthma maybe distinct clinical phenotype (Green *et al.*, 2002b) (Wenzel *et al.*, 1999) (Berry *et al.*, 2007).

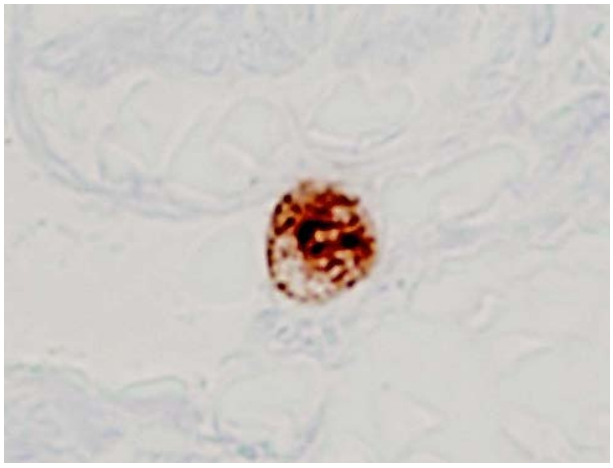


Figure 1.7 Photomicrograph of a neutrophil

Representative photomicrographs of neutrophil elastase positive neutrophil immunostaining in bronchial biopsy obtained from a patient with asthma. X400 magnification.

1.8.6 Smooth Muscle Cells

Airway smooth muscle (ASM) was first described by Reisseisen in 1822 (Reisseisen FD, 1822). It surrounds the lumen of the bronchi and once developed express the markers α-smooth muscle actin, myosin, desmin, tropomyosin, caldesmon and calponin (Low & White, 1998). Smooth muscle plays a central role in asthma and is associated with airway remodelling, AHR and inflammation.

One of the key features of airway remodelling is an increase in smooth muscle mass. The mechanisms underlying ASM remodelling are ASM hyperplasia and hypertrophy and deposition of extracellular matrix proteins (ECM) proteins (Woodruff *et al.*, 2004) (Benayoun *et al.*, 2003).

Analysis of ASM from patients with fatal asthma identified two subgroups; one in which there was ASM hyperplasia and a second where there was evidence of cellular hypertrophy throughout the airway (Ebina *et al.*, 1993). There is limited data on ASM hyperplasia and hypertrophy in non-fatal asthma due to the difficulty in obtaining bronchial biopsies with the full ASM thickness. However one study shows there is an increase in ASM mass in both the large and small airways of fatal and non-fatal asthma compared to controls (Carroll *et al.*, 1993). Airways from severe asthmatics have been shown to have increased ASM cell diameter compared to mild asthmatics and controls suggesting hypertrophy maybe the cause of increased ASM in a subset of patients (Benayoun *et al.*, 2003). A recent study showed ASM hypertrophy was present in both fatal and non-fatal asthma however in ASM hyperplasia was present only in fatal asthma

(James *et al.*, 2012b). Furthermore the group demonstrated in fatal asthma the total amount of extracellular matrix within and around the ASM is increased as disease severity progresses, which is not related to the duration of asthma (James *et al.*, 2012b). In vitro ASM cell proliferation is increased in asthmatic patients compared to healthy controls (Johnson *et al.*, 2001). In a separate study Woodruff *et al.* found no increase in ASM cell size in patients with mild-moderate asthma however they did find a twofold increase in ASM cell number compared to controls (Woodruff *et al.*, 2004). Together these studies suggest how the increase in smooth muscle mass, may differ according to disease severity.

The ASM is the main cell type involved in AHR and is capable of transforming between “contractile” and “synthetic” phenotypes, characterised by alterations in marker expression (Halayko *et al.*, 1997). Direct mediators such as methacholine can directly induce smooth muscle contraction while indirect mediators first stimulate mast cells that release mediators leading to ASM contraction. The mechanisms leading to ASM AHR are unclear, but as discussed above under mast cells, the cross-talk between ASM and mast cells may be a key factor.

1.8.7 T lymphocytes

Historically eosinophils and mast cells had been the main focus of histological studies in asthma (Ellis, 1908). T cells only came into the limelight in asthma with the discovery of the Th1 and Th2 subsets of helper T cells (Mosmann *et al.*, 1986).

T lymphocytes mature in the thymus and are distinguished from other lymphocytes (B cells and natural killer cells) by the expression of the T cell receptor (TCR) on the surface. Upon maturation they migrate to secondary lymphoid organs through the blood and the lymphatic system and depending on the microenvironment differentiate into CD4+ T helper (Th) cells, cytotoxic CD8+ T cells, memory T cells (either CD4+ or CD8+), and natural killer T cells. Once activated CD4+ T cells can be further subdivided into Th1, Th2, Th17 and T regulatory (Treg) subsets depending on their production of signature cytokines (Figure 1.8).

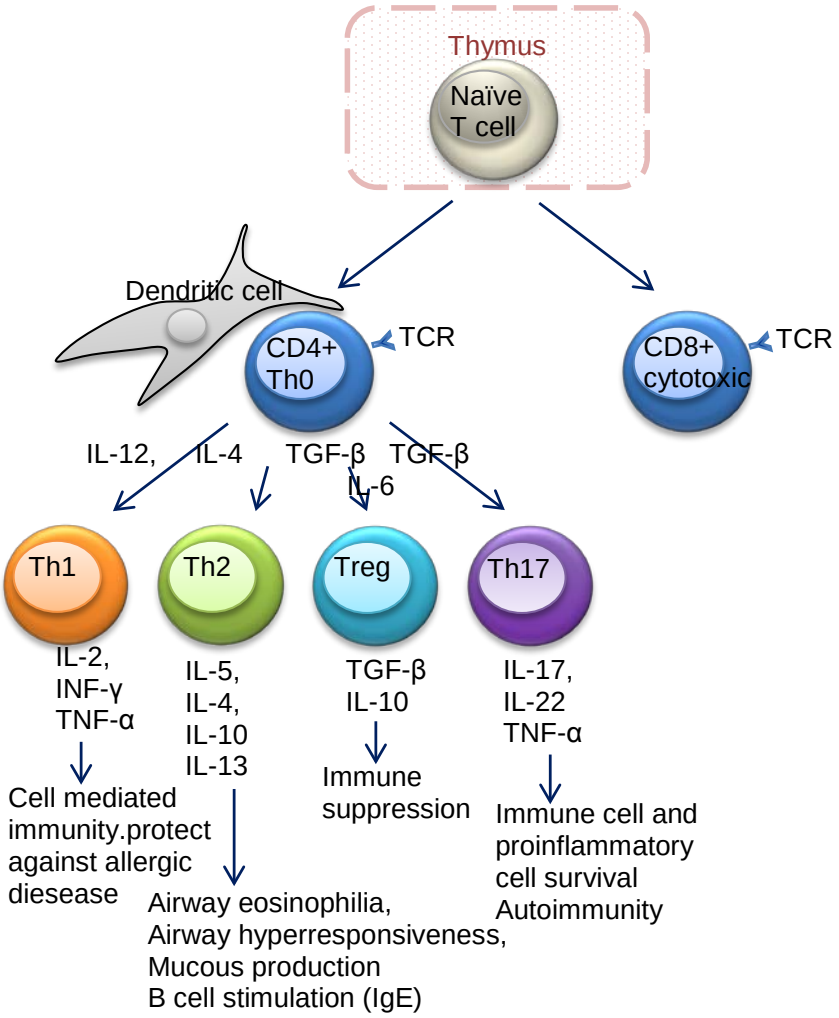


Figure 1.8 Model of T helper cell differentiation

CD4+ T cells differentiate into 4 different cell types each producing specific signature cytokines

1.8.7.1 Th1/Th2 cell profiles

Antigen activated T cells in the presence of IL-12 differentiate into Th1 cells and produce mainly interferon-γ (IFN-γ), but also IL-2, and TNF-α. IFN-γ is important at maximising antigen presentation to macrophages as well as inhibiting IL-4 production. In the presence of IL-4 antigen-activated Th2 cells develop and expand subsequently producing additional IL-4 and IL-13, and IL-5 (Abbas *et al.*, 1996). These Th2 cytokines have a range of effects in particular induction of IgE production by B-cells, mast cell degranulation, activation and recruitment of eosinophils, mucous production, and AHR. Together IFN-γ/Th1 and IL-4/Th2 counter-regulate each other however in allergic inflammation the Th1/Th2 balance is skewed towards a Th2 phenotype.

Facilitating Th1/Th2 imbalance are the recent discovery of T regulatory (Treg) cells which act as suppressor T cells and are involved in down regulating the immune response. Increased expression of the inhibitory cytokines, IL-10 and TGF-β by Treg cells leads towards a Th1 phenotype, whereas inhibition creates a Th2 permissive environment (Akdis *et al.*, 2004). Recent studies suggest immunosuppression of Treg cells in allergic inflammation may augment a Th2-dominant immune response (Lin *et al.*, 2008; Hartl *et al.*, 2007).

1.8.7.2 Th2 Cytokines

IL-4 was first described by a variety of different names B-cell IgG differentiation factor, IgG1 induction factor B-cell stimulating factor 1 and B-cell growth factor 1 (Noma *et al.*, 1986). It was initially recognised for its ability to induce isotype class switching of B cells to IgE synthesis and subsequent up regulation of IgE

receptors on mast cells and basophils (Coffman *et al.*, 1986) (Del Prete *et al.*, 1988). Soon after, it was associated with the Th2 subsets of helper T cells and therefore a crucial facilitator of Th2 development (Mosmann *et al.*, 1986) (Swain *et al.*, 1990).

Th2 cells are a source of IL-4 however these cells require IL-4 to initiate this synthesis. Other cells capable of expressing IL-4 are mast cells, eosinophils basophils and macrophages (Bradding *et al.*, 1994) (Gessner *et al.*, 2005) (Pouliot *et al.*, 2005). IL-4 binds to the IL-4 receptor-α (IL-4Rα) which is expressed on T cells. In addition studies have shown B cells, eosinophils, fibroblasts, smooth muscle cells and bronchial epithelial cells all express the functional receptor (Kotsimbos *et al.*, 1998) (Dubois *et al.*, 1998) (Doucet *et al.*, 1998) (van der Velden *et al.*, 1998). The IL-4Rα is made up of 3 receptor subunits. IL-4Rα/common γ chain (type I) is specific to only IL-4 whereas the other two complexes IL-13Rα1/IL-4 and IL-13Rα1/IL-13 (type II) allow both IL-4 and IL-13 to bind which explains their biological overlap (LaPorte *et al.*, 2008).

In asthma elevated IL-4 protein levels are reported in the serum and bronchiolar lavage fluid (Daher *et al.*, 1995; Walker *et al.*, 1994). Bronchial biopsies have elevated levels of IL-4 protein and mRNA as well as increased expression of IL-4Rα in subjects with asthma (Humbert *et al.*, 1996) (Kotsimbos *et al.*, 1996) (Kotsimbos *et al.*, 1998). In mild asthmatics airway hyper responsiveness and eosinophila were induced upon administering IL-4 and in mouse models IL-4 deficient mice had reduced inflammation and eosinophilia following allergen challenge (Shi *et al.*, 1998; Brusselle *et al.*, 1994).

T lymphocytes were first reported in 1993 to express IL-13 with its position located on the same gene cluster as IL-4, chromosome 5q31-34 (Minty *et al.*, 1993). IL-13 expression is predominantly in T cells but mast cells, eosinophils and more recently epithelial cells have been shown to be vital sources in asthma (Minty *et al.*, 1993) (Jaffe *et al.*, 1996) (Schmid-Grendelmeier *et al.*, 2002) As mentioned earlier, IL-4 and IL-13 functionally overlap as they share a common receptor unit. The IL-13 receptor consists of the same IL-4Rα and an additional IL-13Rα1 subunit. IL-4 can signal through both of these receptor subunits (Wills-Karp & Finkelman, 2008). IL-13Rα1 is expressed by structural cells -epithelial cells, smooth muscle, fibroblasts as well as inflammatory cells-mast cells eosinophils, macrophages (Wang *et al.*, 2004) (Lordan *et al.*, 2002) (Murata *et al.*, 1998) (Laporte *et al.*, 2001).

Two reports revolutionised the study of IL-13 in asthma after demonstrating it to be a ‘central mediator of allergic asthma’ (Wills-Karp *et al.*, 1998) (Grunig *et al.*, 1998). In asthma elevated IL-13 levels are reported in the sputum, bronchial biopsies and bronchiolar lavage fluid (Saha *et al.*, 2008). IL-13 concentrations in peripheral blood are elevated with further increases during exacerbations (Siddiqui *et al.*, 2009) (Lee *et al.*, 2001b). IL-13 mRNA expression is increased in bronchial biopsies from moderate asthmatics and in sputum from mild and moderate asthmatics (Kotsimbos *et al.*, 1996; Berry *et al.*, 2004). In addition BAL cells from mild asthmatics have elevated IL-13 expression (Kroegel *et al.*, 1996). Genome wide association studies (GWAS) have reported bronchial hyper responsiveness, elevated IgE levels and asthma susceptibility are related to single nucleotide polymorphisms (SNP) of the IL-13 gene and the IL-13

receptors genes (Bottema *et al.*, 2010; Li *et al.*, 2010; Howard *et al.*, 2002). The relationship between asthma and IL-13 is further supported by animal models. When IL-13 activity is neutralised, allergen–induced airway hyper responsiveness, mucous production, airway inflammation and eosinophilia are inhibited whereas exogenous IL-13 delivery to the airway restores these effects (Grunig *et al.*, 1998). Together these studies begin to suggest IL-13 is influential in only a subset of asthmatic patients.

1.9 Th2 Cytokines in Severe Asthma

Asthma can be classified in many ways, and it is useful to consider it terms of severity. It is patients with “severe” disease who have the real unmet clinical need. The study of “severe” asthma has been complicated by the lack of a clear definition, but for practical purposes, patients uncontrolled at treatment step 4 or reaching step 5 in the BTS/SIGN Asthma Guideline can be considered to have severe disease. The study of severe asthma has also been hampered by the recognition that asthma is a highly heterogeneous disease both clinically (Haldar *et al.*, 2008) and at the molecular level (Woodruff *et al.*, 2009). For many years research has focussed on the helper cell Th1/Th2 imbalance hypothesis, whereby it is proposed that T cell-derived Th2 cytokines such as IL-4, IL-5 and IL-13 promote airway mucosal eosinophilia and disordered airway function and Th1 cytokines IL-2 and IFN-γ, inhibit T cell proliferation and promote activation induced cell death (Kay, 2006). However, these Th2 cytokines may contribute to disease pathogenesis in only a proportion of patients.

Gene expression microarrays in airway epithelial cells revealed 3 genes, periostin (POSTN), chloride channel regulator 1 (CLCA1), and serpin peptidase inhibitor, clade B, member 2 (SERPINB2) are specifically induced in asthma and are directly regulated by IL-13 (Woodruff *et al.*, 2007). Using these three genes as surrogate markers of Th2 inflammation a recent study of gene arrays from asthmatic tissue suggested that up to 50% of patients do not exhibit evidence of Th2-driven inflammation (Woodruff *et al.*, 2009). Furthermore, IL-13 expression is only detectable in about 50% of patients with severe asthma (Saha *et al.*, 2008), suggesting that IL-13-independent mechanisms drive severe disease in a substantial proportion of patients (Woodruff *et al.*, 2009).

1.10 Novel biological approaches to therapy

The emerging evidence for cytokines as therapeutic targets in asthma management is a new and exciting field giving rise to a new category of drugs. The role of cytokines therefore is important in the pathogenesis of asthma. Restoring the imbalance of dysregulated cytokine expression is a key target for novel asthma treatment. Efforts have been made to either block elevated levels of cytokines or to find alternative cytokines that are insufficiently expressed in asthma. Clinical trials with cytokine inhibitors have had success in some studies but not with others.

Lebrikizumab developed by Genentech Inc. (San Francisco), is a humanised α-IL-13 antibody that binds to IL-13. It is targeted for asthmatics that are not well controlled on inhaled or oral corticosteroids and long-acting β2- agonists. In a phase II trial asthmatic patients with high serum periostin (Th2-high status) had

increased lung function with lebrikizumab compared to those with low periostin (Th2-low status) levels. In addition lebrikizumab reduces exacerbations in severe asthma with a greater reduction in Th2-high status (Corren *et al.*, 2011). Lebrikizumab has recently started phase III trials to ascertain its safety and efficacy. Other humanised IgG1 antibodies that bind to or neutralise IL-13 activity were used to block the binding of IL-13 to the IL-4 receptor α chain. This attenuated allergen-induced bronchoconstriction during the early and late asthmatic responses (Gauvreau *et al.*, 2011).

In addition measuring serum periostin levels maybe a potential biomarker of airway eosinophilia. Periostin is partly regulated by IL-13 and could potential be used in targeting therapy to patients with Th2 inflammation. Patients with airway eosinophilia had increased serum periostin levels despite high dose corticosteroids this correlated with blood and sputum eosinophila. Serum periostin was the best measure of eosinophilia when compared with IgE levels, blood eosinophil numbers and fraction of exhaled nitric oxide (Feno) levels (Jia *et al.*, 2012).

Omalizumab (trade name Xolair) developed by Genentech/Norvartis, is a humanised form of α-IgE antibody approved for treatment for people over 12 years with uncontrolled severe persistent allergic asthma after inhaled corticosteroids, long-acting β-agonists, and leukotriene modifiers fail to work. It selectively binds to an antigenic epitope on IgE which overlaps with FcεRI high affinity IgE receptor preventing crosslinking of IgE in mast cells and basophils (Presta *et al.*, 1993). Reduced exacerbations, reduced hospitalisation, reduced

corticosteroid use and improved quality of life are some of the clinical benefits of omalizumab (Bousquet *et al.*, 2005). However omalizumab treatment has no effect on lung function (Rodrigo *et al.*, 2011).

Mepolizumab (trade name Bosatria) is a humanised α-IL-5 monoclonal antibody manufactured by GlaxoSmithKline which selectively inhibits eosinophilic airway inflammation by preventing IL-5 activated eosinophil production. Therefore it is targeted at a subgroup of patients with severe eosinophilic asthma that suffer from exacerbations despite using high-dose inhaled or oral corticosteroids and long-acting beta-2 agonist use. Studies show mepolizumab reduces lung eosinophils and eliminates blood eosinophil counts. Clinically patients with severe eosinophilic asthma had reduced asthma exacerbations, improved quality of life, and reduced use of prednisone when treated with mepolizumab. However in these studies it did not improve every day symptoms lung function or bronchial hyper-responsiveness (Haldar *et al.*, 2009; Nair *et al.*, 2009). These findings were recently confirmed in a large scale multi-centered trial (DREAM) (Pavord *et al.*, 2012). Mepolizumab has recently started phase III trials to ascertain its safety and efficacy.

Pitrakinra (trade name Aerovant) is an IL-4 receptor antagonist developed by Aerovance. It prevents inflammation induced by IL-4 and IL-13 by selective inhibition of the IL-4Rα subunit thus inhibiting downstream signaling. It is aimed for asthmatics that are not well controlled on inhaled or oral and long-acting β2-agonists. In phase II studies following allergen challenge pitrakinra improved pulmonary function and reduced exhaled nitric oxide, a marker of lung

inflammation. In addition there was a reduction in asthma attacks requiring medical intervention (Wenzel *et al.*, 2007).

Other cytokine inhibitor clinical trials have been less successful. Cytokine treatment AMG317 is another α-IL-4Rα antibody used in phase II, however it had no clinical efficacy (Corren *et al.*, 2010) Altrakincept is a solubilised IL-4 receptor fragment that neutralises IL-4 however it was inefficient in phase III trials (Borish *et al.*, 2001). Pascolizumab an intravenous humanised α-IL-4 monoclonal antibody by GlaxoSmithKline also failed to demonstrate efficacy in phase II trial (Hart *et al.*, 2002).

Other potential cytokine targets in asthma include, TNF-α, α-IL-9 and α-granulocyte-macrophage colony-stimulating factor (GM-CSF) however human trials are in very early stages, or in the case of anti-TNF-α, were ineffective in larger randomised controlled-trials (Wenzel *et al.*, 2009). There is a need for novel potential asthma treatments targeted at specific aspects of the inflammatory response.

Recent evidence suggests the induction and regulation of Th2 cytokine dependent immune responses involve novel epithelial cell derived TSLP, IL-33 and IL-25 (Bousquet *et al.*, 2005). Investigating this triad of non-hematopoietic cell-derived cytokines is an exciting area for emerging asthmatic therapeutic targets.

1.11 Thymic Stromal Lymphopoietin

1.11.1 TSLP Structure

TSLP was originally cloned as a factor for lymphocyte proliferation in conditioned media from a mouse thymic stromal cell line (Friend *et al.*, 1994). It has been found to promote the development of IgM+ B cells. Murine TSLP is four-helix bundle which has three N-linked carbohydrate addition sites and seven cysteine residues (Levin *et al.*, 1999). Using *in silico* methods, a TSLP homolog was identified in humans which is 140 amino acids in length. Similar to murine TSLP, human TSLP is an IL-7 like four-helix-bundle cytokine that has two *N*-glycosylation sites and six cysteine residues. It has a calculated molecular weight of 14.9kDa but TSLP purified from HEK293 cells overexpressing TSLP has a molecular weight of 23kDa suggesting the mature form maybe glycosylated. The human orthologue of TSLP shares a weak sequence homology of only 43% amino acid identity with murine TSLP and there is no cross species reactivity (Reche *et al.*, 2001). Human *TSLP* gene is located on chromosome 5q22.1 which is in close proximity to the Th2 cytokines (IL-4, IL-5, IL-9 and IL-13) gene cluster loci 5q31-34.

1.11.2 TSLP binds to the TSLP Receptor

The TSLP receptor is a heterodimeric receptor complex comprising of two subunits, TSLP-specific receptor chain and IL-7-receptor α chain (IL-7Rα). TSLP-specific receptor subunit is similar to common γ receptor chain and binds to TSLP with low affinity whereas the IL-7Rα subunit does not bind to TSLP. However together, the binding affinity of TSLP to the TSLP-receptor complex (TSLP-R) is considerably increased. TSLP-R contains a single tyrosine residue

and a conserved box 1 motif, the latter being important for Janus tyrosine kinase binding (Figure 1.9) (Park *et al.*, 1990) (Isaksen *et al.*, 2002). Similar to TSLP, human and mouse TSLP-receptor shares a homology of only 39% amino acid identity (Reche *et al.*, 2001; Pandey *et al.*, 2000).

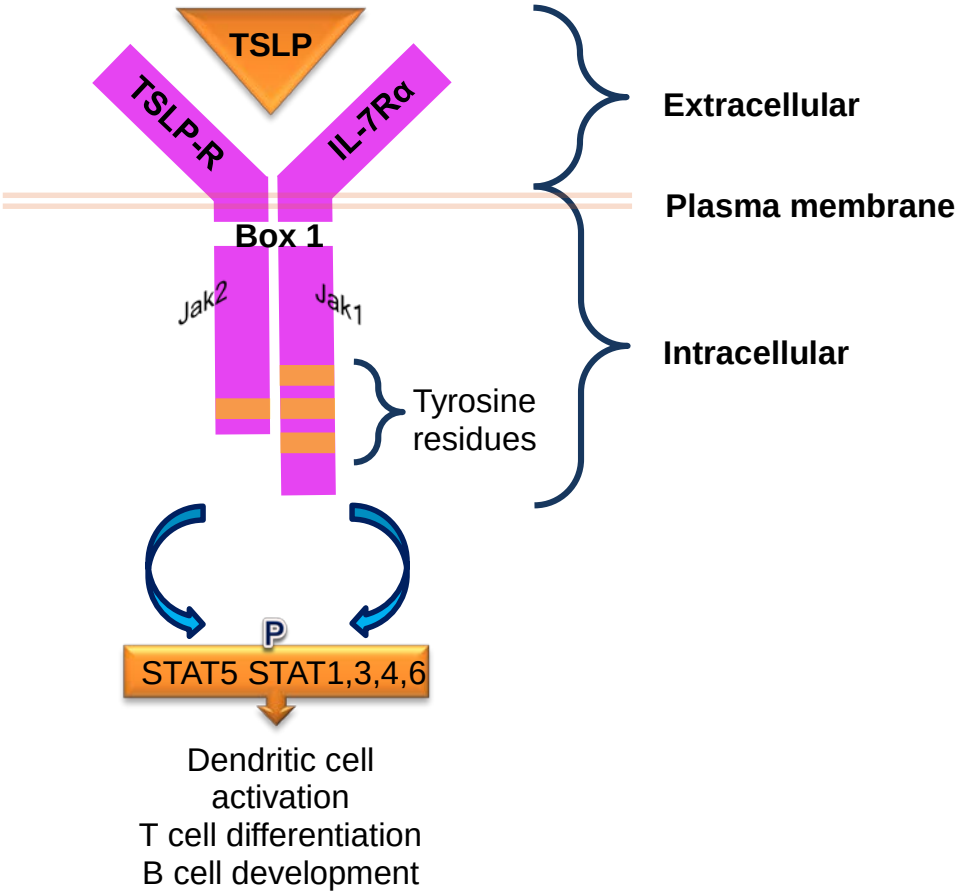


Figure 1.9 Schematic representation of TSLP bound to the TSLP receptor complex.

Intracellular part contains a box 1 motif and tyosine residue. After receptor engagement Jak1 and 2 are activated leading to signal transducer and activator of transcription (STAT) phosphorylation. Some of the outcomes are listed.

TSLP-R is expressed on hematopoietic lineage cell populations such as dendritic cells, T cells, B cells, basophils, and eosinophils (Reche *et al.*, 2001) (Park *et al.*, 1990). In addition mast cells express TSLP-R (Allakhverdi *et al.*, 2007) (Semlali *et al.*, 2010) (Rochman & Leonard, 2008). This expression is important in studying TSLP's primary biological role, which will be discussed in later sections.

1.11.3 TSLP Gene Studies

Genome wide association studies (GWAS) and meta-analysis of GWAS are recently being used to identify pathways related to allergic diseases including asthma.

The GABRIEL study (A multidisciplinary study to identify the genetic and environmental causes of asthma in European community) is a meta-analysis of European populations (Moffatt *et al.*, 2010). A separate meta-analysis, the EVE study is an asthma meta-analysis in ethnically diverse US populations (Torgerson *et al.*, 2011). Both studies consistently identified single nucleotide polymorphisms (SNP) in 7 loci that were highly associated with asthma of which 4 loci (*TSLP, 17q21 locus, the IL-1RL1/IL18R1 locus and IL-33*) met genome wide significance levels with ethnically diverse subjects. These results highlight the importance of epithelial cell derived cytokines (*TSLP, IL-33, IL-1RL1* which encodes IL-33 receptor) in asthma. In addition to asthma GWAS, a GWAS of eosinophilic esophagitis (EoE) an allergic disorder whereby eosinophils accumulate in the oesophagus identified significant SNPs near the *TSLP* loci and that TSLP mRNA expression was increased in subjects with EoE

suggesting a role in other allergic disease (Rothenberg *et al.*, 2010) (Sherrill *et al.*, 2010).

Candidate gene studies are, as the name implies, studies in which a known gene is selected and studied based on their susceptibility to allergic disease. TSLP candidate gene studies have found genetic polymorphisms near or on the *TSLP* loci which are associated with allergic disease.

Allergic rhinitis was associated with a TSLP SNP (rs1837253) in 3 different cohorts of asthmatic subjects (Bunyavanich *et al.*, 2011). In an atopic dermatitis gene study variants in *TSLP* and its receptors were associated with higher risk of atopic dermatitis (Gao *et al.*, 2010)

TSLP has been reported to have 2 splice variants (Roche *et al.*, 1989; Reche *et al.*, 2001). In asthma studies only the long form of TSLP can be induced in normal bronchial epithelial cells when stimulated with double-stranded RNA (mimics viral DNA) and result in TSLP protein expression. This suggests TSLP production by environmental stimuli maybe be affected by genetic factors (Harada *et al.*, 2009). The role of the short form *TSLP* remains unclear. A rs3806933 in the *TSLP* promoter region of the long form, creates an activator protein 1 (AP-1) transcription factor binding site which enhances AP-1 binding to regulatory molecules leading to the downstream effects of TSLP (Harada *et al.*, 2009) (Demoly *et al.*, 1992). In a Japanese population this *TSLP* polymorphism correlated with both childhood and adult asthma (Harada *et al.*, 2011). This same SNP was identified in the genomic TSLP loci by the GWAS.

A sex stratified analysis showed that a TSLP SNP (rs2289276) in Costa Rican asthmatic females was associated with cockroach-specific IgE (Hunninghake *et al.*, 2008). Two separate groups reported asthma was associated with the SNP rs1837253 upstream of the TSLP transcription start site (Moffatt *et al.*, 2010; He *et al.*, 2009). Taken together these genetic studies further implicate the *TSLP* loci in allergic disease especially asthma.

1.11.4 TSLP Signalling

There is little information regarding TSLP's signalling pathway once it engages with the TSLP-R. Early murine studies reported that TSLP could induce tyrosine phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT 5 but not any of the Janus family kinases (JAK) which are known cytokine dependent STAT activators (Pandey *et al.*, 2000) (Reche *et al.*, 2001) (Levin *et al.*, 1999).

However 2 recent studies have shed light on the role of various STAT family members and JAK (Rochman *et al.*, 2010; Arima *et al.*, 2010). In human and murine $CD4^+$ cells, TSLP activates STAT5 via JAK1 and JAK2 which are associated with IL-7Rα and TSLP-R, respectively. The study shows the phosphorylation of JAK1 and JAK2 precedes the activation of STAT5 and that STAT5 signalling is necessary for CD4+ T cell survival and proliferation (Rochman *et al.*, 2010). The second study confirmed these finding in human dendritic cells (Arima *et al.*, 2010).

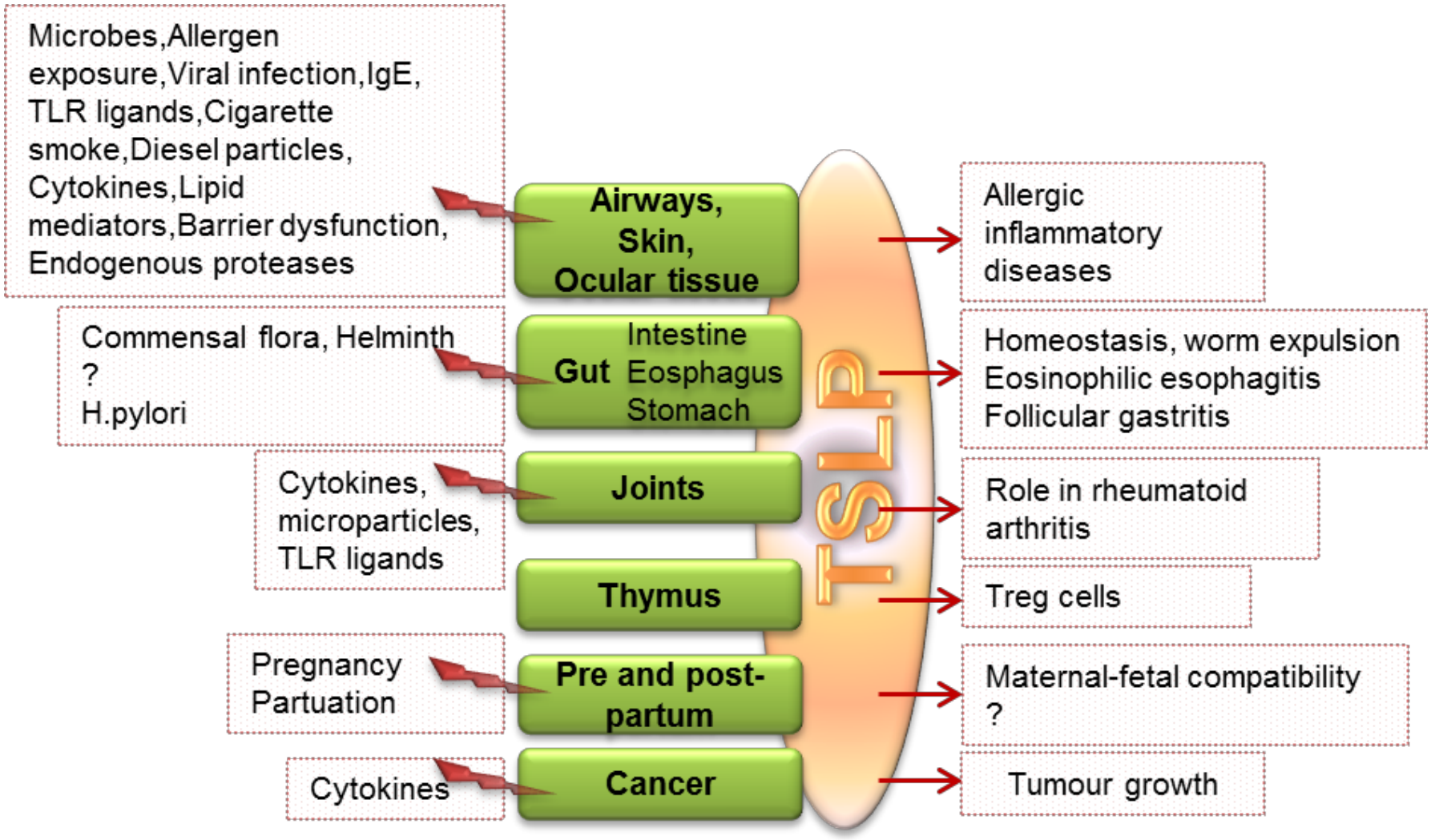
In addition to STAT5 signalling human TSLP-activated dendritic cells were able to phosphorylate a range of STATs including STAT1, STAT3, STAT4, and STAT6. Of particular interest are STAT5 and STAT6. As well as confirming the Rochman study STAT5 and STAT6 are involved in Th2 responses. Phosphorylation of STAT6 results in the production of Th2 cell attracting chemokine thymus and activation-regulated chemokine (TARC /CCL17) by activated dendritic cells, T cells and macrophages (Liddiard *et al.*, 2006; Wirnsberger *et al.*, 2006). TSLP's unique ability to activate this pathway in dendritic cells partly explains how TSLP-activated dendritic cells are able to produce TARC and supports the finding that increased TSLP mRNA expression correlates to increased TARC expression in human bronchial biopsies and in nasal polyps (Soumelis *et al.*, 2002; Ying *et al.*, 2005) (Kimura *et al.*, 2011). In addition TSLP-activated dendritic cells have a distinct feature compared to TLR activated dendritic cells in that they do not stimulate the production of IRF-8 or STAT4 both which are essential for Th1 polarising cell production factors for IL-12 production by dendritic cell (Arima *et al.*, 2010). In addition TNF-α and a combination of Th2 cytokines increase TSLP production but not individually lending further evidence to the role of STAT signalling (Kato *et al.*, 2007).

1.11.5 Induction of TSLP Expression

TSLP is released by cells in response to various environmental and endogenous stimuli. Some of the major environmental contributors induce injury and include microbes, allergen exposure, viral infection, TLR ligands, cigarette smoke and diesel particles. Endogenous contributors include cytokines, lipid mediators, IgE, barrier dysfunction and endogenous proteases (Figure 1.10)

Figure 1.10 Triggers and tissue sources of TSLP

The production of TSLP in response to various environmental and endogenous stimuli contributes to a wide range of diseases and disorders. Modified from (Takai, 2012)


Microbes,Allergen exposure,Viral infection,IgE, TLR ligands,Cigarette smoke,Diesel particles, Cytokines,Lipid mediators,Barrier dysfunction, Endogenous proteases
Airways, Skin, Ocular tissue
Allergic inflammatory diseases
Commensal flora, Helminth ? H.pylori
Gut Intestine Eosphagus Stomach
Homeostasis, worm expulsion Eosinophilic esophagitis Follicular gastritis
Cytokines, microparticles, TLR ligands
Joints
Role in rheumatoid arthritis
Thymus
Treg cells
TSLP
Pregnancy Partuation
Pre and post-partum
Maternal-fetal compatibility ?
Cytokines
Cancer
Tumour growth

1.11.5.1 Microbes

In atopic dermatitis the lesions are extensively colonised with *Staphylococcus aureus* (Kisich *et al.*, 2008). In keratinocytes a *S. aureus* fraction stimulated TSLP production and its mRNA expression was TLR2 and TLR6 dependent (Vu *et al.*, 2010). In mice dermal fibroblasts *S. aureus* vesicles trigger TSLP production (Hong *et al.*, 2011).

1.11.5.2 Allergen Exposure

Alternaria is an airborne fungus that contains prototopic proteases. Exposure to an alternaria extract stimulated TSLP release in a BEAS-2B epithelial cell line and TSLP protein expression in bronchial epithelial cells (Kouzaki *et al.*, 2009). House dustmite extract induces TSLP production by epithelial cells and dendritic cells (Kashyap *et al.*, 2011). In addition TSLP expression is increased in BAL from mice when exposed to house dustmite (Hammad *et al.*, 2009).

1.11.5.3 Virus

Rhinovirus and respiratory syncytial virus (RSV) are known to trigger asthma exacerbations. These viruses can in human bronchial epithelial cells up regulate TSLP mRNA expression thorough a p38 and Jun kinase signalling pathway (Tu *et al.*, 2007). Upon infection with rhinovirus bronchial epithelial cells are reported to increase TSLP mRNA and in bronchial epithelial cells from COPD patients there is an increase in TSLP release (Kato *et al.*, 2007; Calven *et al.*, 2012; Brandelius *et al.*, 2011). TSLP production is increased in airway epithelial cells after infection with RSV through NF-$_K$β / retinoic acid induced gene I (RIG-I) signalling pathway (Lee *et al.*, 2012).

1.11.5.4 TLR ligands

Double stranded RNA (dsRNA) is a molecular pattern that's associated with viral infection. TLR3 recognises this pattern and the commercial analog of dsRNA, polyinosine:polycytidylic acid (Poly I:C) is used to mimic viral dsRNA.

Both Poly I:C and bacterial peptidoglycan (PGN) are able to increase TSLP production in bronchial epithelial cells and small airway epithelial cells (Kato *et al.*, 2007) (Allakhverdi *et al.*, 2007). The ligands for TLR2 (lipoteichoic acid, LTA), TLR8 (ssRNA) and TLR 9 (CpG-B) can also trigger TSLP mRNA expression in airway epithelial cells (Lee & Ziegler, 2007). In addition TSLP mRNA is increased by toll like receptor 3 (TLR3) and this effect is synergistically enhanced with the addition of IL-4 (Kato *et al.*, 2007) (Allakhverdi *et al.*, 2007).

1.11.5.5 Cytokines

As well as working synergistically with TLR, cytokines are known inducers of TSLP production (Okayama *et al.*, 2009; Nagarkar *et al.*, 2012; Bogiatzi *et al.*, 2007). TSLP mRNA expression is significantly up-regulated in normal human bronchial epithelial cells (NHBE) when primed with Th2 cytokines IL-4 and IL-13 (Kato *et al.*, 2007). In atopic dermatitis, high levels of Th2 (IL-4 or IL-13) and proinflammatory (IL-1α or TNF-α) cytokines in lesional skin increases TSLP production by keratinocytes (Bogiatzi *et al.*, 2007).

As well as being associated with asthmatic lung and pulmonary function, TNF-α and IL-1β are known inducers of NF-$_K$β signalling (Matsukura *et al.*, 1999). NF-$_K$β is a ubiquitously expressed transcription factor that adjusts the expression of

cellular genes involved in immune inflammatory processes. Proinflammatory cytokines IL-1β and TNF-α are able to induce TSLP production in NHBE stimulated and mutations in the NF-$_K$β binding sites inhibit TSLP release by IL-1β (Lee & Ziegler, 2007). Equally IL-4, IL-13, TGF-β and IFN-β can up regulate TSLP expression. The expression by IL-4 and IL-13 is enhanced in combination with TNF-α in bronchial epithelial cells (Kato *et al.*, 2007). IL-4 induces TSLP mRNA and protein expression in epithelial cells in the presence of the prototypic proteases either trypsin or papain. Protease-sensing G protein-coupled receptor, protease-activated receptor 2 (PAR-2) was required for this induction (Kouzaki *et al.*, 2009).

These studies suggest innate or adaptive immune cells modulate TSLP expression through inflammatory stimuli or by TLR ligands during inflammation.

1.11.5.6 Lipid Mediators

Asthmatic patients have elevated levels of the bioactive lipid mediator lysophosphatidic acid in the lungs and in bronchial epithelial cells TSLP expression is induced in the presence of lysophosphatidic acid (Medoff *et al.*, 2009).

1.11.5.7 Cigarette Smoke and Diesel Exhaust Particles

Cigarette smoke may also induce TSLP production. In mice intranasal exposure to cigarette smoke increases TSLP protein and mRNA. In human bronchial biopsies and bronchiolar lavage (BAL) fluids TSLP levels are elevated in chronic obstructive pulmonary disease (COPD) with a greater increase in

COPD patients that are smokers (Nakamura *et al.*, 2008) (Ying *et al.*, 2008). TSLP is released in bronchial epithelial cells and mRNA is expressed in an epithelial cell line in response to diesel exhaust particles (Bleck *et al.*, 2010).

1.11.6 TSLP Expression

Since its first discovery, TSLP expression has and still is being extensively studied. Originally TSLP mRNA was expressed predominantly by epithelial cells but dendritic cells, mast cells, lung fibroblasts, macrophages, endothelial cells and neutrophils all have the capability to express it. However other lympho-hematopoietic cells such as neutrophils, T cells, B cells, monocytes and endothelial cells are unable to express TSLP (Allakhverdi *et al.*, 2007; Soumelis *et al.*, 2002; Kato *et al.*, 2007).

More recently studies shows TSLP plays an important role in allergic inflammatory disease in the airway, skin and ocular tissue (Soumelis *et al.*, 2002) and to a lesser extent in other conditions affecting the gut, joints, tumours and pregnant women (Taylor *et al.*, 2009).

1.11.6.1 TSLP and Allergic Inflammation

Elevated TSLP expression has been reported in a number of atopic diseases such as asthma, allergic rhinitis, atopic dermatitis and allergic conjunctivitis (Zhou *et al.*, 2005) (Miyata *et al.*, 2008), (Soumelis *et al.*, 2002) (Ma *et al.*, 2009) (Figure 1.10).

The first study to provide a direct link between allergic inflammation and TSLP is study of Netherton disease. Patients with Netherton disease suffer from severe atopic manifestations caused by a mutation in SPINK5 gene encoding LEKTI. Asthma, persistent atopic dermatitis, food allergies and elevated IgE levels are just a few of the symptoms (Judge *et al.*, 1994). A deficiency in LEKTI a protease inhibitor leads to PAR-2 activation through deregulation of kallekrein 5. TSLP expression in epithelial cells and keratinocytes is directly activated by PAR-2. Therefore a mutation altering TSLP expression has direct consequences on the presence of an atopic disease (Chavanas *et al.*, 2000; Demehri *et al.*, 2009) (Briot *et al.*, 2009). In addition a skin disease with atopic dermatitis like features developed in mice after TSLP induction and in a model of dermatitis TSLP was required for skin inflammation (Yoo *et al.*, 2005; He *et al.*, 2008). Mice that lacked RXRα and RXRβ developed atopic dermatitis like disease in a TSLP dependent manner (Li *et al.*, 2005). In atopic dermatitis lesions TSLP mRNA is located to keratinocytes from the skin but not in non-lesional skin or nickel induced allergy dermatitis (Soumelis *et al.*, 2002). In addition a recent report demonstrated children with atopic dermatitis had elevated serum TSLP (Lee *et al.*, 2010). Taken together these studies highlight the importance of TSLP in skin inflammation.

In the airway TSLP mRNA is expressed in predominantly in the epithelial cells, but is also expressed by dendritic cells, mast cells, fibroblasts, macrophages, endothelial cells and neutrophils suggesting a role in airway inflammation (Allakhverdi *et al.*, 2007; Soumelis *et al.*, 2002; Kato *et al.*, 2007). Initial studies in mice demonstrated allergic airway inflammation requires TSLP and it is

sufficient to sustain Th2 type airway inflammation. In an ovalbumin-induced mouse model, TSLP deficient mice were unable to develop inflammation and this may be due to CD4+ T cells inability to respond to TSLP (Al-Shami *et al.*, 2005). Inflammatory mediators IL-1β and TNF-α which lead to asthma also modulate human TSLP gene expression (Lee & Ziegler, 2007).

TSLP also plays a role in allergic rhinitis. TSLP expression is elevated in nasal epithelial cells when treated with dsRNA (Zhu *et al.*, 2009) as well as in nasal lavage (Xu *et al.*, 2010). Furthermore patients with allergic rhinitis express higher levels of TSLP mRNA in nasal polyps compared to mucosa and this expression is located to epithelial cells, fibroblasts and endothelial cells (Kimura *et al.*, 2011).

1.11.6.2 TSLP and the Atopic March

The atopic march refers to the typical sequence of allergic symptoms and diseases that manifest during childhood and remain for a number of years (Spergel & Paller, 2003). It is described as the progression from atopic dermatitis to the development of asthma and allergic rhinitis (Rhodes *et al.*, 2001). Both AD and asthma have atopic manifestations such as increased IgE levels, Th2 inflammation often with eosinophilia (Spergel & Paller, 2003). TSLP plays a central role in driving inflammation and as a result its involvement in the atopic march is of recent interest.

In an experimental mouse atopic march model, allergen sensitisation was created through damaging the skin, followed by airway challenge. Keratinocyte

derived TSLP promoted skin allergen sensitisation which triggered the atopic march and resulted in allergic asthma (Leyva-Castillo *et al.*, 2012). When sensitised and challenged by OVA, mice with atopic dermatitis overexpressing IL-13 significantly exhibited classic asthma characteristics which were higher in acute atopic dermatitis than chronic atopic dermatitis. Furthermore inhibition of TSLP receptor attenuated IL-13-induced enhanced lung inflammation upon OVA challenge indicating IL-13 can induce atopic dermatitis and trigger the atopic march through a TSLP dependent pathway (Zhu *et al.*, 2011).

Another link between TSLP and the atopic march is a recent study where intradermal treatment with TSLP and OVA in mice resulted in atopic dermatitis-like skin inflammation. Upon subsequent intranasal OVA re-challenge the mice exhibited severe airway inflammation compared to OVA challenge without TSLP present (Han *et al.*, 2012). Moreover, increased circulation of keratinocyte derived TSLP in an atopic dermatitis model was able to exaggerate inflammatory responses to aero-antigens (Zhang *et al.*, 2009) (Demehri *et al.*, 2009).

1.11.6.3 TSLP and other diseases

TSLP predominantly contributes to disorders however in some cases expression contributes to homeostasis. TSLP expression in the thymic medulla by the epithelial cells of Hassall's corpuscles is selectively expressed and is responsible for Treg cell differentiation from CD4+ T cells which regulates thymic dendritic cell activity (Watanabe *et al.*, 2005).

TSLP has been reported in a number of gut related conditions. During helminth infection in the intestinal microenvironment TSLP-TSLPR interactions facilitate the expulsion of parasitic worms by limiting proinflammatory cytokine production and inflammation (Taylor *et al.*, 2009). Similarly TSLP interactions with commensal flora may play a homeostatic role (Tanaka *et al.*, 2010). In the stomach introduction of *Helicobacter pylori* induced TSLP leading to gastritis and in eosinophilic esophagitis there is evidence of elevated TSLP mRNA (Kido *et al.*, 2010) (Rothenberg *et al.*, 2010).

TSLP has also been reported in breast milk and mammary epithelial cells are thought to be the source of it, however how it relates to allergies and gut conditions remains unclear (Macfarlane *et al.*, 2010).

Synovial fluid in rheumatoid arthritis exhibit elevated TSLP levels and this is located to synovial fibroblasts which can release it in response to *in vitro* stimuli (Koyama *et al.*, 2007; Ozawa *et al.*, 2007; Messer *et al.*, 2009).

TSLP expression has also been reported in conjunctival epithelial cells in allergic keratoconjunctivitis (Matsuda *et al.*, 2010). Cancer-associated fibroblasts release TSLP into the tumour microenvironment after activation with tumour-derived Th2 cytokines, indicating a role in cancers (De Monte *et al.*, 2011) (figure 1.10)

TSLP plays a vital role in many conditions but its primary role seems to be in allergic disease namely asthma and atopic dermatitis.

1.11.7 TSLP and its Cellular targets

As well as being expressed in a broad range of tissues and cell types, TSLP exhibits its actions on a number of target cells at the mucosal surface including barrier cells and resident cells. Dendritic cells, T cells, mast cells, epithelial cells, smooth muscle cells, Treg cells, natural killer T cells, B cells, and monocytes all have the potential to interact with TSLP as they all express the receptor (Park *et al.*, 1990; Soumelis *et al.*, 2002). A few of these targets cells relevant to this study are described further. Further discussion of TSLP and other cell types is beyond the scope of this review.

1.11.7.1 TSLP and Dendritic cells

Dendritic cells conditioned by epithelial cell derived TSLP at the tissue site may by recruited to the lymph nodes and participate in T cell activation.

The current paradigm for the role of TSLP is that it activates CD11c+ dendritic cells from peripheral blood by binding to the TSLP receptor expressed on these cells (Reche *et al.*, 2001). Once activated, the fully mature dendritic cells produce IL-8 and eotaxin 2 which attract neutrophils and eosinophils. TSLP-activated dendritic cells (TSLP-dendritic cells) also induce the release of Th2-attracting chemokines, macrophage-derived chemokines (MDC) and thymus- and activation-regulated chemokines (TARC). TSLP-dendritic cells alone do not produce proinflammatory cytokines TNF-α, IL-1β and IL-6 or Th1 cytokine IL-12 (Soumelis *et al.*, 2002) creating a Th2 permissive microenvironment.

TSLP activated mature DCs up regulate expression of MHC class II and co-stimulatory molecules CD40, CD80, CD86 and CD83. More importantly in the absence of IL-12, TSLP-dendritic cells express OX40 ligand (OX40L), the ligand for the cell survival factor OX40. This expression enables mature dendritic cells to induce Th2 cell responses (Ito *et al.*, 2005) (Hoshino *et al.*, 2003). OX40L interacts with OX40 expressed on naïve CD4+ T cells leading to the differentiation of CD4+ T cells into a unique Th2 phenotype producing inflammatory cytokines IL-4, IL-5, IL-13 and TNF-α but little or no IL-10 (Soumelis *et al.*, 2002). Blocking OX40/OX40L interactions results in inhibition of TSLP driven Th2 responses (Seshasayee *et al.*, 2007). Naïve CD8+ T cells are induced by TSLP activated mature DCs to expand and differentiate into cytotoxic effecter cells producing IL-5, IL-13, and IFN-γ (Gilliet *et al.*, 2003). This CD8+ differentiation may amplify the proallergic response therefore further inducing tissue damage (Figure 1.11).

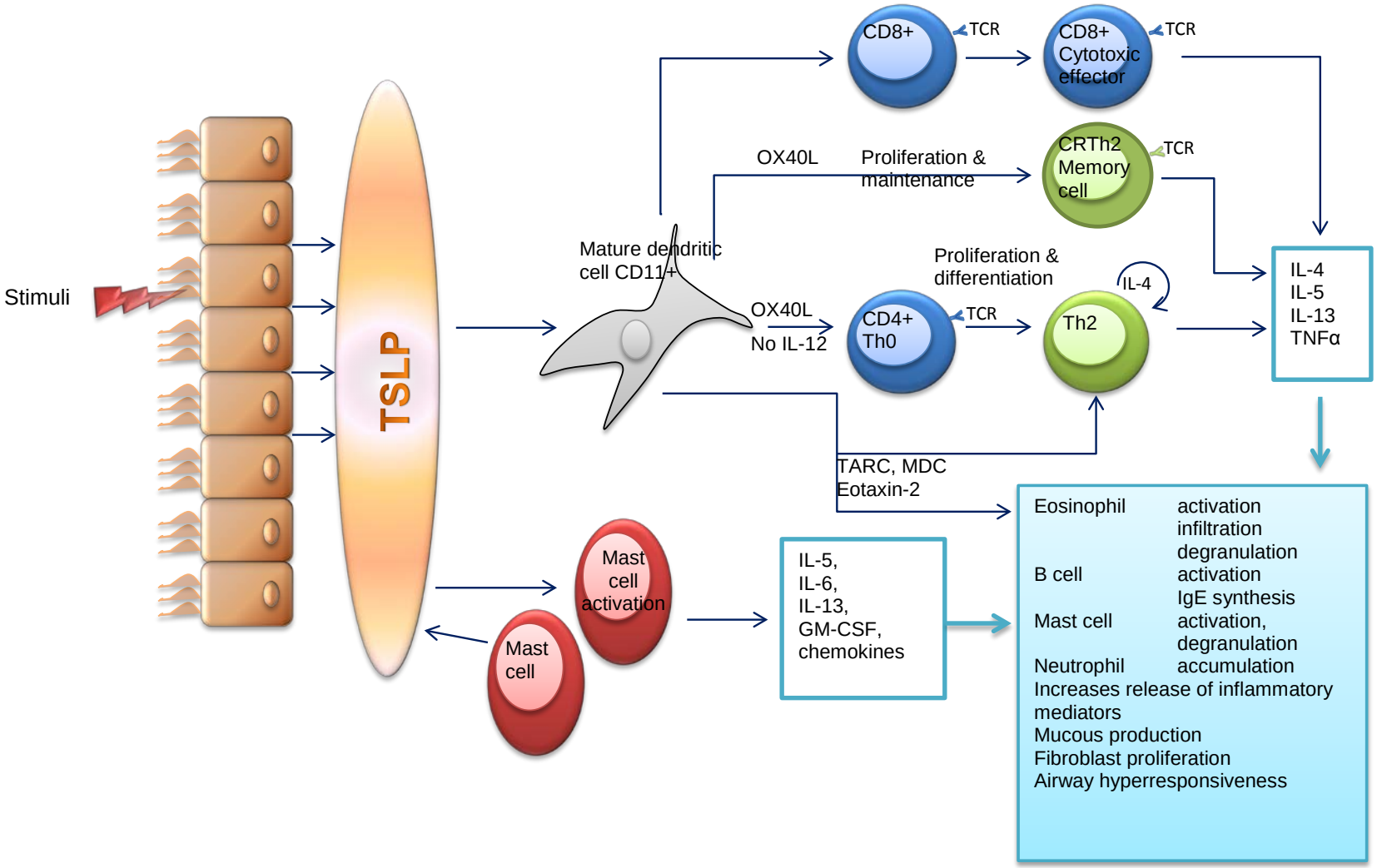
Recent studies suggest the inflammatory response is reinitiated and maintained through the prostaglandin D2 receptor (CRTH2+) expressing memory Th2 cells. In atopic dermatitis, TSLP expressed by keratinocytes activates Langerhan cells (the dendritic cell of the epidermis). Langerhan cells migrate from the epidermis to the dermis and then possibly to the draining lymph nodes where they trigger a Th2 response (Soumelis *et al.*, 2002). Lesional skin and blood from these patients expressing high levels of TSLP are found to be infiltrated with $CRTH2^{+}CD4^{+}$ Th2 memory cells (Soumelis *et al.*, 2002) (Cosmi *et al.*, 2000). TSLP-dendritic cells induce expansion of $CRTH2^{+}CD4^{+}$ Th2 memory cells whilst retaining their memory phenotype. This expansion is maintained through

OX40/OX40L interactions and can be enhanced by a second epithelial cell derived cytokine IL-25 (Wang *et al.*, 2006; Wang *et al.*, 2007). (Wang *et al.*, 2006)

Thus together epithelial cell derived cytokines drive Th2 cell mediated inflammation (Figure 1.11).

Figure 1.11 TSLP mediated allergic inflammation upon allergen challenge.

Schematic representation of TSLP-OX40L-T cell axis and a TSLP-mast axis. Epithelial cells produce biologically active TSLP in response to allergen challenge. This activates dendritic cells, which polarize naive T cells toward the Th2 pathway via OX40L/OX40 interaction, stimulating production of cytokines, such as IL-4 and IL-13, as well as TNF-α. IgE production is triggered; which in turn activates mast cells. In addition human TSLP directly activates mast cells leading selective cytokine production. Both pathways initiate the inflammatory cascade.


Stimuli
TSLP
Mature dendritic cell CD11+
CD8+
TCR
CD8+ Cytotoxic effector
TCR
OX40L
Proliferation & maintenance
CRTh2 Memory cell
TCR
Proliferation & differentiation
IL-4
OX40L
No IL-12
CD4+ Th0
TCR
Th2
IL-4
IL-5
IL-13
TNFα
TARC, MDC
Eotaxin-2
Mast cell activation
Mast cell
IL-5,
IL-6,
IL-13,
GM-CSF,
chemokines
Eosinophil activation
infiltration
degranulation
B cell activation
IgE synthesis
Mast cell activation,
degranulation
Neutrophil accumulation
Increases release of inflammatory mediators
Mucous production
Fibroblast proliferation
Airway hyperresponsiveness

Interestingly a recent study revisited TSLP production by dendritic cells (Kashyap *et al.*, 2011). TLR9 (CpG) in the presence of IL-4 activated both myeloid dendritic cells and to a lesser extent plasmacytoid dendritic cells suggesting dendritic cells may bypass epithelial cell derived TSLP activation and create a positive feedback loop, whilst acting as a TSLP source to other cells (Kashyap *et al.*, 2011).

1.11.7.2 TSLP and T cells

In addition to T cells being activated by TSLP through dendritic cells, T cells may also be directly activated to a Th2 phenotype by TSLP.

CD4+ T cells stimulated with TSLP in the presence of T cell receptor (α-CD3 antibody) directly promoted proliferation and differentiation through IL-4 mRNA and protein production (Omori & Ziegler, 2007). IL-4 then further stimulated TSLP-R production creating a positive feedback loop. TSLP-R expression levels are higher in Th2 cells compared to Th1 or Th17 cells and can be maintained in the presence of IL-4. However, only Th2 cell proliferation could be induced by TSLP through a STAT 5 dependent pathway (Kitajima *et al.*, 2011). Two separate studies demonstrated TSLP-R mRNA and protein expression was not detectable in naïve human CD4+ and CD8+ T cells isolated from peripheral blood. However upon co-activation with α-CD3 antibody and α-CD28 antibody (activates T cell receptor) TSLPR mRNA and protein expression was increased in addition to an increase in CD4+ proliferation and CD8+ cell expansion (Rochman *et al.*, 2007) (Akamatsu *et al.*, 2008).

However there are conflicting results regarding the direct effects of TSLP on CD4+ T cells. Lu *et al*. reported CD4+ T cells express TSLP-R but the levels reported were very low and STAT5 was only weakly induced by TSLP with marginal T cell proliferation and survival compared to myeloid dendritic cells (Lu *et al.*, 2009). Furthermore, TSLP-R expression was not detected on CD4+ T cells in a study of late phase cutaneous responses (Corrigan *et al.*, 2009).

1.11.7.3 TSLP and Mast cells

It has been shown dermatitis or asthma can be induced in TSLP transgenic mice lacking T cells (TCRβ-/- or RAG-/-) showing that allergic disease can occur in animals lacking T cells and IgE suggesting an alternative pathway targeting important cells in addition to dendritic cells and T-cells (Liu *et al.*, 2007; Zhou *et al.*, 2005; Yoo *et al.*, 2005). Recent evidence indicates that a TSLP-mast cell axis may mediate these changes (Figure 1.11).

Human peripheral blood derived mast cells express the TSLP-R and respond to TSLP in the presence of IL-1β and TNF by releasing pro-inflammatory Th2 cytokines (IL-13, IL-5, IL-6, and GM-CSF) suggesting that during inflammation TSLP is a potent activator of mast cells. TSLP alone cannot stimulate mast cells but in the presence of IL-1 and TNF Th2 cytokine release is dose dependent and this activity can be suppressed by the addition of neutralising antibody to TSLP or TSLP-R. IL-13 production by mast cells is inhibited by the blockage of TSLP release from activated epithelial cells suggesting an IL-13/TSLP pathway (Allakhverdi *et al.*, 2007).

Similarly, a recent study confirmed TSLP alone did not stimulate Th2 cytokine production by mast cells. However Th2 cytokine production from mast cells was elevated by the addition of epithelial cell supernatant pre-treated with IL-4 and dsRNA. This process was dependent on TSLP and IL-1 as this effect was supressed by neutralising antibodies to TSLP and IL-1. TSLP production by epithelial cells positively correlated with IL-5 production by mast cells. Therefore epithelial cell derived TSLP may activate mast cells to facilitate a Th2 microenvironment in response to viral infection (Nagarkar *et al.*, 2012).

Mast cell activation following IgE-cross-linking by allergen is mediated through the high-affinity IgE receptor (FcεRI) expressed on mast cells and plays vital role results in degranulation and cytokine release (Cruse *et al.*, 2005; Bradding P., 2008; Brown *et al.*, 2008). In a mouse model of allergic rhinitis, the high affinity FcεRI receptor in mast cells is required for the increased TSLP production by airway epithelium (Miyata *et al.*, 2008). TSLP expression was upregulated in mice after ovalbumin challenge however this was abolished in mast cell deficient mice. Similarly epithelial cells in FcγR chain deficient mice have reduced TSLP production compared wild type. In addition neutralising TSLP during OVA challenge suppressed the development of allergic rhinitis suggesting antigen alone may not be sufficient to induce TSLP expression in epithelial cells (Miyata *et al.*, 2008).

Mast cells have a unique ability to both respond to TSLP and to produce it. Initial studies showed mast cells to be a cellular source for TSLP mRNA in the bronchial submucosa and isolated from blood (Ying *et al.*, 2005) (Soumelis *et*

al., 2002). Mast cells derived from peripheral blood have been reported to express low levels of TSLP mRNA and this mRNA expression is up regulated upon FcεRI activation (Okayama *et al.*, 2009). Preincubation with IL-4 enhances TSLP mRNA production and TSLP protein in cell pellets suggesting mast cells can store TSLP as well as produce it in response to activation and Th2 stimuli (Okayama *et al.*, 2009). A recent letter reported addition of supernatant from FcεRI activated mast cells stimulated bronchial smooth muscle cells to produce TSLP. However the amount of TSLP present in the activated mast cell supernatant was not shown therefore based on Okayama *et al.*'s findings it possible the TSLP release was from mast cells and not the smooth muscle cells (Allakhverdi *et al.*, 2009).

1.11.7.4 TSLP and Epithelial cells

Extensive studies show epithelial cells express TSLP however a recent study showed epithelial cells are able to respond to TSLP (Semlali *et al.*, 2010). TSLP receptor was expressed in the epithelium of bronchial biopsies from asthmatics and corticosteroid treatment showed no difference in the expression pattern. *In vitro*, epithelial cells expressed the TSLP-R at both the mRNA and protein level. Furthermore addition of TSLP resulted in IL-13 release by epithelial cells through a STAT3 and STAT5 dependent pathway. Exogenous TSLP also aided wound repair in epithelial cells suggesting TSLP plays a homeostatic role in regulating epithelial injury and repair (Semlali *et al.*, 2010).

1.11.7.5 TSLP and Smooth Muscle cells

Human airway smooth muscle cells isolated from healthy lung resection express low levels of TSLP in the presence of TNF-α and IL-1β with greater immunoreactivity in COPD airway smooth muscle (Zhang *et al.*, 2007). Following exposure to cigarette smoke extract airway smooth muscle cells have been reported to express both TSLP and TSLP-R. In addition airway smooth muscle exposed to TSLP exhibit a higher Ca^{2+} peak response to histamine than untreated (Smelter *et al.*, 2010). A recent study reported that TNF-α can stimulate airway smooth muscle cells to produce TSLP protein through NF-$_K$β and AP-1 pathway however the amounts were minimal and close to the assay detection limit (Redhu *et al.*, 2011).

1.12 Innate Lymphoid cells

Since the completion of the experimental work in this thesis a number of innate cell populations which were initially thought to be different have been classified into groups of innate lymphoid cells (ILC).

ILC are morphologically similar to lymphocytes but do not express the cell surface markers that are associated with immune cell linages. ILC have been likened to the innate counterparts of Th cells and the recent discovery of various ILCs with a range of names has led to new nomenclature (Spits *et al.*, 2013). ILC populations have been classified into three groups based on their phonotype and functional characteristics;

- Group 1 ILCs – (ILC1) consist of natural killer cells and ILC1s which produce high levels of produce interferon-γ (IFNγ) but not any of the other any of the Th2 or Th17 cell-associated cytokines. (Bernink *et al.*, 2013; Gordon *et al.*, 2012)

- Group 2 ILCs – (ILC2) are able to produce Th2 cell associated cytokines IL-5 and IL-13 in response to the cytokines IL-25, IL-33 and TSLP. Transcription factor GATA3 and RORα are required for this process. (Fort *et al.*, 2001; Hurst *et al.*, 2002) (Halim *et al.*, 2012).

- Group 3 ILCs – (ILC3) require transcription factor RORγt for their development and are capable of producing Th17 cell associates cytokines IL-17 and IL-22 (Takatori *et al.*, 2009) (Luci *et al.*, 2009).

1.12.1 ILC2

Four independent centres have described innate cell populations termed nuocytes, natural helper cells (NHC), innate type 2 helper helper cells (Ih2) and multi-potent progenitor type 2 (MPP^{type2}) cells which have been assigned the nomenclature ILC2 (Neill *et al.*, 2010) (Moro *et al.*, 2010) (Price *et al.*, 2010; Saenz *et al.*, 2010). The work in this thesis has primarily focused on the cell type originally identified as nuocytes.

Nuocytes named after the 13^{th} letter of the Greek alphabet are innate immune cells which provide an early source of IL-13 during murine infection with parasite *Nippostrongylus brasiliensis*. They have been termed lineage negative

as the population lacks expression of surface markers associated with most hematopoietic cell lineages such as T cells, B cells, macrophages, dendritic cells, natural killer cells, lymphoid tissue inducer cells and neutrophils. Nuocytes express high levels of the cell surface marker CD45 as well IL-7Rα, co stimulatory molecules ICOS and MHC class II. Nuocytes expressing the IL-33 receptor T1/ST2 and IL-25 receptor IL-17BR can be detected predominantly in the gut but also in lungs, blood, mesenteric lymph nodes, spleen and intestines in mice when administered with IL-25 or IL-33, both potent initiators of type 2 immunity. In addition to all nuocytes secreting IL-13, the majority of nuocytes also secrete IL-5 but very few produce IL-4. Physiologically nuocytes can induce goblet cell hyperplasia and enhance T cell responses (Neill *et al.*, 2010). Nuocytes originate in the bone barrow and differentiate from lymphoid progenitor cells. In vitro their development requires IL-13, IL-7 and notch signalling. RAR-related orphan receptor alpha (RORα) is a transcription factor that is part of the nuclear receptor superfamily. The presence of RORα is critical for nuocyte development (Wong *et al.*, 2012).

Whether nuocytes and other ILC2s are identical or the same cell at different stages of development or even different subsets of innate lymphoid cells is still unclear. In this thesis nuocytes will be referred to by their new nomenclature ILC2. Table 1.3 compares nuocytes with other ILC2s and their cellular markers.

Group 2 ILCs	**Nuocytes**	**NHC**	**Ih2**	**MPPtype2**
Linage	negative	negative	negative	negative
Tissue localisation	MLN, Spleen, Lung	FALC	MLN, Spleen, Bone marrow	MLN, Gut
Cell Surface Marker				
CD45	positive			
IL-7Rα	positive	positive	ND	negative
CD117 (KIT)	positive	positive/negative	low	negative
IL-33R (ST2)	positive	positive	positive	negative
IL-25R (IL-17BR)	positive	ND	positive	
ICOS	positive	ND (cells are IL-25 responsive)	positive	
SCA1	positive	positive	positive	positive
CD90 (THY1)	positive	positive	positive	negative
CD25	positive	positive	positive	
Cell activation	IL-25, IL-33, helminth infection	IL-25, IL-33, helminth infection	IL-25, IL-33, helminth infection	IL-25, helminth infection
Signature cytokines	IL4, IL-5, IL-13	IL4, IL-5, IL-6, IL-13	IL4, IL-5, IL-13	IL-5, IL-13
Cell differentiation potential	Not to T cells	Not to T cells, mast cells, basophils	ND	Yes to mast cells, basophils and macrophages
Reference	(Neill *et al.*, 2010) (Barlow *et al.*, 2012)	(Moro *et al.*, 2010)	(Price *et al.*, 2010)	(Saenz *et al.*, 2010)
	Innate Lymphoid Cells 2			
Tissue localisation	MLN, Lung, Gut, FALC, Spleen			
Cell activation	TSLP, IL-25, IL-33, helminth infection, viruses, allergen, fungi			
Effects	Th2 cytokine secretion, AHR, mucous hypersecretion, eosinophilia			

Table 1.3 Comparison of innate lymphoid cells

ND = not determined. NHC = natural helper cells, Ih2 = innate helper 2 cells, MPP^{type2} = multi-potent progenitor type 2, MLC = mediastinal lymph nodes

MPP^{type2} cells cultured with SCF and IL-3 have been shown to differentiate into mast cells, macrophages and basophils and therefore may potentially be precursor myeloid cells (Saenz *et al.*, 2010). To date only one subset of ILC2 has been described in humans. The human equivalent cells been reported to be linage negative and are capable of producing high levels of IL-13 in response to IL-25 and IL-33. In addition to ST2 and IL-17BR, human ILC2 express the prostaglandin D2 receptor, CRTH2 (Mjosberg *et al.*, 2011).

1.12.2 ILC2 and allergic inflammation

The expression of ILC2 has been reported in the gut, lungs and mediastinal lymph nodes by a various groups (Mjosberg *et al.*, 2011) (Monticelli *et al.*, 2011) (Chang *et al.*, 2011) (Barlow *et al.*, 2012). Although their functions have not been fully studied, given their cytokine profile, initial studies implicate ILC2 in Th2 inflammation.

ILC2 when stimulated with IL-33 in the presence of TSLP, IL-2 or IL-7 are capable of producing significant levels of Th2 cytokines IL-5 and IL-13. Exposure to protease allergen papain resulted infiltration of eosinophils and mucous hypersecretion which was abolished in ILC2 deficient mice (Halim *et al.*, 2012). Monticelli *et al*. demonstrated ILC2 was expressed in mouse and human lung tissue which influenced the repair of the epithelium upon exposure to influenza virus (Monticelli *et al.*, 2011). A separate study showed virally induced AHR was dependent on IL-13 and ILC2 secreting IL-13 were able to induce AHR in IL-13 knockout mice (Chang *et al.*, 2011). Furthermore upon exposure to fungal allergen, *Alternaria alternata* increased production of IL-33

and subsequent IL-5, IL-13 expression in BAL and induced airway eosinophilia which was abolished in IL-33 and ILC2 deficient mice (Bartemes *et al.*, 2012). ILC2 contribute to allergic asthma. In a classic model of ovalbumin-induced allergic lung inflammation BAL and lung tissue was shown to express ILC2. In IL-13 deficient mice, airway hyper responsiveness and eosinophilia was induced with the addition of isolated ILC2 (Barlow *et al.*, 2012).

The identification of ILC2 in the lung during allergic lung inflammation has attracted a lot of attention and whether human ILC2 are as important as animal ones in triggering type 2 responses is unclear however the activation of ILC2 by various stimuli including epithelial cell derived cytokines TSLP, IL-33 and IL-25 suggests the epithelium may closely interact with these cells.

1.13 Summary

Taken together, these studies suggest the presence of two distinct TSLP-driven pathways that may drive asthma pathophysiology: a TSLP-OX40L-T cell axis and a TSLP-mast axis. Whether these pathways are operating in human asthma is unknown. Increased expression of TSLP mRNA has been reported in both the airway epithelium and lamina propria in a group of asthmatic subjects with disease of varying severity, although expression in relation to severity was not presented. TSLP mRNA was expressed in airway epithelial cells, mast cells, macrophages, endothelial cells and neutrophils (Ying *et al.*, 2005). A further study demonstrated increased concentrations of TSLP in BAL in subjects with moderate to severe asthma compared to normal controls (Ying *et al.*, 2008). Whether the increased expression of TSLP mRNA and BAL protein extends to mucosal protein expression is not known. One study however demonstrated increased numbers of mast cells expressing immunoreactive TSLP in the airway mucosa of subjects with predominantly mild to moderate asthma (Okayama *et al.*, 2009).

There is therefore, little data regarding TSLP protein expression in asthmatic airways, or how TSLP expression relates to downstream effector pathways, disease severity and phenotype. Understanding these has the potential to target these pathways in patients with severe asthma. Importantly, we need to understand whether TSLP drives severe asthma in all patients, or a subset, to ensure that future clinical trials targeting TSLP deliver treatment to the right patients.

1.14 Hypothesis

That airway mucosal TSLP expression relates to distinct immunologic pathways and asthma severity.

1.15 Aims

Overall aim:

- Examine the expression and cellular provenance of immunoreactive TSLP and the downstream cellular networks associated with TSLP overexpression in subjects with well-characterised asthma of varying severity.

Specific aims:

- Investigate whether there is a difference in inflammatory cell and Th2 cytokine expression between healthy controls and asthmatic subjects with disease of varying severity.
- Investigate whether the expression of TSLP increases across asthma severity and phenotype.
- Investigate the cellular provenance of TSLP in asthmatic airways
- Investigate whether the TSLP/dendritic cell/OX40L pathway and/or TSLP-mast cell axis are up regulated in asthmatic bronchial biopsies.
- Investigate the cross-talk between cultured human lung mast cells and primary human airway epithelial cells with respect to TSLP and IL-13 production.

CHAPTER 2.

Materials and Methods

2.1 Subjects selection criteria

Asthmatic subjects (n=36) and healthy volunteers (n=12) were recruited from respiratory clinics including the Leicester Difficult Asthma Clinic, from staff at Glenfield Hospital, and from the general population through advertisement. Of the 36 asthmatics 16 were female. The ratio was slightly lower in this cohort compared to some studies which maybe a limitation of this study. Subjects with asthma gave a suggestive history and had objective evidence of variable airflow obstruction as indicated by one or more of the following: (1) methacholine airway hyperresponsiveness (PC20 FEV1 < 8 mg/mL), (2) >15% improvement in FEV1 10 min after 200 µg inhaled salbutamol, (3) peak expiratory flow (> 20% maximum within-day amplitude from twice-daily peak expiratory flow measurements over a period of 14 days).

The study was approved by the Research Ethics Committee of study institutions (Leicester - Leicestershire, Northamptonshire, & Rutland Research Ethics Committee reference 04/Q2502/74. Written informed consent was gained from all participants prior to their involvement.

2.2 Clinical Characterisation

Subjects underwent spirometry; allergen skin prick tests for Dermatophagoides pteronyssinus, dog, cat, grass pollen and Aspergillus fumigatus; a methacholine inhalation test using the tidal breathing method; and sputum induction using incremental concentrations of nebulised hypertonic saline (i.e., 3%, 4%, and 5%, each for 5 min) (Brightling *et al.*, 2000). Subjects with asthma also kept a diary card for 2 weeks prior to bronchoscopy, recording daytime and night-time

symptoms, daily short-acting β_2-agonist use and twice daily peak expiratory flow (PEF). Asthma severity was defined by British Guideline on the Management of Asthma treatment steps (mild = step 1, β_2-agonist only; moderate = steps 2 and 3, inhaled corticosteroid ≤800 mg beclomethasone equivalent per day ± long-acting β_2-agonist; severe = step 4 and 5) (British Thoracic Society Bronchoscopy Guidelines Committee,a Subcommittee of Standards of Care Committee of British Thoracic Society, 2001). Of the 16 severe patients at step 4/5, 13 met the American Thoracic Society criteria for refractory asthma (Proceedings of the ATS workshop on refractory asthma, 2000).

2.3 Fibre-optic Bronchoscopy

Subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines (British Thoracic Society Bronchoscopy Guidelines Committee,a Subcommittee of Standards of Care Committee of British Thoracic Society, 2001). Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe.

2.4 Biopsy processing in Glycolmethacrylate (GMA) resin

Biopsies were fixed in ice cold acetone containing 2 mM phenylmethylsulfonyl fluoride and 20 mM iodoacetamide overnight at -20ºC. The fixative was replaced with acetone at room temperature for 15 min and transferred to methyl benzoate at room temperature for 15 min. Biopsy was immersed in GMA monomer (JB4 Kit, Park Sciences Ltd., U.K.) containing 5 % methyl benzoate. GMA solution was changed 3 times over 6 h at 4ºC. Biopsy was embedded in

GMA embedding resin (70 mg Benzoyl peroxide dissolved in 10 mL GMA solution A and 250 µl GMA solution B, Park Sciences Ltd, UK) in polythene capsules with lids and polymerised at 4ºC for 16 h. Blocks stored at -20ºC in an airtight container (Britten *et al.*, 1993).

2.5 Silane coating glass slides

Tissue sections have to adhere firmly to glass slides. Usually optimal adhesion occurs when there are two flat surfaces, however tissue sections are never flat and glass slide are very rarely completely clean. To prevent the loss of tissue sections during immunohistochemical staining the slides were pre-treated with 3-Aminopropyltriethoxysilane (Sigma, UK).

Microscope slides (76 X 26 X 1 mm size) were immersed in 2% solution of 3-aminopropyltriethoxysilane in acetone for 5 sec. The slides were then transferred to acetone (pure) for 5 sec (2X). Next the slides were immersed in distilled water for 5 sec to wash the excess. This step was repeated and slides were dried overnight at 20˚C. Once dried the coated slides are stable for several months at room temperature.

2.6 Haematoxylin and eosin stain

Haematoxylin and eosin was used to stain bronchial tissue and used to examine the morphology. Slides were kindly stained by the histology department at University Hospitals of Leicester using an automated Leica Stainer.

Briefly slides were immersed in xylene for 1 min, fresh xylene for 30 sec, 2X 99% industrial methylated spirit (IMS) for 20 sec, distilled water 20 sec, 2X haematoxylin 2.15 min each, 2X distilled water 1 min, eosin 40 sec, water 30 sec, 4X IMS 20 sec each and 5X xylene for 30 sec.

2.7 Immunohistochemistry (IHC)

2 µm sections were cut using a Leica RM 2155 microtome (Figure 2.1) and floated onto 0.2 % ammonia water for 60 sec and slides dried at room temperature for 1-4 h. Figure 2.2 shows an overview of the method.

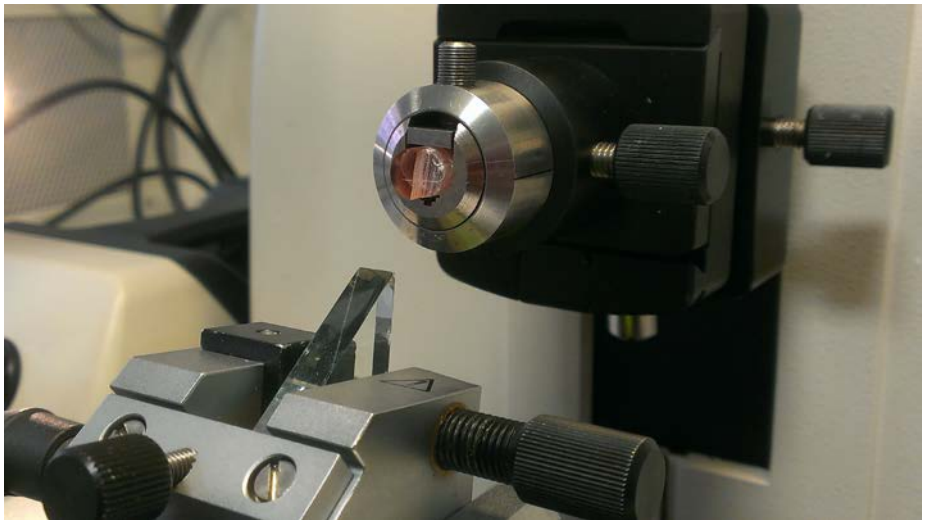


Figure 2.1 Photographs of Leica microtome

To inhibit endogenous peroxidise activity sections were incubated with 0.1 % sodium azide and 100 µL 0.3% hydrogen peroxide solution for 30 min. Sections were washed 3 X for 5 min using tris buffer saline (TBS) pH 7.6 before treating with blocking medium (DMEM, fetal calf serum and bovine serum albumin (BSA)). Isotype controls and antibodies were added to appropriate slides overnight. Sections were washed and an appropriate biotinylated second stage was added for 2 h. Sections were incubated with streptavidin-biotin peroxidise complex detection system (Dako, UK) for 2 h. Sections were treated with chromogen aminoethylcarbazole (AEC) for 10 min giving a red reaction product and counterstained with Mayer's haematoxylin for 5 min. Table 2.1 shows the primary antibodies used. Tonsil tissue obtained from Scottish Biomedical (Glasgow, UK) and embedded in GMA resin, was used as a positive control.

Figure 2.2 Flowchart of immunohistochemical process

Immunohistochemistry was first reported in 1942 by Albert Coons where he and his colleagues demonstrated the presence of a pneumococcal antigen in liver tissues using a fluorescent labelled antibody (Coons, 1942). Since this pioneering experiment, immunohistochemistry has become valuable tool used to visualise tissue components whereby target antigens interact with specific antibodies that can be colorimetrically determined by quantitative methods. The distribution and localisation of specific cells or biological markers can be identified in the correct tissue context using immunohistochemistry resulting in greater understanding of tissue component interactions.

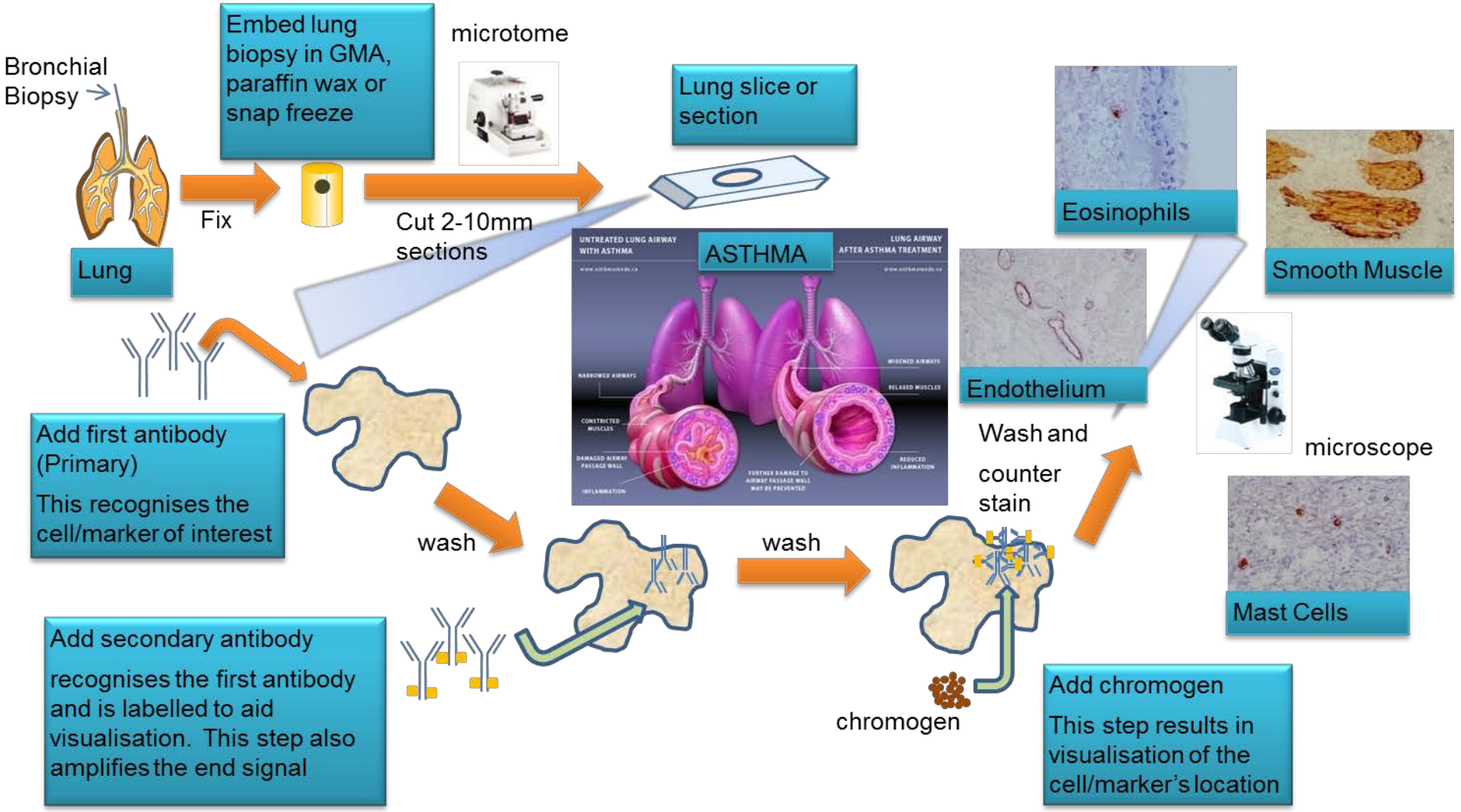

Bronchial Biopsy
Lung
Fix
Embed lung biopsy in GMA, paraffin wax or snap freeze
microtome
Cut 2-10mm sections
Lung slice or section
ASTHMA
Add first antibody (Primary)
This recognises the cell/marker of interest
wash
Add secondary antibody
recognises the first antibody and is labelled to aid visualisation. This step also amplifies the end signal
wash
chromogen
Add chromogen
This step results in visualisation of the cell/marker's location
Wash and counter stain
Eosinophils
Endothelium
Smooth Muscle
Mast Cells
microscope

Table 2.1 Antibodies used for immunohistochemistry

Primary antibodies concentrations and sources used for IHC with the appropriate isotype controls and secondary antibodies.

Primary antibody	Clone	Working Concentration	Company	Isotype Control	Company	Secondary Antibody	Working Concentration	Company
TSLP		7.5 µg/mL	R &D Systems, UK	Sheep IgG	R &D Systems Abingdon, UK	donkey anti-sheep IgG biotinylated affinity purified	0.4 µg/mL	R &D, UK
Mast cell tryptase	AA1	0.1 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD3	UCHT1	3 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD4	MT310	10 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD8	DK25	0.5 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD68	PG-M1	0.6 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
Neutrophil Elastase	NP57	0.04 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
Major Basic Protein	BMK-13	0.4 µg/mL	Monosan, The Netherlands	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
OX40	ACT35	5 µg/mL	BD Biosciences Europe	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
OX40 Ligand	159403	10 µg/mL	R &D Systems, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD83	HB15e	1 µg/mL	Serotec, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD1a	O10	26 µg/mL	Dako, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
IL-13	fixed	5 µg/mL	Genentech, San Francisco	Rabbit IgG	BD biosciences Europe	polyclonal swine anti rabbit F(ab)2 biotinylated	1.8 µg/mL	Dako, UK
IL-4	3H4	12.5 µg/mL	Gift Dr C.Heusser, Novartis, Switzerland	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
CD45	2D-1	2 µg/mL	Santa Cruz biotechnology, UK	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
ICOS (CD278)	DX29	15 µg/mL	BD Biosciences Europe	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
IL-17BR	170220	5 µg/mL	R &D Systems, UK	Mouse IgG2b	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
ST2	HB12	15 µg/mL	MBL International	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK
C-Kit	YB5.B8	5 µg/mL	BD Biosciences Europe	Mouse IgG1	Dako, UK	polyclonal rabbit anti mouse F(ab)2 biotinylated	2.5 µg/mL	Dako, UK

2.8 Absorption Control

Absorption control assay prior to IHC was used to show that antibodies bind to specific targets in the tissue. Recombinant TSLP and IL-13 (R and D systems, UK) were pre-incubated 4°C overnight with TSLP and IL-13 antibodies respectively, at the working dilution with a 10:1 molar ratio. The pre-absorbed antibody was added to the bronchial tissue section. The isotype controls and non-pre-absorbed antibody were added to sequential sections on either side of the pre-absorbed antibody. IHC was carried out as described. The tissue staining in absorption control was compared with the control and with antibody staining.

2.9 Assessment and quantification of Immunohistochemical staining

Epithelial, submucosal and airway smooth muscle (ASM) bundle areas in sections were identified and measured using a computer analysis system (analySIS docu. Olympus, Germany). The numbers of positively stained nucleated cells in epithelial, sub mucosal and smooth muscle bundle areas were counted and expressed per mm^2. All counts were performed blind to clinical characteristics. Areas of ASM < 0.1 mm^2 were considered insufficient to quantify. Cells staining in sequential sections were co-localised using the computer analysis system.

Two methods were used for the quantitative assessment of TSLP expression in the epithelium. First, a thresholding technique was developed based on the hue, saturation, intensity (HSI) of TSLP staining. The HSI colour system was defined by a scale of 0 to 255 for HSI. Sections were acquired at X200 magnification

and white balance corrected, and pixels of representative TSLP staining selected in the epithelium were measured.

A minimum of 2 non-contiguous sections were measured for each patient. The median of the lower and upper limit of the HSI was then defined from 10 validation patients. The HSI was then selected as the final threshold to produce a threshold that appropriately captured highly saturated red light. All biopsy specimens were subsequently measured by using this final threshold. The mean percentage area of TSLP staining in 10 areas in the epithelium from 2 sections was taken as the final measurement. The final threshold measurement was tested by comparing with a semi quantitative score of staining (0, none; 1, weak intermittent; 2, strong intermittent or weak generalised; and 3, strong generalised).

2.10 Statistical Analysis

Prism 5 was used for statistical analysis of immunohistochemical staining. Group comparisons were made using the Kruskal-Wallis test; non-parametric data comparisons between groups were made using the Mann–Whitney U test and unpaired t-tests were used for parametric data. The spearman rank correlation test was used for correlations. A P value <0.05 was considered statistically significant.

2.11 Sputum processing

Sputum was collected on ice and processed at 4˚C within 2 h of expectoration. Sputum plugs were selected from the saliva and using larger blunt ended

forceps the sputum plugs were gathered into one mass and moved around the petri dish with small circular motions. This technique spreads the saliva across the dish but keeps sputum in one mass. The selection procedure and condensation/removal of saliva are important in reducing squamous cell contamination. The sputum mass was then transferred to a falcon tube and weighed. Freshly diluted 0.1% mucolytic dithiothrietol (DTT) in PBS was added to the sputum plugs using 4 X weight/volume (e.g. 4 ml DTT per gram of selected sputum).The sputum mixture was dispersed by gentle aspiration using 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 µm nylon gauze pre-wet with PBS. The total cell counts, squamous cell contamination and viability were assessed using a trypan blue stain. The suspension was centrifuged at 2000 rpm for 10 min and the supernatant stored at -80˚C until required for ELISA analysis.

The cell pellet was resuspended in 0.5-3 mL PBS. Cytospins were prepared from the cell suspension and stained in neat Romanowski stain for 5 min and fixed in dilute stain for 25 min. A differential cell count was obtained by counting 400 non-squamous cells on a Romanowski stained cytospin. These counts were used in the clinical characterisation of subjects.

2.12 Cells

2.12.1 Human Lung Mast Cell Isolation

Human lung mast cells (HLMC) were dispersed from macroscopically normal lung obtained within 1 h of resection for lung cancer. For enzymatic dispersal, lung tissue was finely chopped and filtered through 100 µm nylon gauze to remove macrophages and blood cells. The tissue was washed twice with Dulbecco's Modified Eagles Medium (DMEM) with GlutaMAX^{TM-}I, 4500 mg/L D-glucose and 25 mM HEPES (Invitrogen) supplemented with 2% foetal calf serum (FCS) (Promocell). The filtrate was discarded and lung tissue was stored overnight at 4°C in DMEM with 10% FCS (Promocell), 1% antibiotic / antimycotic (containing 100 U/mL penicillin, 100 mg/mL streptomycin sulphate and 0.25 µg/mL amphotericin B) (Sigma) and 1% non-essential amino acids (NEAA) (Invitrogen).

For cell dispersion, collagenase (12 mg/10 g lung tissue) and hyaluronidase (37.5 mg/10 g lung tissue) (Sigma) were added to the lung tissue and incubated at 37°C for 75 min whist stirring. Connective tissue was removed by washing the dispersed cells through 50 µm gauze followed by 3 X centrifugation washes at 1300 rpm for 8 min. Lung cells were incubated with blocking buffer (HBSS (Hanks balanced salt solution) (Invitrogen), 10% horse serum, 2% FCS and 1% bovine serum albumin (Sigma) for 30 min at 4°C to inhibit nonspecific binding.

Cells were washed through a 70 µm cell-strainer (Beckton Dickenson) and centrifuged. A pan sheep α-mouse IgG coated magnetic dynabeads (Invitrogen UK) conjugated with mouse α-human CD117 antibody (Biosciences UK) was

added to the lung cells for 90 min at 4°C. Beaded cells were positively selected and removed using MPC magnet (Invitrogen, UK).

Purified HLMCs were cultured in Dulbecco's modified essential medium (DMEM) / Glutamax / HEPES containing 10% FBS, 1% NEAA, 1% antibiotic / antimycotic solution (Sigma, UK), 100ng/mL recombinant human stem cell factor (SCF), 50 ng/mL recombinant IL-6 and 10 ng/mL recombinant IL-10 (R and D systems, UK) at 37°C in a humidified incubator 5% CO_2. Half of the medium was changed every 7 days.

2.12.2 Epithelial cell culture

Cells were obtained from the bronchus by brushings during bronchoscopy and stored overnight at 4°C. The relevant wells in a 12 well plate were coated with 1mL of 1% collagen (Nutacon) in PBS for 1 h at room temperature. The cells from the brushings were shaken off and centrifuged at 1200 rpm for 5 min. Cells were resuspended in 1mL bronchial epithelial growth media (BEGM) consisting of bronchial epithelial basal media (BEBM), bullet kit (Clonetics) penicillin / streptomycin, fungizone (Invitrogen) and added to the collagen coated wells for 24 h.

Detached cells were transferred to a fresh collagen coated well. Fresh media (1 mL) was added to all wells. At 90-100% confluence cells were passaged into T75 flasks. Media was removed, cells were washed with 1 mL HBSS and replaced with 1mL trypsin/EDTA (Sigma) until the cells had detached. Trypsin was in activated with 1mL BEGM and washed 3 X. The cell suspension

centrifuged at 1200 rpm for 5 min. The cells were resuspended in 1 mL BEGM and cells from each collagen coated well were transferred to a collagen coated T75 flask containing 9 mL BEGM. Upon 90–100 % confluence, cells were passage into fresh T75 flasks. The media was replaced every 2 days.

2.13 Basal epithelial cell/Human lung mast cell Co-culture

Basal epithelial cells were grown to confluence in a 24 well plate. All the wells were washed with 400 µL Hanks balanced salt solution (HBSS, Gibco UK) and all conditions were conducted in duplicate. HLMC (6 $X10^4$ cells /300 µL) or serum free, SCF free media alone were cultured with epithelial cells for 16 h at 37°C. As a third condition HLMC were cultured alone (Table 2.2 shows plate plan). The media was centrifuged at 1300 rpm for 5 min and the supernatant stored at -20°C.

Epithelial cells alone	Epithelial cells alone
HLMC alone	HLMC alone
Epithelial cells + HLMC	Epithelial cells + HLMC

Table 2.2 Mast cell and epithelial cell coculture plate plan

2.14 TSLP recovery from Human lung mast cells

HLMC (6 $X10^4$) were cultured in a 24 well plate with 300 µL serum free mast cell media alone or containing either 1000 pg/mL or 100 pg/mL recombinant TSLP. The same was repeated containing α-FcεRI antibody to activate the cells. The media and cells were collected at 12 h. The media was centrifuged at 1300 rpm for 5 min and the supernatant stored at -20°C. 300 µL cell dissociation solution was added. Once the cells had detached the cell dissociation solution was inactivated with serum free mast cell media (300 µL). The cell suspension was centrifuged at 1300 rpm for 7 min 4°C. Supernatant was discarded and the pellet was stored at -20°C.

2.15 Mast cell degranulation

β-Hexosaminidase assay was used to assess mast cell activity. The reagents were made as described in Table 2.3.

Reagent	Composition	Storage
0.05 M Citric Acid	1.9 g citric acid / 500 mL dH20	4°C
0.05 M Trisodium Citrate	2.9 g trisodium citrate / 500 mL dH20	4°C
0.05 M Na2C03	1.06 g sodium carbonate Na2C03 / 200 mL dH20	Room Temp
0.05 M NaHC03	0.84g Sodium bicarbonate NaHC03 / 200 mL dH20	Room Temp
Citrate Buffer (pH 4.5)	49.5 mL (0.05M) citric acid + 50.5 mL (0.05M) trisodium citrate	4°C
Sodium Carbonate Buffer (pH 10.0)	60 mL (0.05M) Na2C03 + 40 mL (0.05M) NaHC03	Room Temp
2 mM Substrate	13.6 mg pNAG (p-nitrophenyl N-acetyl β-D-glucosamine) / 20 mL citrate buffer	-20°C

Table 2.3 Composition of reagents required for β-Hexosaminidase assay

40 µL sample supernatant were added to a flat bottomed 96 well plate (triplicate). 40 µL serum free mast cell media was used as a control. 80 µL substrate solution was added to each well and incubated at 37°C, for 75 min. 200 µL Sodium carbonate buffer was added to stop the reaction and the plate read at 405 nm.

2.16 Enzyme Linked Immunoabsorbant Assay (ELISA)

2.16.1 TSLP

TSLP in sputum and in cultured cells was measured by ELISA using human TSLP DuoSet (R and D systems, UK). A 96 well microplate was coated with 100 µL per well of 0.8 µg/mL of sheep anti-human TSLP (capture antibody), sealed and incubated at room temperature overnight. The microplate was washed 3 X with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) ensuring buffer was completely removed after each wash. The plate was then blocked with 300 µL per well of reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2µm filtered) for 1 h. The microplate was incubated with 100 µL of sample or standards (serial dilutions ranging from 31.25–2000 pg/mL) made up in reagent diluent per well. The plate was covered and incubated at room temperature for 2 h. Following 3 washes the plate was incubated at room temperature with 100 µL per well of detection antibody (400 ng/mL biotinylated sheep anti-human TSLP) for 2 h. The plate was washed and 100µL of streptavidin conjugated to horseradish peroxidise (HRP) was added to each well, covered and incubated in the dark for 20 min at room temperature. After the final wash 100 µL of TMB substrate solution (1:1 mixture of colour reagent A (H_2SO_4) and colour reagent B (tetramethylbenzindine) was added to each well for 20 min. To inhibit the colour change reaction, 50 µL of 2N H_2SO_4 was gently mixed into each well. The microplate reader set at 450 nm was used to determine the optical density of each well.

To validate the TSLP ELISA for TSLP in sputum supernatants, the effect of DTT on the recovery of TSLP standards was determined. The optical density of a

standard curve without DTT was compared to standards with varying concentrations of DTT (0.00044-0.022%). To further validate the ELISA, sputum supernatants processed with DTT were spiked with known concentrations of TSLP prior to the ELISA and then recovery of spiked TSLP was determined. In addition the actual concentration of the standard curve was compared to that suggested in the product datasheet.

2.16.2 Interleukin-13

Sputum IL-13 was measured using IL-13 ELISA (EBiosciences).The kit has previously been validated by our group (Berry *et al.*, 2004). Therefore sputum IL-13 concentration was measured in the samples and standards after dialysis. A microplate was coated with 100 µL per well of 2.5 µg/mL monoclonal coating antibody to human IL-13, sealed and incubated at room temperature overnight. The microplate was washed 3 X with wash buffer (0.05% Tween 20 in phosphate buffer saline (PBS). 250 µL of assay buffer (0.5% BSA, 0.05% Tween 20 in PBS) was added to each well for 2 h at room temperature for blocking. The plate was washed then 100 µL of assay buffer in duplicate was added to all standard wells. The standards were prepared by adding 100 µL of IL-13 standard containing 0.022% DTT (previously determined (Berry *et al.*, 2004)) in duplicate using serial dilutions ranging from 100-1.56 pg/mL. Samples were diluted 1/50 with assay buffer total 100 µL per well. 50 µL of conjugate mixture (Biotin-Conjugate anti-human IL-13 monoclonal antibody, Streptavidin-HRP, assay buffer) was added per well. The plate was covered and incubated at room temperature for 2 h on a rotator set at 100 rpm. The plate was then washed as before and 100 µL of TMB substrate solution was added for 10 min

on a rotator 100 rpm. 100 µL of stop solution (4 N H_2SO_4) was gently mixed into each well. The microplate reader set at 450 nm was used to determine the optical density of each well.

The efficiency of the IL-13 ELISA DuoSet (R and D systems, UK) was compared to the kit purchased from BD Biosciences when measuring IL-13 in cultured cells. A 96 well microplate was coated with 100 µL per well of 2 µg/mL of mouse anti-human IL-13 (capture antibody), and incubated at room temperature overnight. The microplate was washed 3 X with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) and blocked with 300 µL per well of reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2µm filtered) for 1 h. The microplate was incubated with 100 µL undiluted supernatant or standards (serial dilutions ranging from 93.8 – 6000 pg/mL) in reagent diluent per well, covered and incubated at room temperature for 2 h. The plate was washed and incubated at room temperature with 100 µL per well of detection antibody (150 ng/mL biotinylated goat anti-human IL-13) for 2 h. The plate was then washed again and 100 µL of streptavidin conjugated to horseradish peroxidise (HRP) was added to each well, covered and incubated in the dark for 20 min at room temperature. After the final wash 100 µL of TMB substrate solution (1:1 mixture of colour reagent A (H_2SO_4) and colour reagent B (tetramethylbenzindine) was added to each well for 20 min. To stop the reaction 50 µL of stop solution (2 N sulphuric acid H_2SO_4) was gently mixed into each well. The microplate reader set at 450 nm was used to determine the optical density of each well.

2.17 Dialysis

Our group has previously used 10kDa dialyser (Sigma, Poole, UK) which was discontinued and replaced with Spectra/Por Float-a-lyzer G2 (Figure 2.3). Samples were dialysed using these Float-a-lyzer G2 dialyzers.

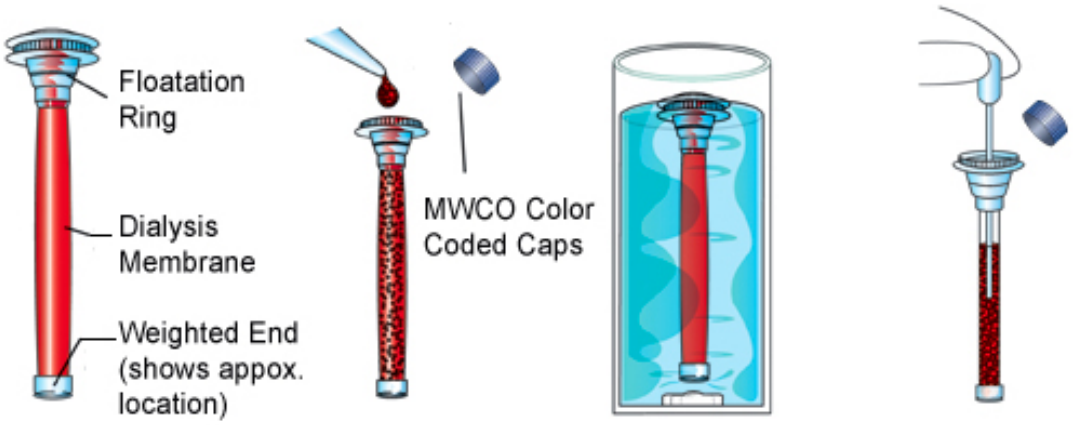


Figure 2.3 Components of a Float-a-lyzer G2.

http://eu.spectrumlabs.com/dialysis/FloatALyzer.html

The Float-A-Lyzer was carefully removed out of the tube to avoid damaging the membrane. The membrane was impregnated with glycerine for protection and submerged in phosphate buffer saline (PBS). 100-120 µL of each sample was added using gel loading tips without damaging the membrane. The cap was screwed on to create a closed seal and the body of the dialyser was placed in a floatation ring. The dialyser was floated vertically in 7 L PBS that was stirred to form a gentle rotating current for 18 h at 4°C. After dialysis the sample volume was retrieved. The total volume was recorded and compared to the initial amount added. Samples recovered were then used at 1/2 dilutions.

CHAPTER 3.

Immunopathology of the lower airways in asthma

3.1 Demographic data

Twelve healthy control subjects and 36 asthmatics subjects were recruited to the study. The asthmatic subjects were subdivided by severity using the SIGN/British Thoracic Society (BTS) guidelines for the classification and management of asthma in adults (BTS/SIGN Asthma Guideline, 2012). Severity was classified by SIGN/BTS treatment steps (Figure 1.1) Of the 36 asthmatics 8 were classified as mild, 12 moderate and 16 severe. The demographic data of the asthmatic subjects are shown in Table 3.1. Of the 16 patients with severe asthma, 13 met the American Thoracic Society criteria for refractory asthma (Proceedings of the ATS workshop on refractory asthma, 2000).

All of the participants were current non-smokers with no upper or lower respiratory tract infection in the 6 weeks prior to their bronchoscopy. All asthmatic participants were clinically stable on their usual medication at the time of bronchoscopy.

Table 3.1 Demographic data of the asthmatic subjects according to disease severity.

Significant values are shown in boldfaced text. BTS, British Thoracic Society; NA, not applicable; PEF, peak expiratory flow.

* Thirteen of 16 meet the American Thoracic Society criteria for refractory asthma.

† Statistical analysis across asthma groups unless otherwise stated.

‡ Ratio for budesonide Turbuhaler calculated as 1.5.

§ Moderate compared with severe asthma

	Healthy control subjects	Patients with mild asthma (BTS step 1)	Patients with moderate asthma (BTS steps 2 and 3)	Patients with severe asthma* (BTS steps 4 and 5)	P value†
No.	12	8	12	16	—
Age (y), mean ± SEM	34.7 ± 5.2	36.4 ± 5.0	30.8 ± 2.5	40.9 ± 2.4	0.056
Sex (M/F)	5/7	2/6	7/5	11/5	0.123
Asthma duration (y), mean ± SEM	NA	12.9 ± 4.6	13.1 ± 3.2	25.5 ± 3.7	**0.017**
Inhaled corticosteroid dose (μg), beclomethasone equivalents‡	0	0	668 ± 114	2325 ± 277	**.0001§**
No. at BTS step 5	0	0	0	8	—
No. taking long-acting β-agonist	0	0	9	16	—
Exacerbations in last year, median (range)	NA	0 (0-2)	0 (0-3)	3 (0-12)	**0.001**
Mean daytime symptom score, median (range)	NA	0.28 (0-1.25)	0.1 (0-1.14)	1.18 (0-3)	0.091
Mean daily nighttime symptom score, median (range)	NA	0.07 (0-1.14)	0 (0-0.14)	0.32 (0-3)	0.064
Reliever use/wk, median (range)	NA	2.0 (0-10)	0.5 (0-70)	30 (0-112)	**0.005**
Sputum eosinophil count (%), geometric mean (95% CI)	0.39 (0.25-0.6)	2.77 (0.96-7.99)	3.1 (1.05-9.15)	2.85 (1.09-7.45)	
PEF amplitude (% of the mean), mean ± SEM	NA	31.6 ± 7.2	19.4 ± 5.37	27.9 ± 4.24	0.296
FEV_1 (% predicted)	99.8 ± 3.2	98.3 ± 3.5	86.3 ± 5.3	72.6 ± 3.8	**0.002**
FEV_1/FVC ratio (%)	81.7 ± 3.6	77.0 ± 2.0	73.5 ± 3.58	64.5 ± 3.1	**0.029**
PC_{20} methacholine (mg/mL), geometric mean (95% CI)	>16	0.84 (0.10-7.11)	2.63 (0.87-7.97)	0.16 (0.05-0.53)	**0.004**
Serum IgE (kU/L), geometric mean (95% CI)	32.5 (18.1-58.6)	68.6 (25.7-183.1)	228.8 (80.0-654.1)	200.5 (80.2-500.8)	0.22
No. with positive skin prick test response	6	4	11	13 (n = 15)	0.051
No. with positive skin prick test response to *Aspergillus fumigatus*	0	1	1	4	0.42

3.2 Inflammatory cell infiltration

Asthma is characterised by the presence of inflammation and airway remodelling. The first aim of this study was to investigate whether there is a difference in inflammatory cell expression between healthy controls and asthmatic subjects with disease of varying severity. To address this, bronchial biopsies embedded in GMA were cut in 2 μm sections and placed onto a glass slide. Each slide had two sections which were immunostained for inflammatory cell markers. Figure 3.1 demonstrates the histology of the bronchial tissue.

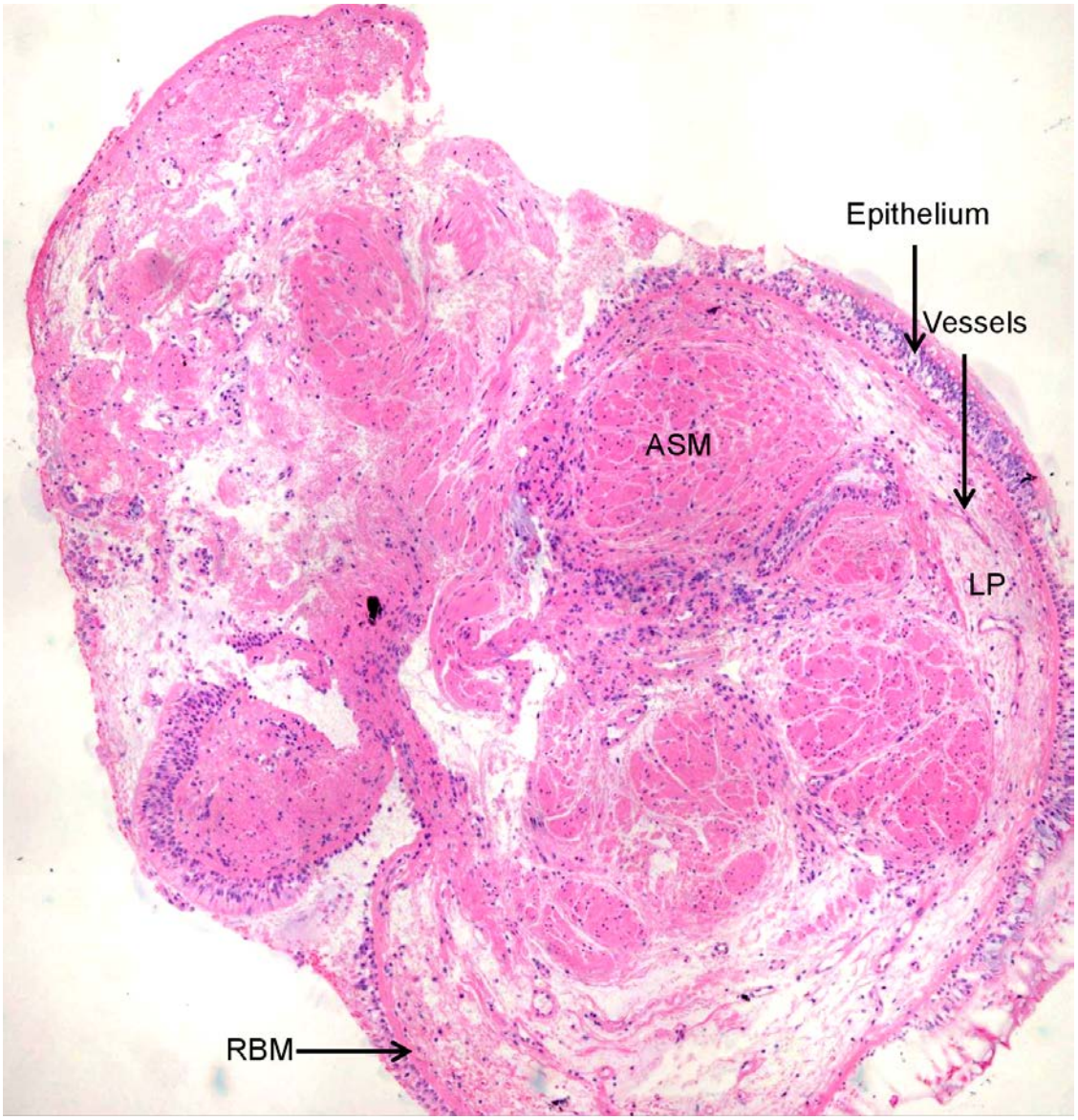


Figure 3.1 H & E asthmatic bronchial biopsy

Representative photomicrograph of haematoxylin and eosin stain showing the structure of a typical bronchial biopsy from a patient with moderate asthma. Black arrows highlight tissue structures. RBM Reticular basement membrane, ASM airway smooth muscle, LP lamina propria X100 magnification.

3.2.1 Assessment and quantification of immunohistochemical staining

Epithelial tissue for analysis was available from 11 healthy subjects, and 5 mild, 8 moderate and 14 severe asthmatic subjects. Lamina propria tissue was available for analysis from 12 healthy and 6 mild, 11 moderate and 15 severe asthmatic subjects.

The values for tryptase-positive mast cells, CD68-positive macrophages, major basic protein (MBP) positive eosinophils, elastase-positive neutrophils, and T cells within the epithelium and lamina propria are presented in summary Table 3.2. These are discussed further individually.

	Healthy control	Mild asthma	Moderate asthma	Severe asthma
Epithelium cells/mm^2				
Tryptase	2.8 [0-7.1]	**31.7*** [16.7-40.1]	9.2 [4.1-18.1]	3.2 [1.3-10.4]
MBP	0.0 [0-10.0]	13.5 [4.4-18.8]	1.7 [0.0-5.7]	3.2 [0.0-14.0]
CD68	0.0 [0-6.7]	**10.0*** [6.3-23.1]	2.4 [0.0-4.8]	15.3 [0.0-31.4]
Neutrophil elastase	2.8 [0-16.7]	0.0 [0.0-4.7]	3.3 [0.0-9.2]	5.6 [2.8-10.8]
CD3	33.3 [11.1-70.2]	60.0 [16.3-132.7]	31.1 [1.3-100.1]	44.7 [8.2-99.4]
CD4	3.3 [2.8-13.9]	10.0 [1.5-32.7]	5.0 [0.0-21.2]	9.8 [0.0-43.3]
CD8	14.7 [8.3-76.7]	30.2 [13.2-103.8]	9.2 [0.0-52.6]	29.1 [2.3-59.1]
Lamina propria cells/mm^2				
Tryptase	14.5 [10.6-19.0]	12.5 [6.8-26.9]	15.1 [8.9-31.4]	8.4 [5.4-15.6]
MBP	2.4 [1.4-5.6]	**11.2*** [9.8-12.7]	4.4 [2.7-17.5]	5.3 [1.3-24.4]
CD68	4.0 [2.3-5.1]	4.7 [3.6-6.5]	2.7 [1.4-7.2]	4.5 [1.4-11.2]
Neutrophil elastase	9.4 [3.7-20.2]	3.4 [1.1-8.6]	8.4 [3.0-17.1]	8.9 [1.9-15.7]
CD3	31.0 [12.0-45.8]	41.0 [32.2-49.6]	19.9 [9.5-29.3]	30.0 [18.4-43.2]
CD4	9.7 [5.4-14.6]	11.5 [8.9-21.6]	7.22 [2.1-11.9]	11.7 [5.1-15.7]
CD8	20.3 [10.9-31.0]	**30.2*** [26.8-37.1]	11.3 [6.1-18.6]	13.3 [7.4-26.1]

Table 3.2 Summary of inflammatory cell counts in the airway epithelium and lamina propria.

Median [interquartile range]. * $p<0.05$ compared to healthy control subjects

3.2.2 Mast cells

The number of epithelial and lamina propria tryptase positive mast cells, CD68 positive macrophages, MBP positive eosinophils and elastase positive neutrophils are shown in Figure 3.2, 3.3, 3.4 and 3.5 respectively.

Figure 3.2.A is a representative photomicrograph of tryptase positive mast cells in both the epithelium and lamina propria of bronchial biopsies. The section is counterstained with haematoxylin and the AEC red colour highlights the granular tryptase content.

Immunohistochemical analysis showed mast cell numbers were increased significantly in the airway epithelium in patients with mild asthma (Figure 3.2.B), which is consistent with previous reports describing intraepithelial mast cells associated with Th2 inflammation in patients with mild asthma (Dougherty *et al.*, 2010; Bradding *et al.*, 1994). Mast cell epithelial density was not increased in moderate or severe asthmatic subjects compared to healthy controls (Figure 3.2). ()In addition there were no significant differences among the groups in the lamina propria mast-cell counts (Figure 3.2.C), and no differences when compared to healthy controls, in keeping with previous findings (Bradding *et al.*, 1994)

Smooth muscle cells were identified based on their morphological appearance and Figure 3.3.A shows an example of a mast cell located within the airway smooth muscle bundles in an asthmatic subject. Mast cells were increased in the airway smooth muscle in asthma (median 4.0 [interquartile range 0.8-9.0])

compared to healthy controls (median 0.0 [0-1.6], p=0.029), but there was no difference across asthma severity (Figure 3.3.B).

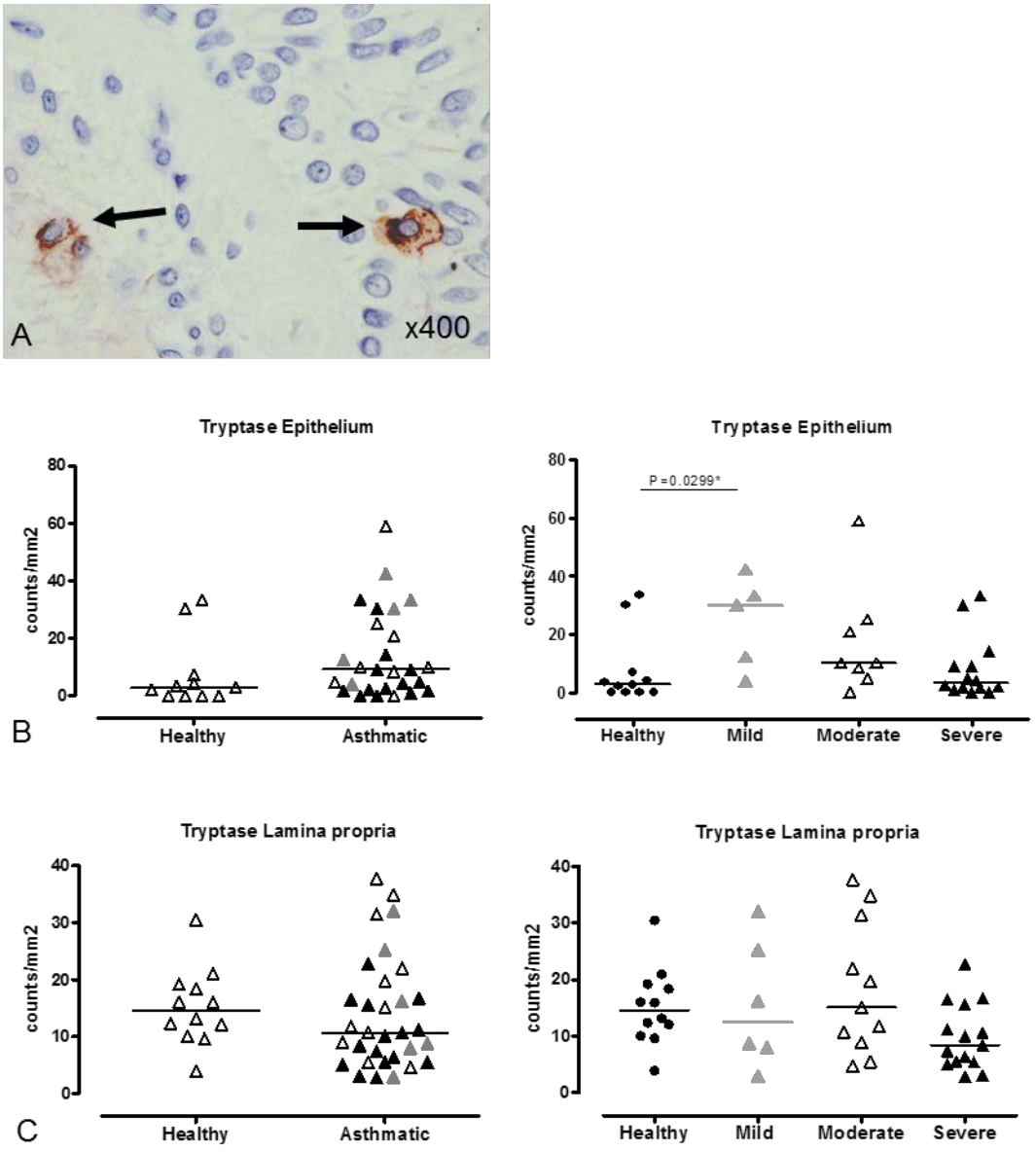


Figure 3.2 The expression of mast cells in bronchial biopsies

A) Representative photomicrographs of IHC showing mast cells (black arrow) in epithelium and lamina propria in bronchial tissue X400 magnification. Tryptase positive mast cell counts in B) epithelium and C) lamina propria in healthy controls verses asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

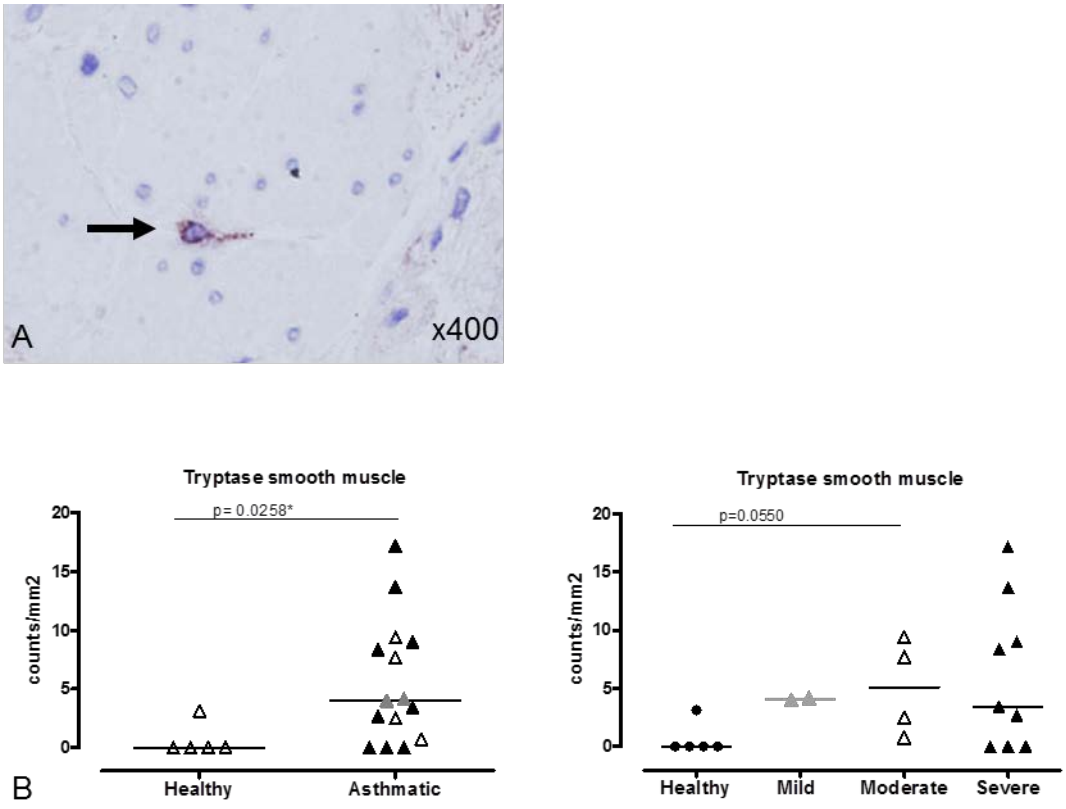


Figure 3.3 Infiltration of mast cells in airway smooth muscle

A) Representative photomicrographs of IHC showing mast cells within the smooth muscle in bronchial tissue (black arrow) X400 magnification. B) Tryptase positive mast cell counts in smooth muscle in healthy controls verses asthmatics as a whole (p=0.0258) and as subgroups (p=0.0550). Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

3.2.3 Eosinophils

Eosinophil major basic protein (MBP) is a potentially toxic protein found in the crystalloid core of the eosinophil granule. It is implicated in allergy and inflammation and is a powerful tool in immunohistochemistry as a marker of eosinophils. Therefore a commercial MBP antibody (Monosan) was used to investigate the expression of eosinophils in bronchial biopsies. An example of an eosinophil immunostained with MBP antibody is shown in Figure 3.4.A.

Eosinophil counts were increased in the airway lamina propria of asthmatic patients compared with those seen in healthy subjects, accounted for in part by a significant increase in the mild group (Figure 3.4.C and Table 3.2). However, 4 patients in each of the groups with moderate and severe asthma had lamina propria eosinophil counts higher than those in the mild asthma group, suggesting the presence of steroid-refractory eosinophilic inflammation. Lamina propria and epithelial eosinophil counts correlated significantly within the asthmatic population ($r_s = 0.45$, $p=0.018$), but consistent with previous reports, (Lemiere *et al.*, 2006) neither correlated with sputum eosinophil counts. The presence of eosinophils in the ASM bundles was not observed in healthy controls or subjects with asthma.

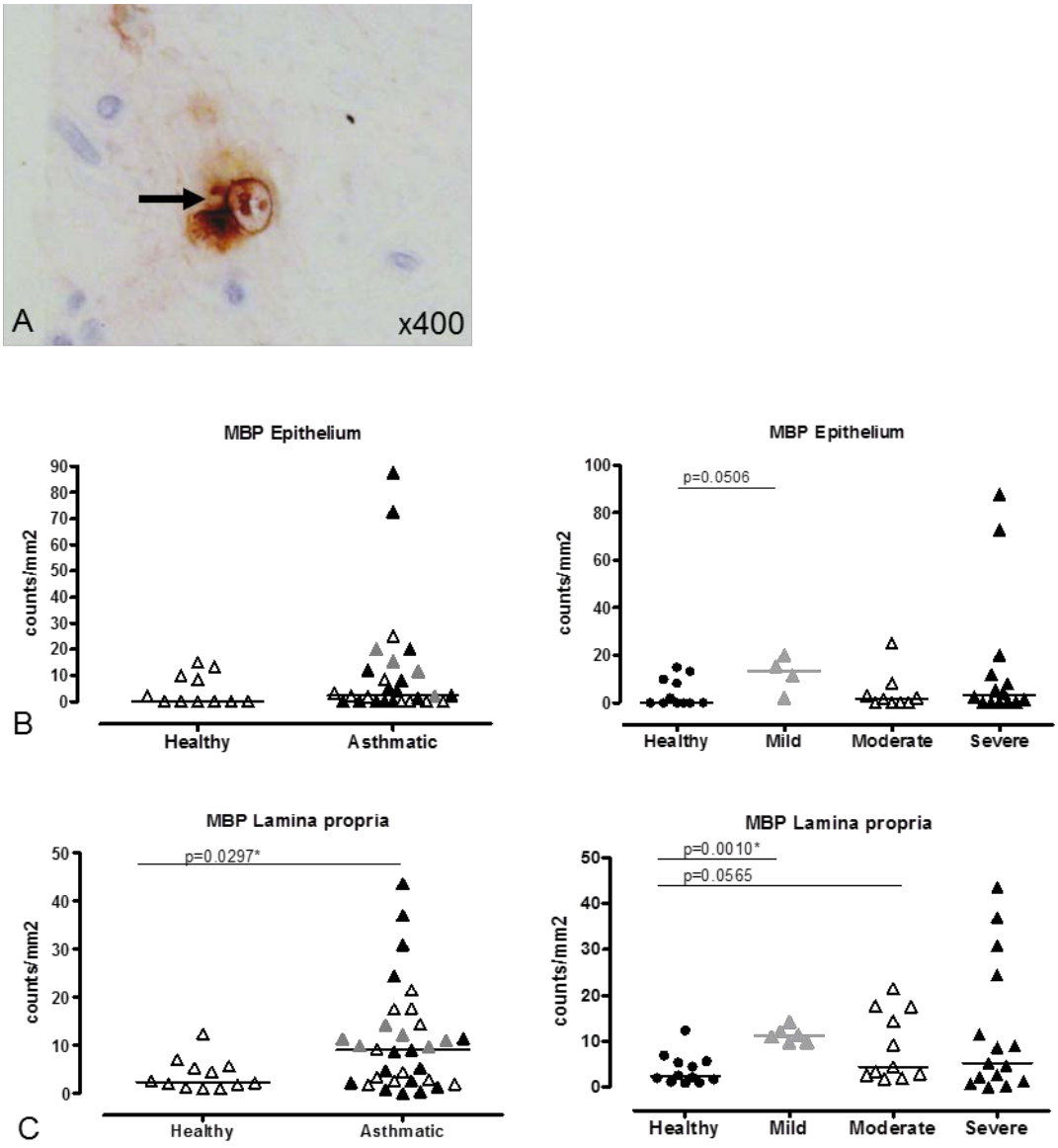


Figure 3.4 The expression of eosinophils in bronchial biopsies

A) Representative photomicrographs of IHC showing MBP+ eosinophils (black arrow) in human bronchial tissue X400 magnification. B) number of eosinophils within the epithelium in healthy controls and asthmatics as a whole and as subgroups. C) number of eosinophils within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

3.2.4 Macrophages and Neutrophils

The glycoprotein CD68 is expressed by macrophages therefore it is widely used in immunohistochemistry to identify these cells. A representative photomicrograph shows the typical immunostaining observed with a CD68 antibody (Dako, UK) (Figure 3.5.A). CD68 positive macrophage density was increased in the airway epithelium of patients with mild asthma compared with that seen in healthy control subjects but not between asthmatic patients and healthy subjects as a whole (Figure 3.5.B). There was no difference between healthy control subjects and asthmatic patients in the lamina propria (Figure 3.5.C).

Neutrophilic infiltration was assessed using a monoclonal antibody to neutrophil elastase. The antibody gave clear cellular positive immunostaining in all the biopsies however there was no difference in elastase positive neutrophils in the epithelium and lamina propria between healthy control subjects and asthmatics or in the subgroups which reflects findings in previous studies (Brightling *et al.*, 2003a) (Siddiqui *et al.*, 2008) (Figure 3.6). The presence of macrophages and neutrophils were not observed in the ASM bundles in healthy controls or subjects with asthma.

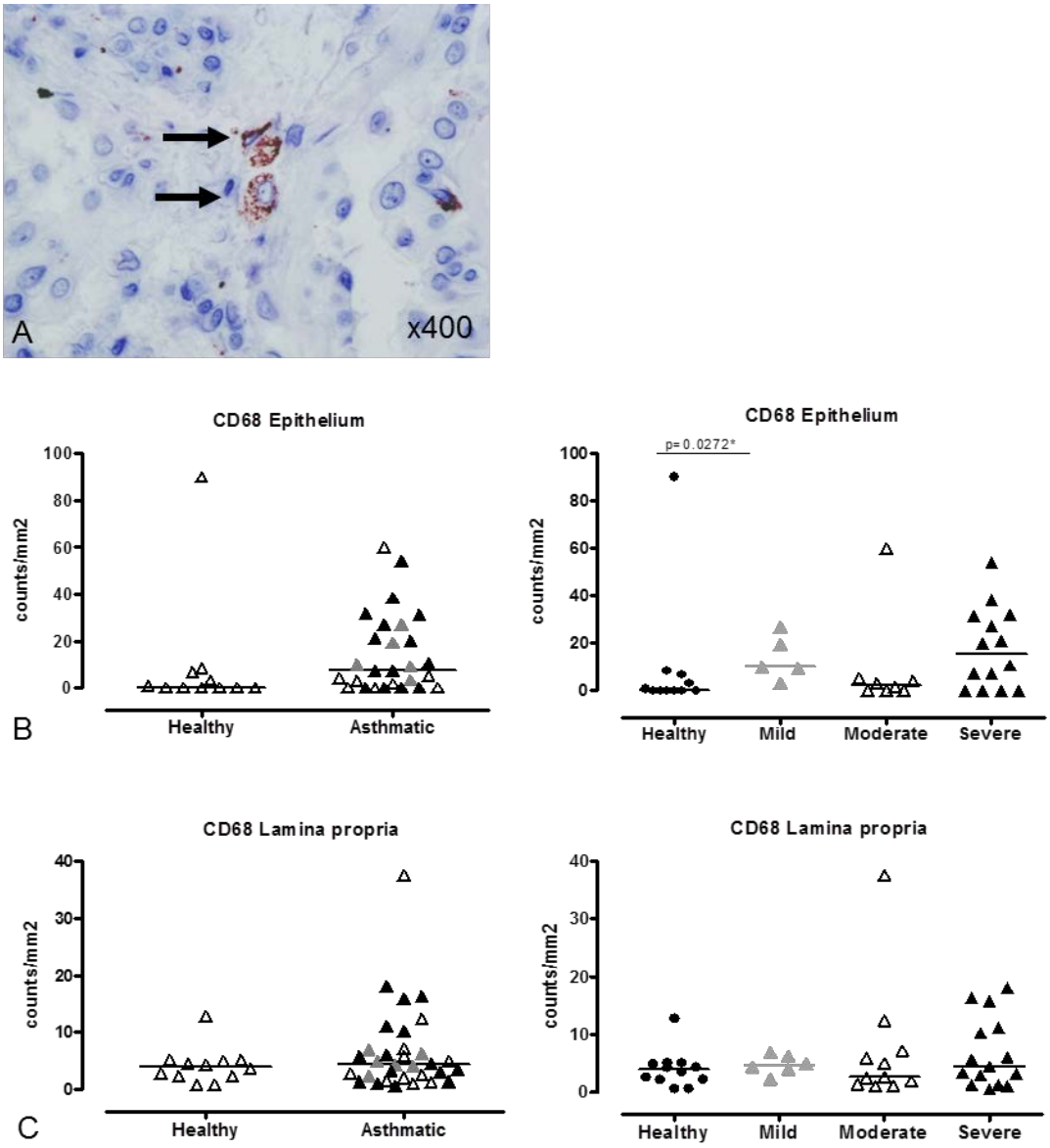


Figure 3.5 The expression of macrophages in bronchial biopsies

A) Representative photomicrographs of IHC showing CD68+ macrophages (black arrow) in human bronchial tissue X400 magnification. B) number of CD68+ macrophages within the epithelium in healthy controls and asthmatics as a whole and as subgroups. C) number of CD68+ macrophages within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma.

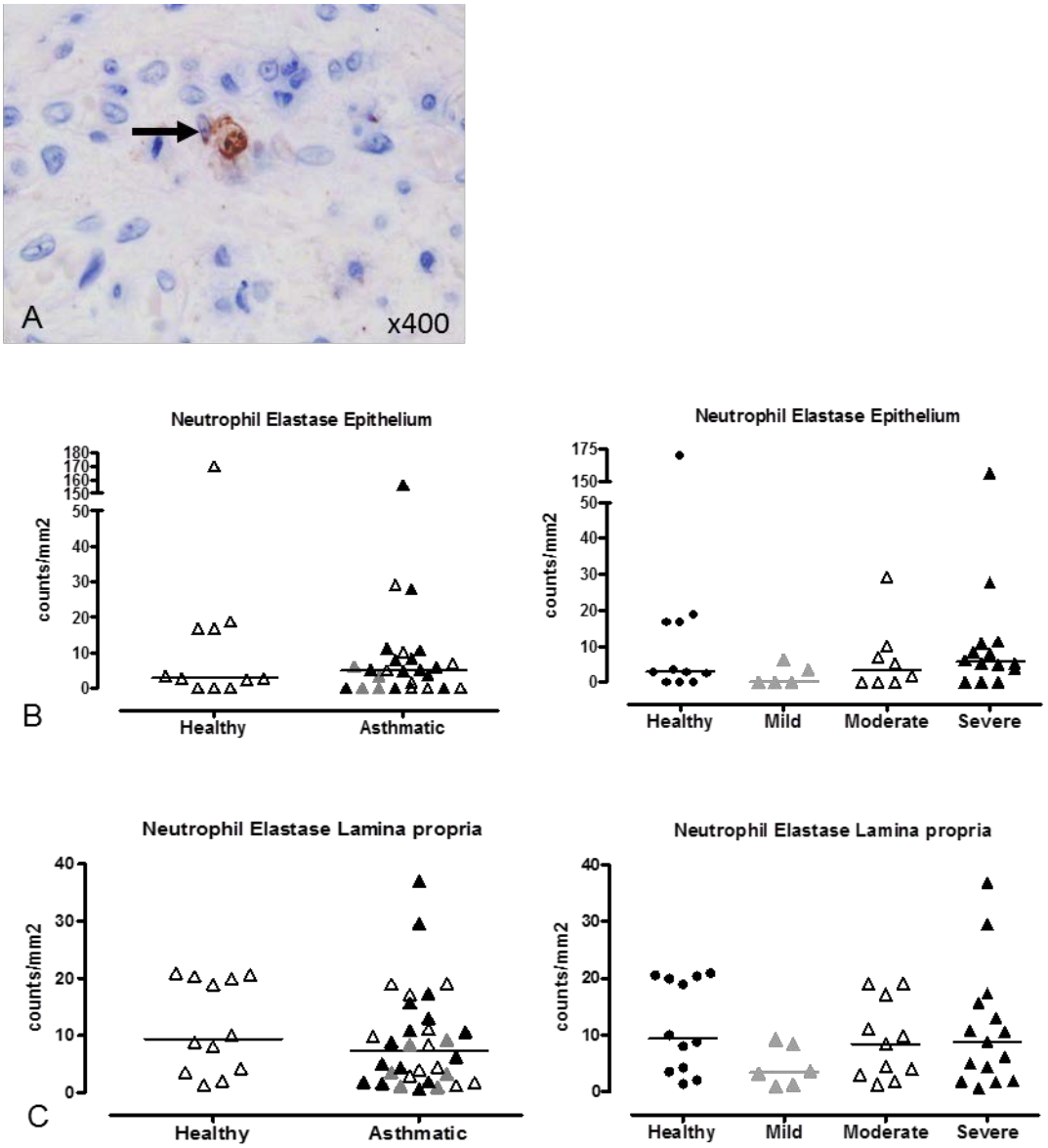


Figure 3.6 The expression of neutrophils in bronchial biopsies

A) Representative photomicrographs of IHC showing neutrophils (black arrow) in human bronchial tissue X400 magnification. B) number of neutrophils within the epithelium in healthy controls and asthmatics as a whole and as subgroups. C) number of neutrophils within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

3.2.5 T cells

CD3 forms part of the T cell receptor which is found almost exclusively on all T cells. Whilst maintaining their CD3 expression they differentiate into CD4+ T helper (Th) cells or cytotoxic CD8+ T cells subsets depending on their microenvironment. The expression of CD3, CD4 and CD8 make them very valuable T cell markers for immunohistochemistry.

Antibodies to CD3, CD4 and CD8 produced a predominantly peripheral ring staining of cells in healthy controls and asthmatic subjects (Figure 3.7.A, 3.8.A and 3.9.A). The number of epithelial and lamina propria T cells are shown in Figures 3.7 to 3.9 and Table 3.2. In keeping with previous studies of asthma (Bradding *et al.*, 1994), there was no significant difference in the number of epithelial or lamina propria CD3+ or CD4+ T cells in asthmatic compared to healthy controls (Figure 3.7.B, C and Figure 3.7.B, C). There was a significant increase in the number of CD8+ T cells in the lamina propria of mild asthma however this is of uncertain clinical significance (Figure 3.8.C).

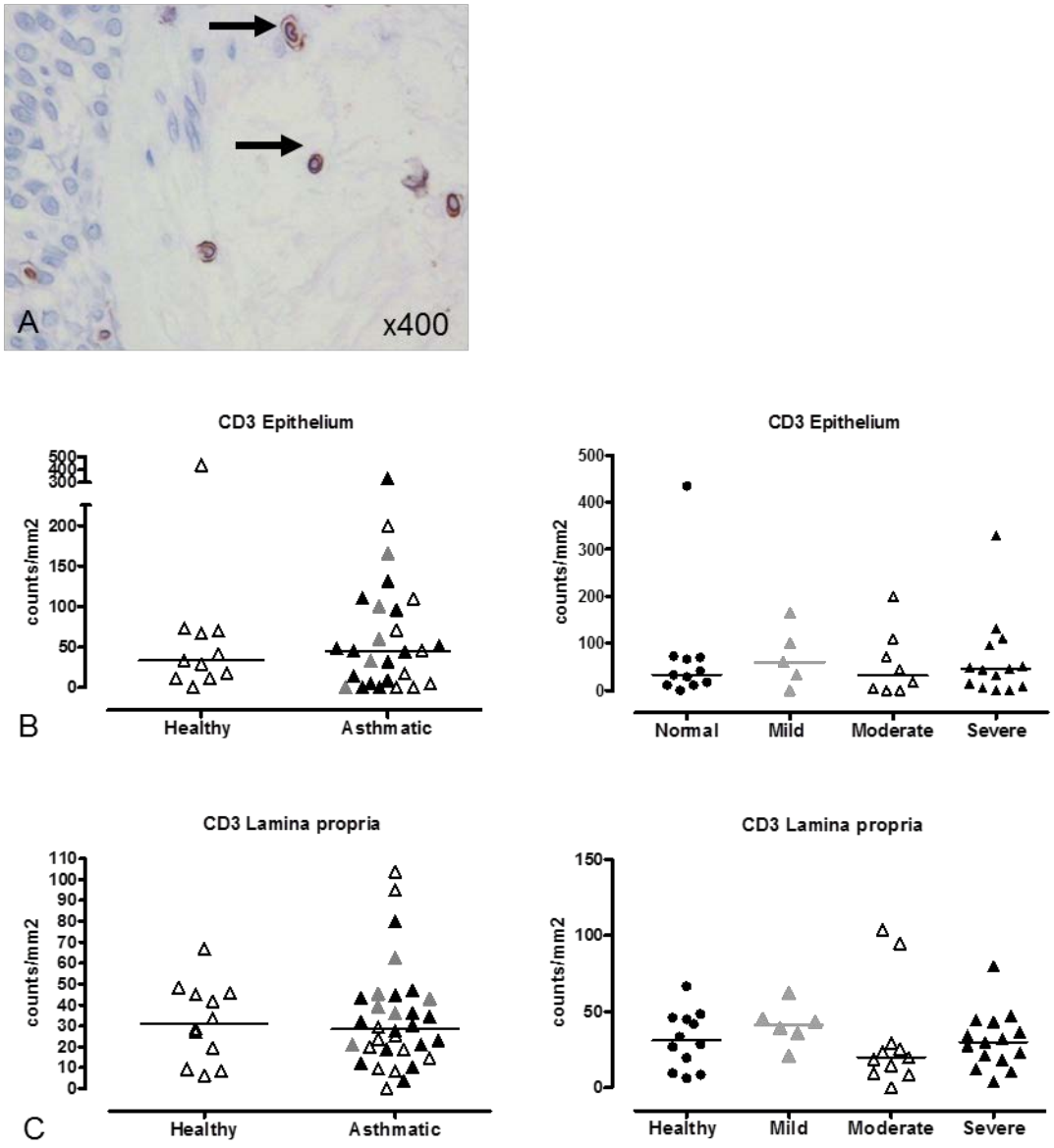


Figure 3.7 The expression of CD3+ T cells in bronchial biopsies

A) Representative photomicrographs of IHC showing CD3+ T cell expression (black arrow) in human bronchial tissue x400 magnification. B) number of CD3+ cells within the epithelium in healthy controls and asthmatics as a whole and as subgroups. C) number of CD3+ cells within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

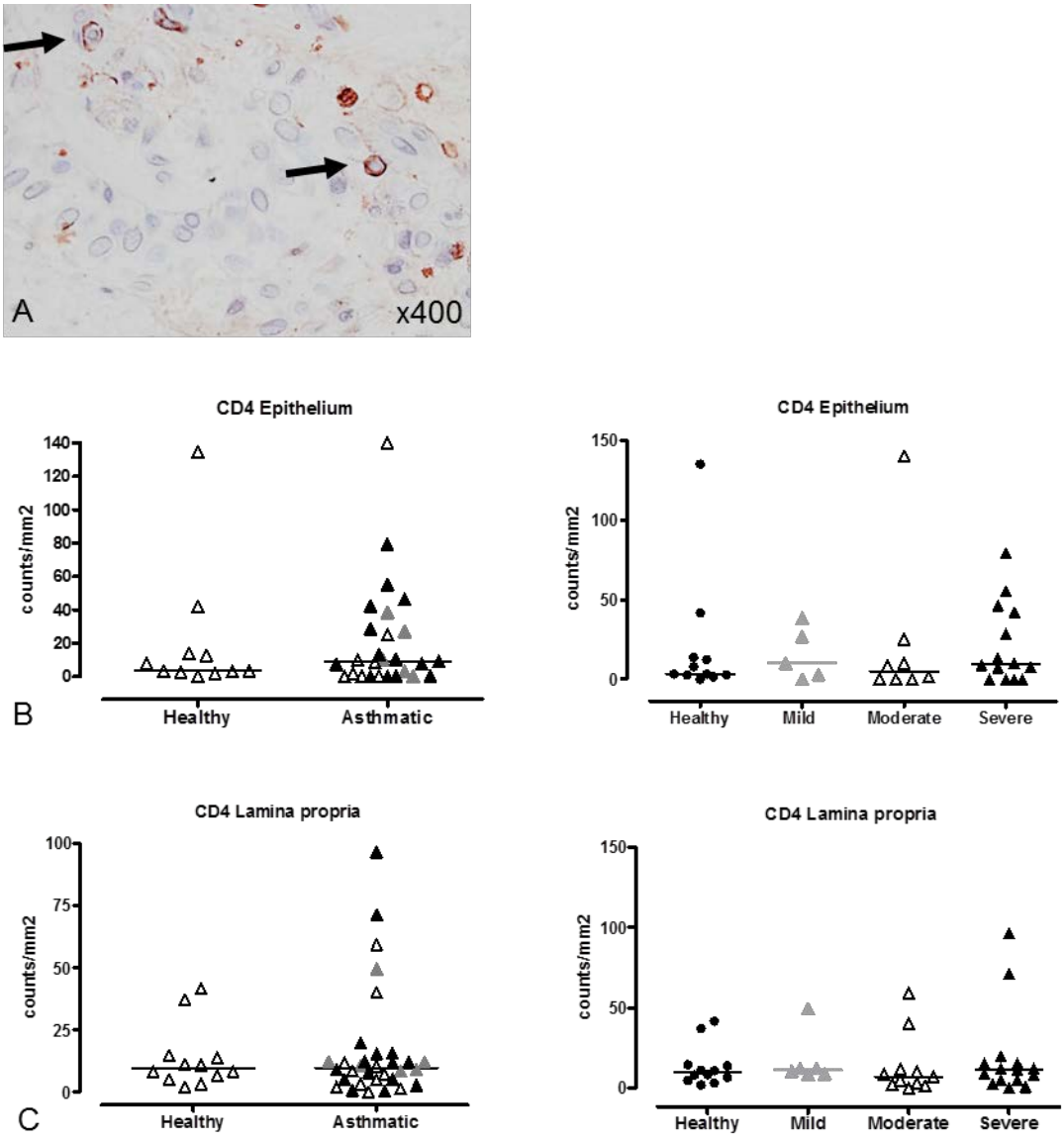


Figure 3.8 The expression of CD4+ helper T cells in bronchial biopsies

A) Representative photomicrographs of IHC showing CD4+ T cell expression (black arrow) in human bronchial tissue X400 magnification. B) number of CD4+ cells within the epithelium in healthy controls and asthmatics as a whole and as subgroups. C) number of CD4+ cells within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

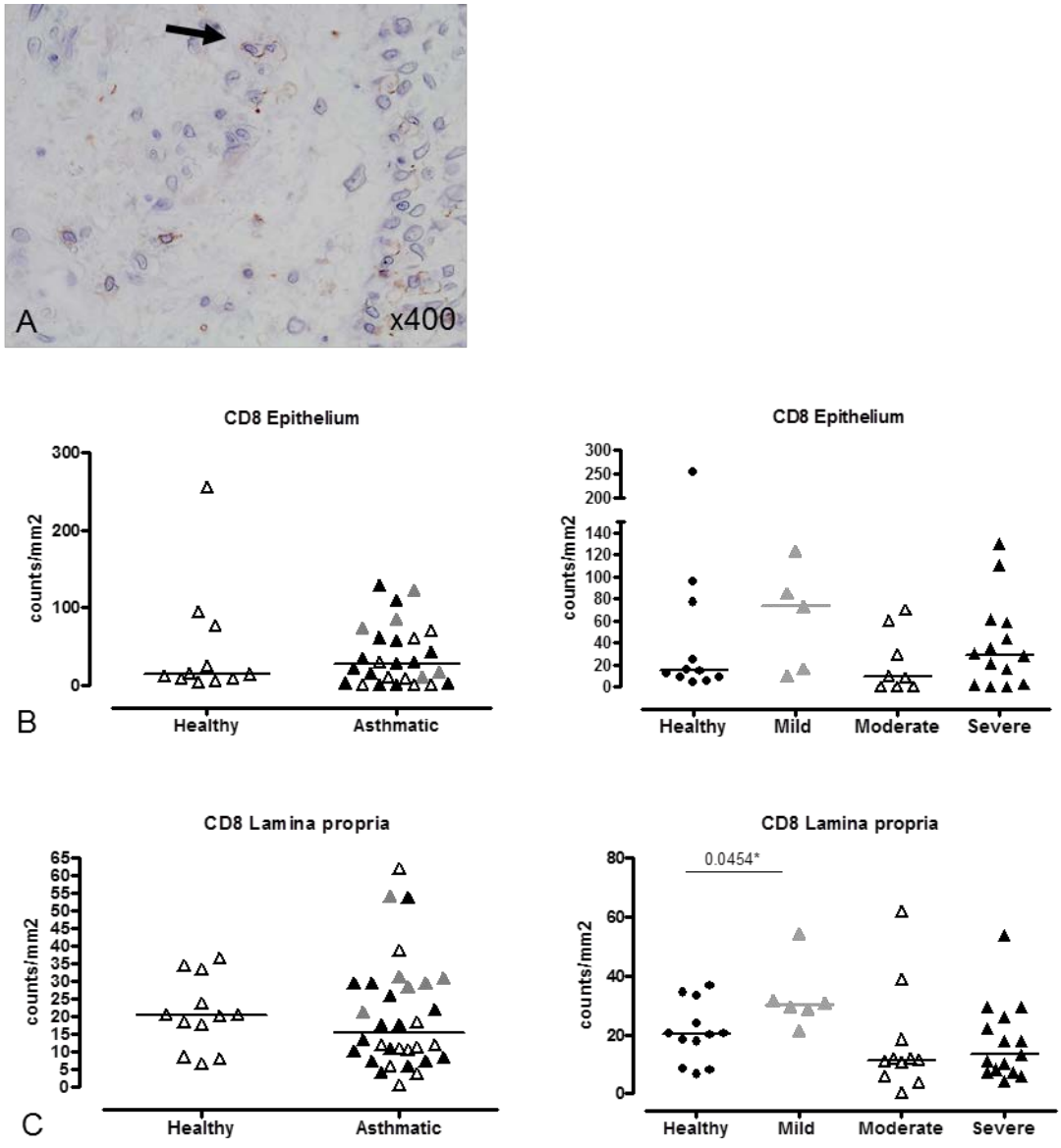


Figure 3.9 The expression of CD8+ cytotoxic T cells in bronchial biopsies

A) Representative photomicrographs of IHC showing CD8+ T cell expression (black arrow) in human bronchial tissue X400 magnification. B) number of CD8+ cells within the epithelium in healthy controls and asthmatics as a whole and as subgroups. C) number of CD8+ cells within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Kruskal-Wallis test p=0.0390. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

3.3 Discussion

In this study I report that inflammatory cells were localised to various airway structures including epithelium, lamina propria and airway smooth muscle as described previously. Interestingly the pattern of inflammatory cell localisation varied for each cell type. This suggests the recruitment and localisation of inflammatory cells is specific and highly regulated (Siddiqui *et al.*, 2007).

This study demonstrates that the number of mast cells in the lamina propria was similar in asthmatic subjects and healthy controls which is in keeping with previous studies (Brightling *et al.*, 2002a) (Bradding *et al.*, 1994) Mast cell numbers were higher in the epithelium of mild asthmatics in this and previous studies (Bradding 1994) when compared to healthy controls. I found that the number of tryptase positive mast cells within the epithelium reduced with increasing disease severity, compatible with previous studies showing that inhaled corticosteroids suppress epithelial mast cell numbers (James *et al.*, 2012a).

In keeping with previous reports, mast cells numbers were increased in smooth muscle bundles of asthmatic patients providing further support to the pathophysiological role of mast cells in the ASM in disease (Brightling *et al.*, 2002a; Siddiqui *et al.*, 2008; Saha *et al.*, 2008). Here the mast cell counts in the ASM were consistent across the spectrum of disease severity which is contrast with the work of Siddiqui *et al*. (Siddiqui *et al.*, 2008) where mast cells numbers in the ASM were associated with increasing disease severity. A possible explanation for the differences between these two studies is the number of

samples that were assessed. A limiting factor in my study was the availability of bronchial tissue with a sufficient volume of smooth muscle for analysis. Siddiqui *et al.* assessed ASM from 19 healthy controls and 42 asthmatic subjects whereas I assessed ASM from 5 healthy controls and 15 asthmatic subjects. In addition ASM mast cell numbers did not correlate with PC_{20} methacholine, a measure of BHR, which may be confounded by steroids when compared to the work of Brightling *et al*. in 2002 (Brightling *et al.*, 2002a), and again different to Siddiqui *et al.* due to reduced sample numbers. However, the finding of increased numbers of mast cells within the airway smooth muscle bundles in asthma is a consistent finding, and supports the view that mast cell infiltration of the smooth muscle is central to asthma pathophysiology.

Eosinophils were increased in the airway lamina propria of asthmatic patients and this was significant in the cohort of mild asthmatics. However, a small number of patients in each of the moderate and severe asthma groups had lamina propria eosinophil counts higher than those in the mild group suggesting the presence of steroid-refractory eosinophilic inflammation. In asthma, oral and inhaled corticosteroids reduce eosinophil numbers in the epithelium and lamina propria (Laitinen *et al.*, 1992) (Djukanovic *et al.*, 1997) and therefore it is highly likely that the relatively low counts observed in moderate and severe asthmatic subgroups reflect steroid treatment. Consistent with the data in this study, a number of research groups have demonstrated elevated eosinophil numbers in bronchial biopsies, induced sputum and bronchiolar lavage (BAL) fluids in steroid-naïve asthmatics (Frigas *et al.*, 1981) (Broide *et al.*, 1991) (Bradding *et al.*, 1994).

There was no difference in neutrophils in asthma and healthy controls similar to previous studies (Siddiqui *et al.*, 2008). This is very interesting as it suggests that idea of neutrophil-driven severe asthma may be misplaced (Wenzel *et al.*, 1999). The number of CD68+ macrophages remained similar between asthmatics and healthy controls. Consistent with previous studies (Bradding *et al.*, 1994) there was no difference in T cell infiltration between asthmatics and healthy controls.

CHAPTER 4.

TSLP immunoreactivity is increased in both the airway epithelium and lamina propria in severe asthma

4.1 TSLP in the airways

TSLP has been implicated in the pathogenesis of asthma, but its protein expression and relationship to immunological pathways in patients with severe disease is unknown. Therefore the next stage of this study focuses on TSLP protein expression. The first aim was to investigate whether the expression of TSLP increases across asthma severity and phenotype. The second aim was to investigate the cellular provenance of TSLP in asthmatic airways using co-localising techniques.

4.2 TSLP validation for immunohistochemistry

After consulting the literature on TSLP, human TSLP affinity purified polyclonal antibody was purchased from R and D systems, UK (product code AF1398). It had not previously been used for immunohistochemistry therefore required validation.

4.2.1 TSLP antibody validation using human tonsil tissue

Tonsils are the main immunocompetent tissue in the upper respiratory tract. B cells, T cells, mast cells, dendritic cells, macrophages and plasma cells are present, and the tonsillar tissue is covered by an epithelial layer. TSLP immunostaining of tonsillar epithelium has been described previously, Tonsil tissue was therefore chosen as a positive control for TSLP and immune cell immunostaining. In this study, tonsil tissue was obtained from Scottish Biomedical (Glasgow, UK), who obtained it with Ethical Approval and written informed consent. It was processed and embedded in GMA resin.

To determine the optimal concentration of anti-TSLP antibody that gave the best signal to noise ratio it was titrated in tonsil tissue using a concentration range from 1-10 µg/mL. An isotype control was used in the same concentration range. TSLP could be detected at 5 µg/mL however the staining was relatively weak suggesting the antibody concentration was too low. At the higher 10 µg/mL concentration TSLP was detected but the tissue was possibly overstained with high “background”, although the isotype controls were negative. Lowering the antibody concentration to 7.5 µg/mL gave good clear TSLP staining with no background, and I chose this for subsequent studies. The TSLP immunostaining in the control human tonsil tissue showed the typical epithelial distribution described previously (Soumelis *et al.*, 2002) (Figure 4.1).

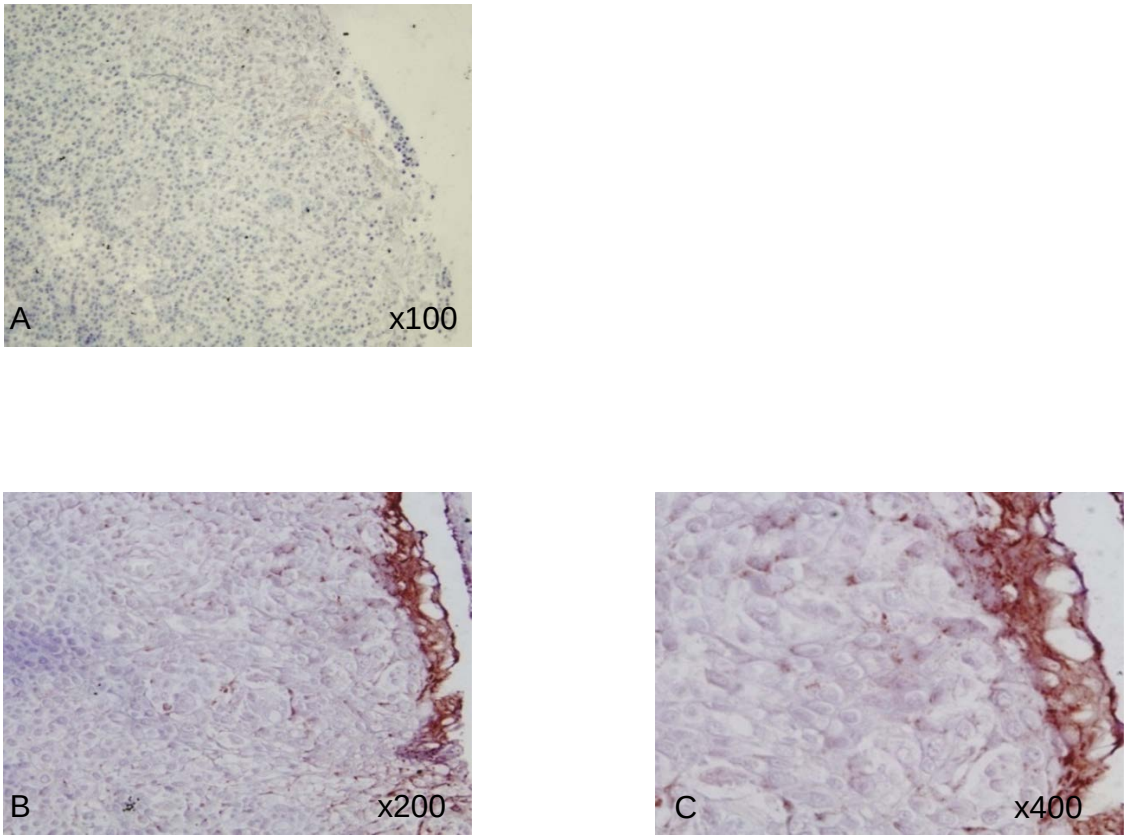


Figure 4.1 TSLP expression in human tonsil tissue

A) Representative photomicrographs of negative immunostaining in tonsil tissue with sheep IgG isotype control X100 magnification and B), C) positive control immunostaining for TSLP in human tonsil tissue (7.5 µg/mL) at X200, X400 magnification respectively.

4.2.2 TSLP antibody validation using recombinant TSLP

Next the specificity of TSLP antibody was assessed. Antibody activity was neutralised by incubating TSLP with excess recombinant protein (10:1 molar ratio) prior to incubating it with the bronchial tissue. Bronchial tissue sections probed with the TSLP/recombinant TSLP were compared with the tissue sections probed with the antibody alone. With the antibody alone, TSLP immunoreactivity was evident in both the airway epithelium and lamina propria of both healthy and asthmatic subjects (Figure 4.2.A-C) whereas the immunostaining was not evident using the TSLP antibody/recombinant TSLP mixture (Figure 4.2.D-F). Therefore the TSLP antibody immunostaining was specific.

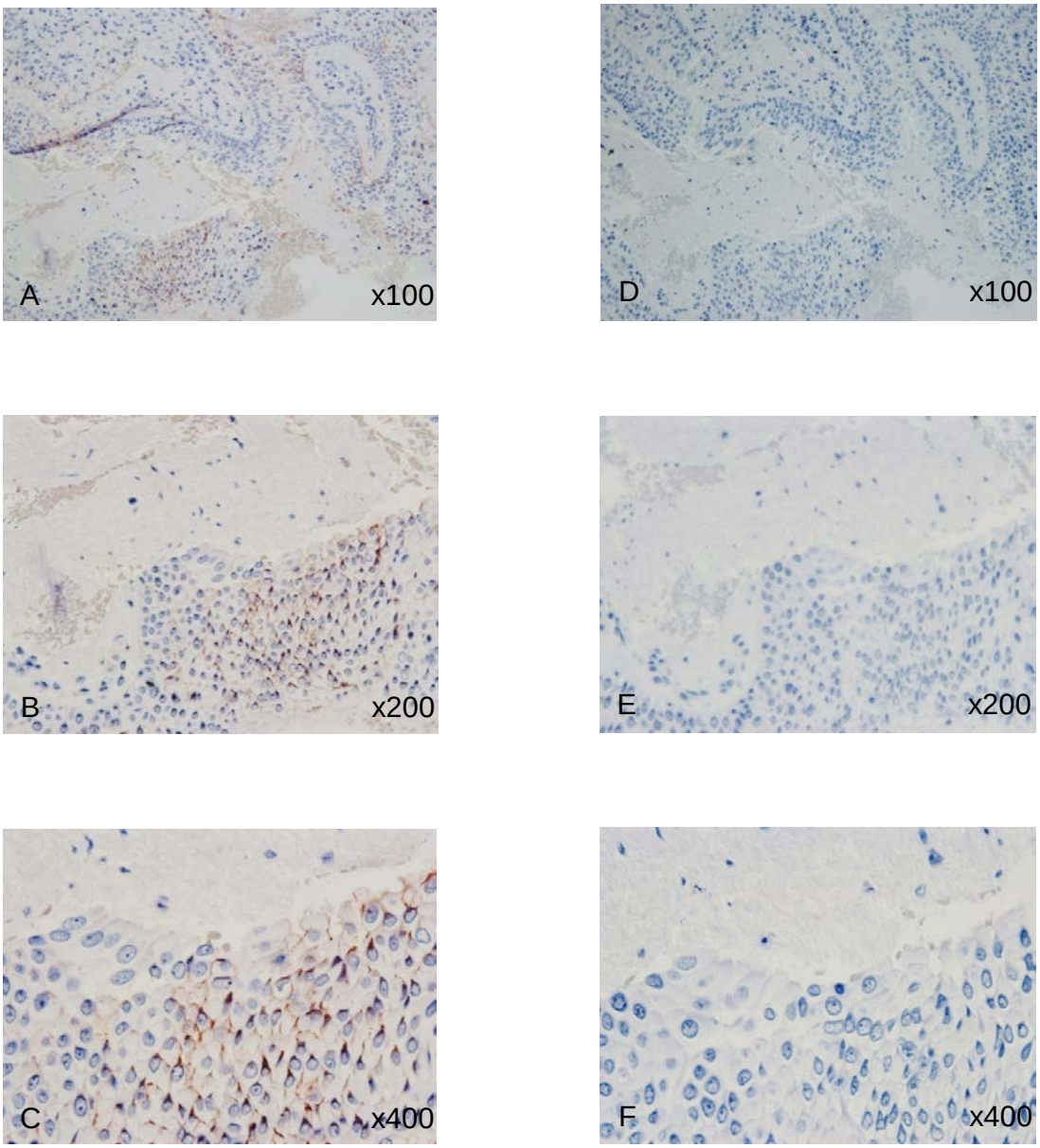


Figure 4.2 TSLP immunoreactivity in the asthmatic airway epithelium.

A-C) TSLP immunostaining in bronchial epithelium and lamina propria of asthmatic bronchial tissue. D-F) shows inhibition of this staining by preincubating with recombinant TSLP. Images taken at A), D) X100, B), E) X200, C), F) X400 magnification, respectively.

4.3 Assessment and quantification of TSLP immunostaining

The TSLP antibody at a concentration of 7.5 µg/mL was used to assess the immunoreactivity in bronchial biopsies from healthy and asthmatic subjects using immunohistochemistry. Epithelial tissue for analysis was available from 11 healthy subjects, and 5 mild, 8 moderate and 14 severe asthmatic subjects. Lamina propria tissue was available for analysis from 12 healthy and 6 mild, 11 moderate and 15 severe asthmatic subjects.

As mentioned above TSLP immunoreactivity was evident consistently in both the airway epithelium and lamina propria of both healthy and asthmatic subjects. To quantify TSLP expression in the epithelium two different methods were applied. First, a thresholding technique was developed based on the hue saturation and intensity (HSI) of TSLP staining which has previously been validated by our group (Siddiqui *et al.*, 2008). Secondly a semi quantitative score of staining was used, whereby staining was scored based on 0, none; 1, weak intermittent; 2, strong intermittent or weak generalised; and 3, strong generalised criteria. The results were compared and I found a strong correlation between the 2 methods ($r_s = 0.837$, $p<0.0001$) (Figure 4.3).

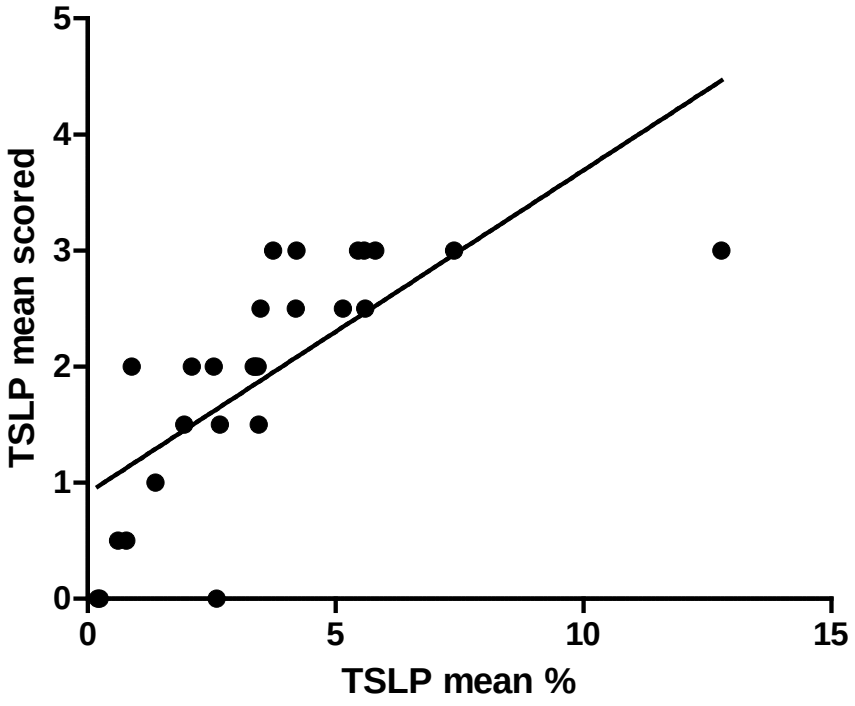


Figure 4.3 Correlation between intensity of staining threshold measurements and semi quantitative scoring method

(rs = 0.837, p<0.0001).

4.4 TSLP immunostaining is elevated in asthmatic bronchial biopsies

TSLP epithelial expression was significantly elevated in asthmatic subjects as a whole compared to healthy controls (p=0.0005), whether measured semi-quantitatively or by threshold intensity of staining (Figure 4.4).

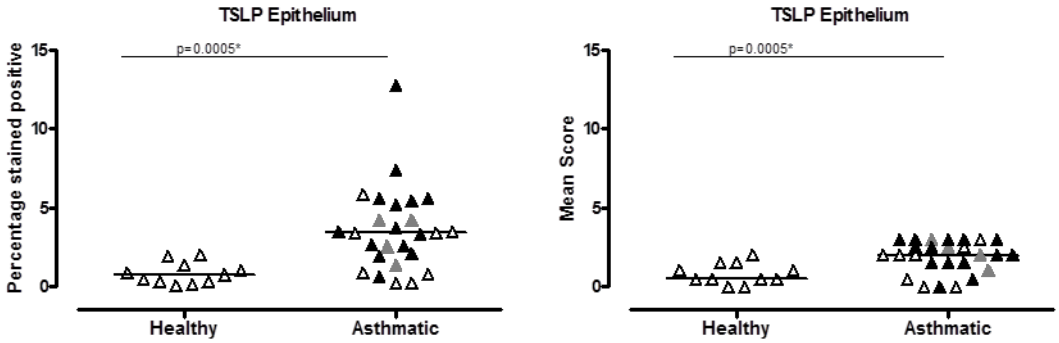


Figure 4.4 TSLP expression in airway epithelium

The extent of TSLP immunostaining in airway epithelium assessed based on threshold measurements and scoring p <0.0005, compared with healthy control subjects as a A) percentage and B) mean score. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

Subgroup analysis demonstrated significant increases in the epithelium of both patients with mild and patients with severe asthma compared with that seen in healthy control subjects by using the threshold assessment and in all asthmatic groups when scored semi quantitatively (Figure 4.5).

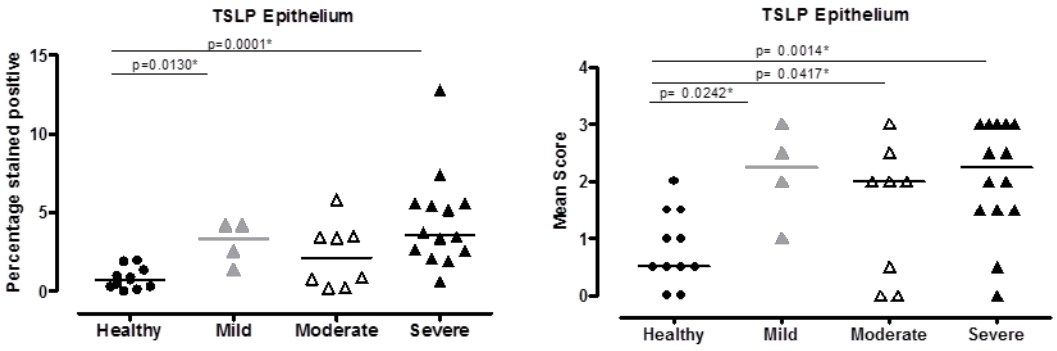


Figure 4.5 Subgroup analysis of TSLP expression in airway epithelium

Extent of TSLP immunostaining in airway epithelium in subgroups assessed based on threshold measurements and scoring. Mild and moderate $p<0.05$, severe (percentage) $p=0.0001$, and severe (scored) $p=0.0014$ compared with healthy control subjects. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

To identify other cellular sources of TSLP within the epithelium, TSLP was colocalised in sequential sections with mast cells, macrophages and neutrophils using the computer analysis system (Olympus, Germany). However, TSLP immunostaining in the airway epithelium was localised predominantly to epithelial cells with only occasional mast cells, macrophages and neutrophils being identified as sources of TSLP (Figure 4.6).

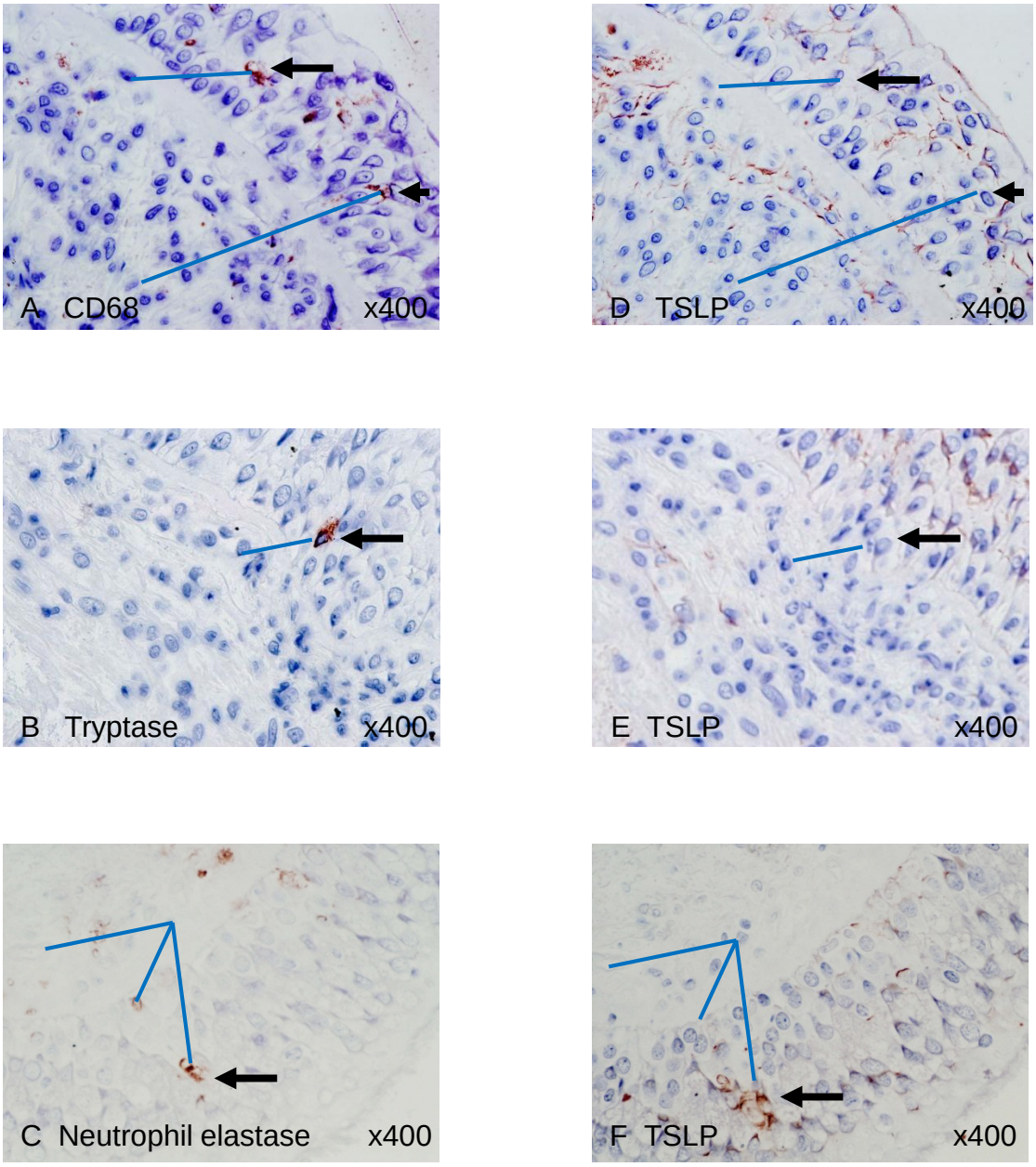


Figure 4.6 Cellular sources of TSLP within the airway epithelium

Representative photomicrographs of TSLP colocalised to inflammatory cells within the epithelium. Two sequential 2 μm sections demonstrating limited colocalisation of TSLP with A) and D)macrophage, B) and E) mast cell, C) and F) neutrophil. Lines demonstrate local landmarks for reference. Colocalised cells are indicated with black arrows.

The same method of counting cells used to quantify the inflammatory cell markers was used to assess TSLP immunoreactivity in the lamina propria. The numbers of positively stained nucleated cells in each compartment were counted and expressed per square millimetre and areas of less than 0.1 mm^2 were considered insufficient to quantify. Numerous cells staining for TSLP were evident in the bronchial lamina propria (Figure 4.7.A). The number of TSLP positive cells in the lamina propria was higher in asthma as a whole (p=0.043) (Figure 4.7.B), but interestingly this was accounted for by a significant increase only in the group of patients with severe asthma (p=0.0058) compared to healthy control subjects (Figure 4.7.C).

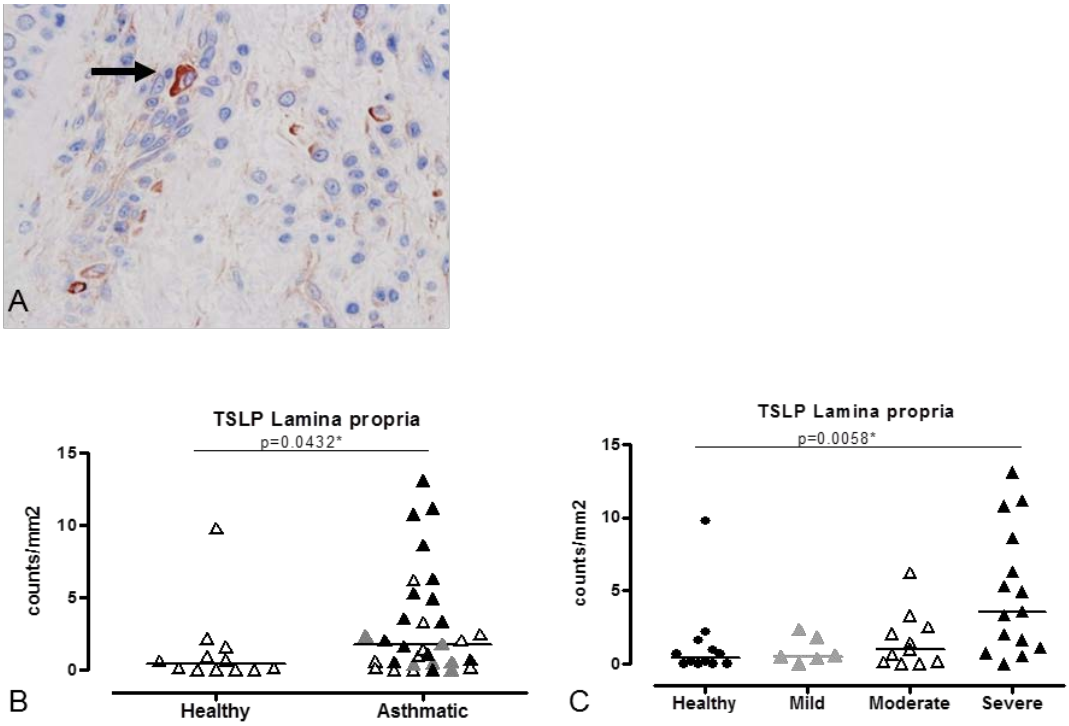


Figure 4.7 TSLP immunostaining in airway lamina propria

A) Representative photomicrographs of IHC showing TSLP (black arrow) immunostaining in the lamina propria of bronchial tissue X400 magnification. B) TSLP positive cell counts in the lamina propria p=0.0432 total asthmatics compared with healthy controls and C) p=0.0058 severe asthmatics compared with healthy control subjects. Kruskal-Wallis test p=0.0121. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

Interestingly, there was evidence of both endothelial cell and mononuclear cell staining in the lamina propria (Figure 4.7.A). To identify the cell types expressing TSLP, sequential 2 µm sections from a subset of 6 severe asthmatic subjects were taken and immunostained for CD68 positive macrophages (Figure 4.8.A), tryptase positive mast cells (Figure 4.8.B) and neutrophil elastase positive neutrophils (not shown). Each was then colocalised with TSLP positive cells. In addition TSLP was colocalised with endothelial cells which were identified based on their morphology and location (Figure 4.8.C).

Endothelial cells accounted for 42.0% ± 5.0% of TSLP positive cells. Further analysis showed approximately 5% of TSLP positive cells in the lamina propria were CD68 positive macrophages and 20% were tryptase positive mast cells (Figure 4.9). Neutrophils however accounted for only occasional TSLP positive cells (data not shown). Appropriate antibody isotype control sections were negative for TSLP immunostaining.

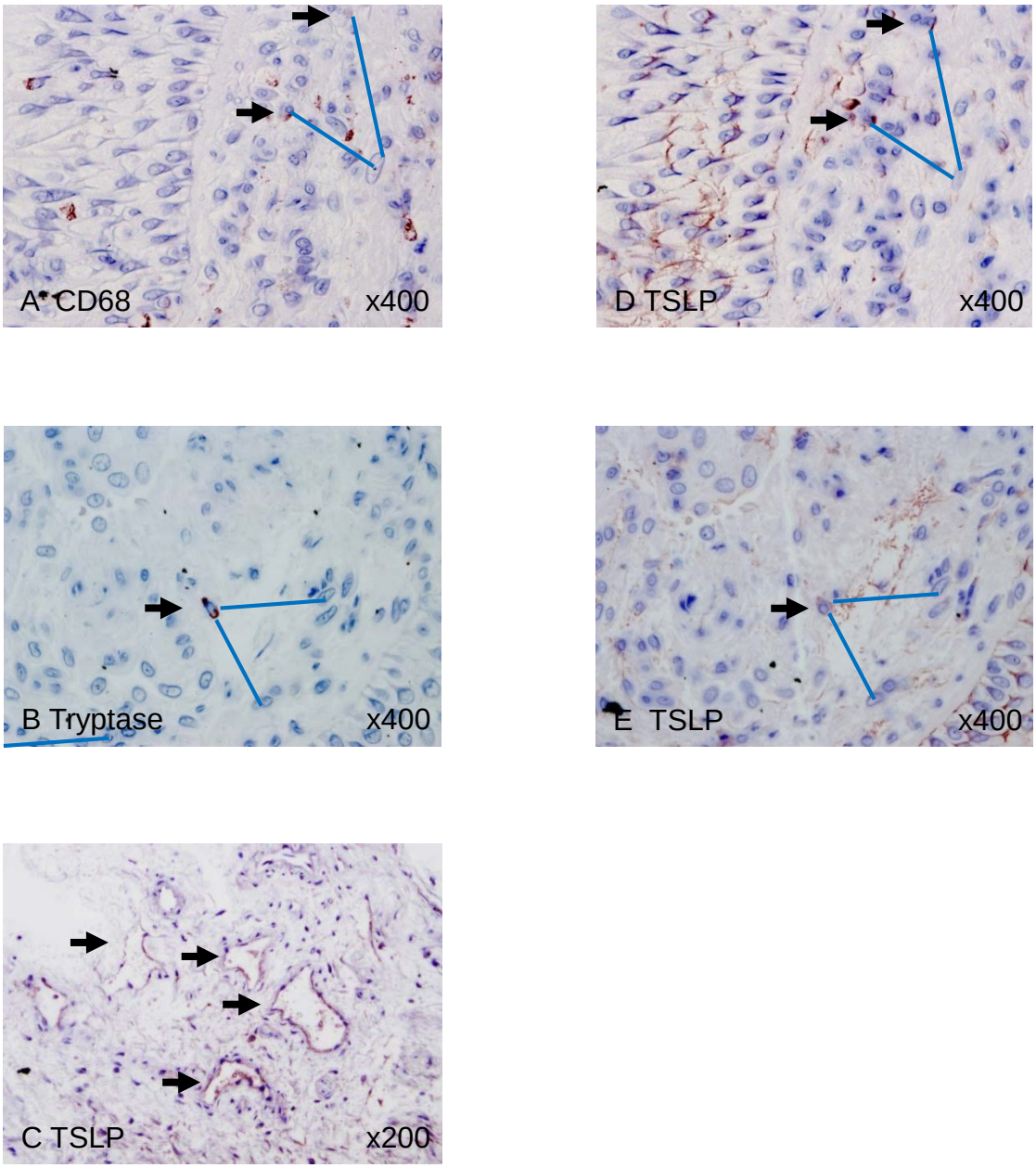


Figure 4.8 Photomicrographs of the cellular sources of TSLP in the lamina propria of asthmatic airways

Representative photomicrographs of TSLP colocalisation to macrophages and mast cells within the lamina propria. A) and D) two sequential 2 µm sections demonstrating colocalisation of macrophages and TSLP B) and E) mast cell tryptase and TSLP. C) highlights TSLP expression by endothelial cell (indicated by black arrows) Lines demonstrate local landmarks for reference. Immunohistology is shown at X400 and X200 magnification.

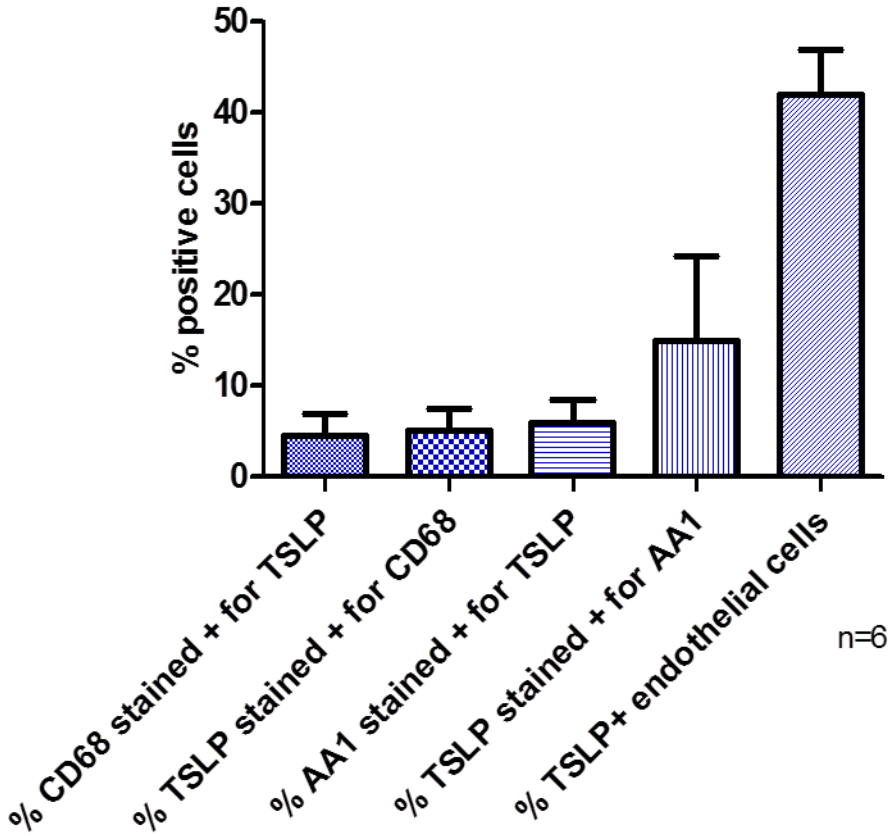


Figure 4.9 Cellular sources of TSLP within the lamina propria of asthmatic airways

The percentage of macrophages, mast cells and endothelial cells expressing TSLP and vice versa in a subset of 6 patients with severe asthma. (Mean ± SEM)

4.5 TSLP protein expression is related to airflow obstruction

To help elucidate potential roles of TSLP in asthma, cell counts were correlated with the asthmatic subject's demographic data (Table 3.1). The FEV1/FVC ratio is routinely used as a measure of airflow obstruction. It distinguishes between reduced FEV1 due to restricted lung volume and that due to obstruction. If the FEV1/FVC ratio is <70% airflow obstruction is present. There were significant inverse correlations between TSLP counts in both the asthmatic bronchial lamina propria and epithelium with the FEV1/FVC ratio ($r_s = -0.53$, $p = 0.002$ and $r_s = -0.40$, $p = 0.037$). There was also a significant correlation between lamina propria TSLP count and reliever use/week ($r_s = 0.40$, $p = 0.045$). No other significant correlations between TSLP staining and clinical parameters were evident.

4.6 TSLP is undetectable in sputum from asthmatic subjects

TSLP protein expression was elevated in bronchial tissue in asthma compared to healthy control subjects, so I next wanted to assess whether TSLP was elevated in induced sputum supernatant in asthma. TSLP was measured in sputum samples from the subjects that underwent bronchoscopy using a commercial ELISA kit (R & D systems, UK). The kit was validated using a calibration curve performance test which is fully described in Chapter 8.

Sputum is liquefied using dithiothreitol (DTT). This allows the cells that are released to be prepared into cytospins for differential cell counting. However, DTT has reducing and denaturing effects and as a result the detectable levels of various proteins are significantly reduced (Woolhouse *et al.*, 2002). Therefore

the effect of DTT on TSLP recovery was assessed by comparing the standard curve concentrations. The TSLP standard was reconstituted with increasing concentrations of DTT (0-0.022%). A top concentration of 0.022% was used as this is the final concentration in sputum supernatant. At any of the concentrations the mucolytic DTT did not affect the recovery of TSLP. Therefore to account for DTT in the sputum supernatants, the standards were reconstituted with a final volume of 0.022% (1:2) DTT (Figure 4.10).

Sputum samples from 4 subjects processed with dithiothreitol were divided into non-spiked and spiked (250 pg/mL TSLP standard) as a positive control. Although the exogenous TSLP spike could be recovered in sputum samples (mean 142.2 pg/mL) there was no measureable TSLP protein in severe asthmatic samples and these undetectable levels of cytokine were assigned the concentration of zero (Figure 4.11).

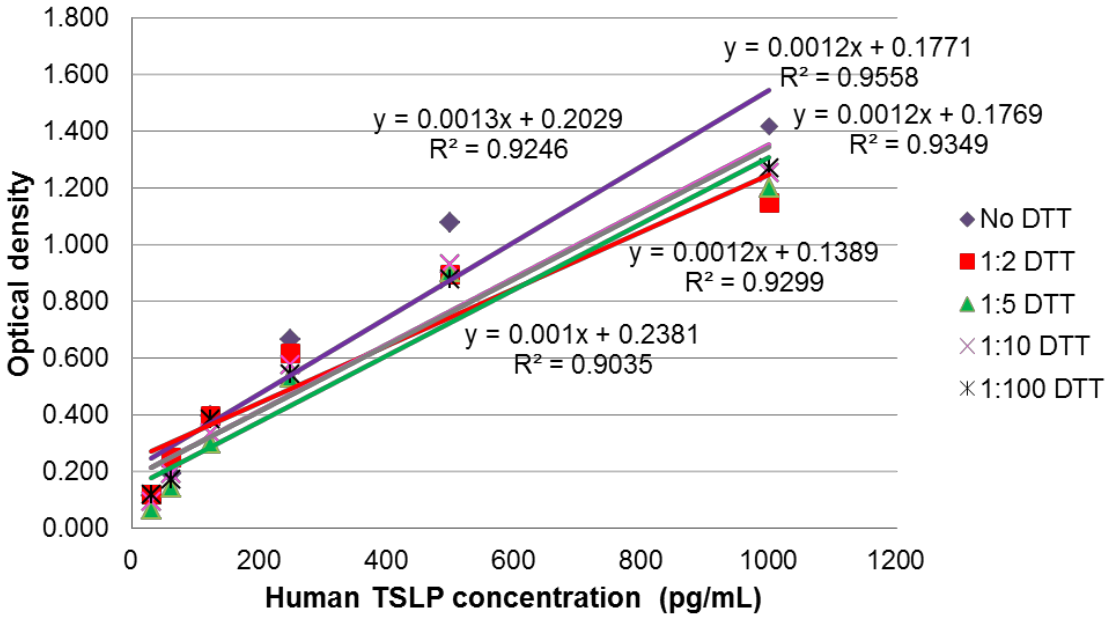


Figure 4.10 The effect of dithiothreitol on the recovery of TSLP

TSLP duoset ELISA standards were reconstituted in reagent diluent containing DTT in a range of 0-0.022%. The effect of DTT on the standard curve was measured at 450 nm.

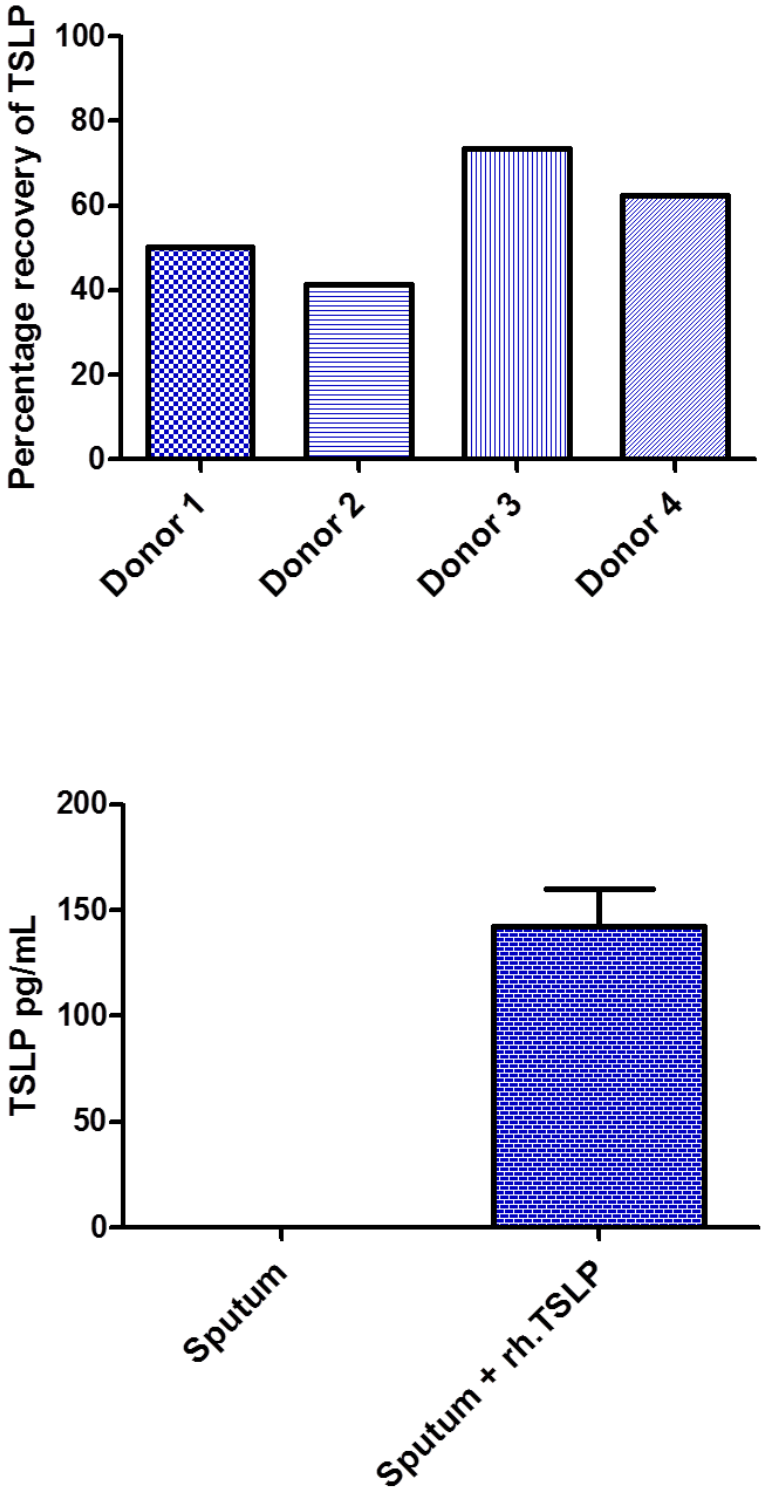


Figure 4.11 Recovery of TSLP in sputum

TSLP protein was measured in asthmatic sputum samples and in the samples spiked with 250 pg/mL of TSLP from the standard n = 4. (Mean ± SEM)

4.7 Discussion

This is the first study to investigate the protein expression of TSLP in bronchial biopsies using immunohistochemistry. The antibody used was validated in human tonsil as a positive control, and the specificity of immunostaining was confirmed by using both isotype controls and immunoabsorption with recombinant human TSLP.

This study shows that expression of TSLP protein is upregulated in the airways of patients with asthma and that this persists in a cohort of patients with severe asthma despite high-dose corticosteroid treatment at steps 4 and 5 of the British asthma treatment guidelines. TSLP expression in the airway epithelium was increased across the disease severity as defined by BTS/SIGN treatment step, although to a lesser extent in patients with relatively well-controlled moderate asthma. However, in the airway lamina propria, increased expression was confined to the group with severe disease. Since the completion of this study, Ferreira *et al*. (Ferreira *et al.*, 2012) showed TSLP expression was upregulated in the large airways of patients with fatal asthma but not in mild asthmatics compared to control subjects, supporting the data presented here.

This study extends previous work demonstrating increased TSLP mRNA expression in asthmatic epithelium and airway mucosa and increased TSLP protein expression in the BAL of 13 patients with moderate-to-severe asthma (Ying *et al.*, 2005; Ying *et al.*, 2008) .I have demonstrated TSLP protein is upregulated in the asthmatic epithelium in particular severe asthma and in support of this finding and those reported by Ying *et al.* I have shown cultured

primary bronchial epithelial cells from asthmatic subjects overexpress TSLP. The findings here extend our understanding of the potential roles of TSLP in asthmatic patients through the co-examination of downstream TSLP-dependent cellular networks. These findings suggest TSLP might be a key mediator of severe chronic asthma and that its anatomic site of expression might be related to asthma severity.

Several cell types including mast cells, epithelial cells and endothelial cells are a potential source of TSLP in the airways (Okayama *et al.*, 2009; Ying *et al.*, 2008). Using sequential sections, TSLP positive cells were therefore colocalised to mast cells, eosinophils, T cells and endothelial cells. This technique was chosen as the doublestain kits used in our laboratories were for only mouse monoclonal and rabbit polyclonal antibodies, and the method of colocalisation using sequential 2 µm GMA sections has been thoroughly validated previously (Bradding *et al.*, 1994; Bradding *et al.*, 1992). Another important reason to avoid double staining was that the available Kits use Fast Red (red reaction product) and DAB+ 3, 3'-diaminobenzidine (brown reaction product) which make it relatively difficult to determine whether the same cell has dual-stained.

Using sequential sections I confirmed that mast cells are a source of TSLP and found that 5% of mast cells in patients with severe asthma express TSLP protein, which in turn accounted for 20% of TSLP positive cells in the lamina propria. These findings are in broad agreement with the data of Ying *et al.* (Ying *et al.*, 2008) with respect to TSLP mRNA expression in resident airway mast cells, but in marked disagreement with the data of Okayama *et al,* (Okayama *et*

al., 2009) who studied TSLP protein. Ying *et al*. found that 20% of mast cells express TSLP mRNA whereas Okayama *et al.* found 90% of the bronchial mucosal TSLP positive cells were mast cells. One speculation for the discrepancy may be due to variations in the methods used for tissue preparation, for example tissue fixation, staining techniques and cell counting methodology.

Ying *et al.* (Ying *et al.*, 2008) also demonstrated TSLP mRNA expression in endothelial cells, neutrophils, CD68 positive macrophages, and CD3 positive T cells. I identified endothelial cell TSLP immunoreactivity by means of morphology and have confirmed the presence of TSLP protein within CD68+ macrophages but have not investigated T-cell colocalisation. Since publishing this data, Ferreira *et al.* confirmed these findings by reporting TSLP was predominantly located in the epithelium, endothelial cells, mononuclear cells and polymorphonuclear cells (Ferreira *et al.*, 2012).

My data demonstrate that DTT does not affect the recovery of recombinant TSLP in induced sputum samples, and that TSLP protein is not detectable in induced sputum obtained from asthmatic subjects. Since the completion of this study a clinical trial on the effects of prednisone on potential biomarkers of allergen responses in asthmatics has reported its findings. Consistent with the data in this study, they also found TSLP levels were too low to detect in sputum and nasal exudates, therefore analysis was abandoned (Merck, Updated 27th June 2012). Recent work has shown that mast cell proteases rapidly degrade

TSLP, which means it might not reach the airway lumen following its release in the airway wall (Okayama *et al.*, 2009).

Ying *et al.* (Ying *et al.*, 2008) found a significant inverse correlation between TSLP mRNA expression in both the airway epithelium and lamina propria with FEV1 percent predicted. I did not find a significant correlation with FEV1 percent predicted but did find significant inverse correlations between TSLP protein expression in both the airway epithelium and lamina propria with the FEV1/FVC ratio. Because there is wide variability between subjects in predicted FEV1 (Falaschetti *et al.*, 2004), the FEV1/FVC ratio provides a more accurate measure of airflow obstruction. This finding suggests that in addition to promoting airway inflammation, TSLP overproduction might play an important role in the remodelling of asthmatic airways and the subsequent development of fixed airflow obstruction.

CHAPTER 5.

The TSLP-dendritic cell-OX40L axis is not evident in human asthmatic airway tissue

5.1 CD1a and CD83 validation for immunohistochemistry

To investigate whether the TSLP/dendritic cell/OX40L pathway is present in asthmatic airway tissue, bronchial biopsies were immunostained for the presence of activated and non-activated dendritic cells.

Human dendritic cells originate from CD34 hematopoietic stem cells. They express a range markers including CD1a. CD1a is expressed by human dendritic cells early in their development and is therefore widely used as an immature DC marker in humans (Brigl & Brenner, 2004). It is known to be expressed in the interdigitating stratified squamous epithelium of the tonsil.

CD83 is a member of the Ig superfamily and is expressed as a cell surface protein. It is selectively expressed and up-regulated upon dendritic cell maturation and is one of the best-known markers for immunostaining mature dendritic cells (Lechmann *et al.*, 2002). Furthermore TSLP activated mature DCs up regulate expression of CD83 (Soumelis *et al.*, 2002).

Using a range of concentrations, antibodies to CD1a and CD83 antibodies were titrated on human tonsil tissue (Figure 5.1.A). The optimal concentration for CD1a was 26 µg/mL for CD1a (Figure 5.1.A and B). This is higher than I would usually find, but the isotype controls were negative, and the immunostaining looked specific. The optimal concentration for CD83 was 1 µg/mL (Figure 5.2.A and B). In addition the mouse isotype control at the corresponding concentrations was clear showing there were no interactions of the immunoglobulin molecules with the tissue and the secondary antibody was at

the optimal concentration. The background staining was negligible. For further details on antibody concentrations please refer to Table 2.1.

5.2 Dendritic cell numbers in asthmatic airway tissue

In the airways, immunostaining for each of these antigens was relatively sparse (Figure 5.1 and Figure 5.2). There was no significant difference in CD1a immunostaining between groups in the airway epithelium. There was a significant increase in the number of CD1a positive dendritic cells in the lamina propria in asthma as a whole but this was not related to any specific disease severity subset (Figure 5.1). There were no significant differences across groups for CD83 staining in the airway epithelium and lamina propria (Figure 5.2 and Table 5.1).

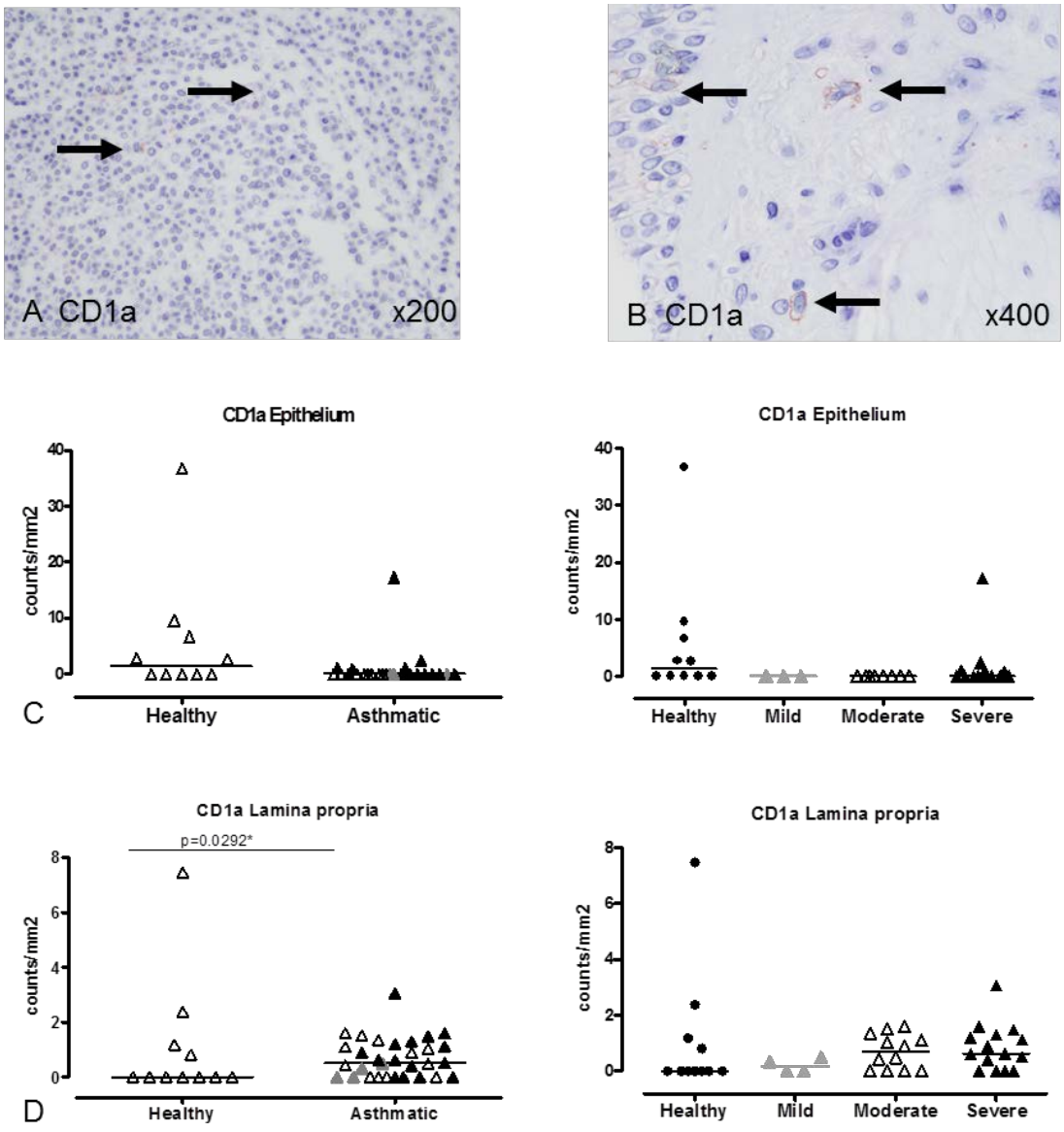


Figure 5.1 The expression of CD1a+ dendritic cells in bronchial biopsies

A) Representative photomicrographs of CD1a+ dendritic cell immunostaining in control tonsil tissue and B) in normal bronchial epithelium and lamina propria (black arrow) X200, X400 magnification. C) number of CD1a+ cells within the epithelium in healthy controls and asthmatics as a whole and as subgroups, D) number of CD1a+ cells within the lamina propria in healthy controls and asthmatics as a whole p=0.0292 and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

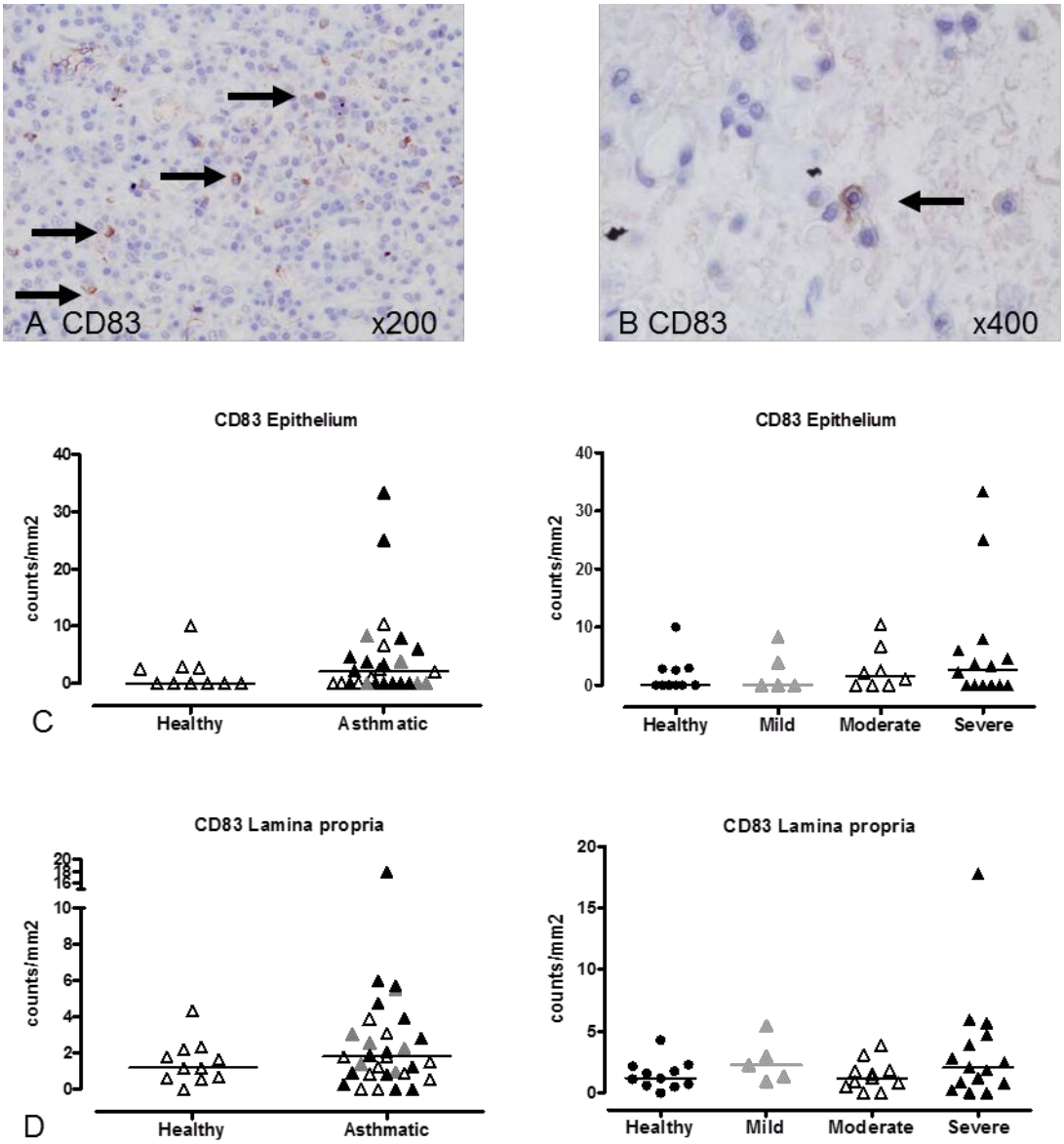


Figure 5.2 The expression of CD83+ dendritic cells in bronchial biopsies

A) Representative photomicrographs of CD83+ dendritic cell immunostaining in control tonsil tissue and B) in normal bronchial epithelium and lamina propria (black arrow) X200, X400 magnification. C) number of CD83+ cells within the epithelium in healthy controls and asthmatics as a whole and as subgroups, D) number of CD83+ cells within the lamina propria in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

5.3 OX40L and OX40 validation for immunohistochemistry

In response to allergen challenge TSLP is released by the epithelium which in turn activates naïve dendritic cells to mature and polarise naïve T cells towards a Th2 phenotype. The cell surface molecule, OX40 ligand (OX40L) is expressed on dendritic cells and OX40 (CD134) is expressed on T cells. The interaction of OX40L with its receptor OX40 is thought to be important in T cell activation by dendritic cells. To determine whether this T cell/dendritic cell interaction exists in bronchial tissue; mouse monoclonal antibodies to OX40 and OX40L were used to investigate expression in healthy subjects and asthmatics.

Human tonsil tissue was used to titrate antibodies against OX40 and OX40L. The optimal concentration for OX40 was determined at 5 µg/mL and 10 µg/mL for OX40L. Figure 5.3 shows the immunostaining for OX40 and OX40L in tonsil tissue. The mouse isotype control at the corresponding concentrations was clear.

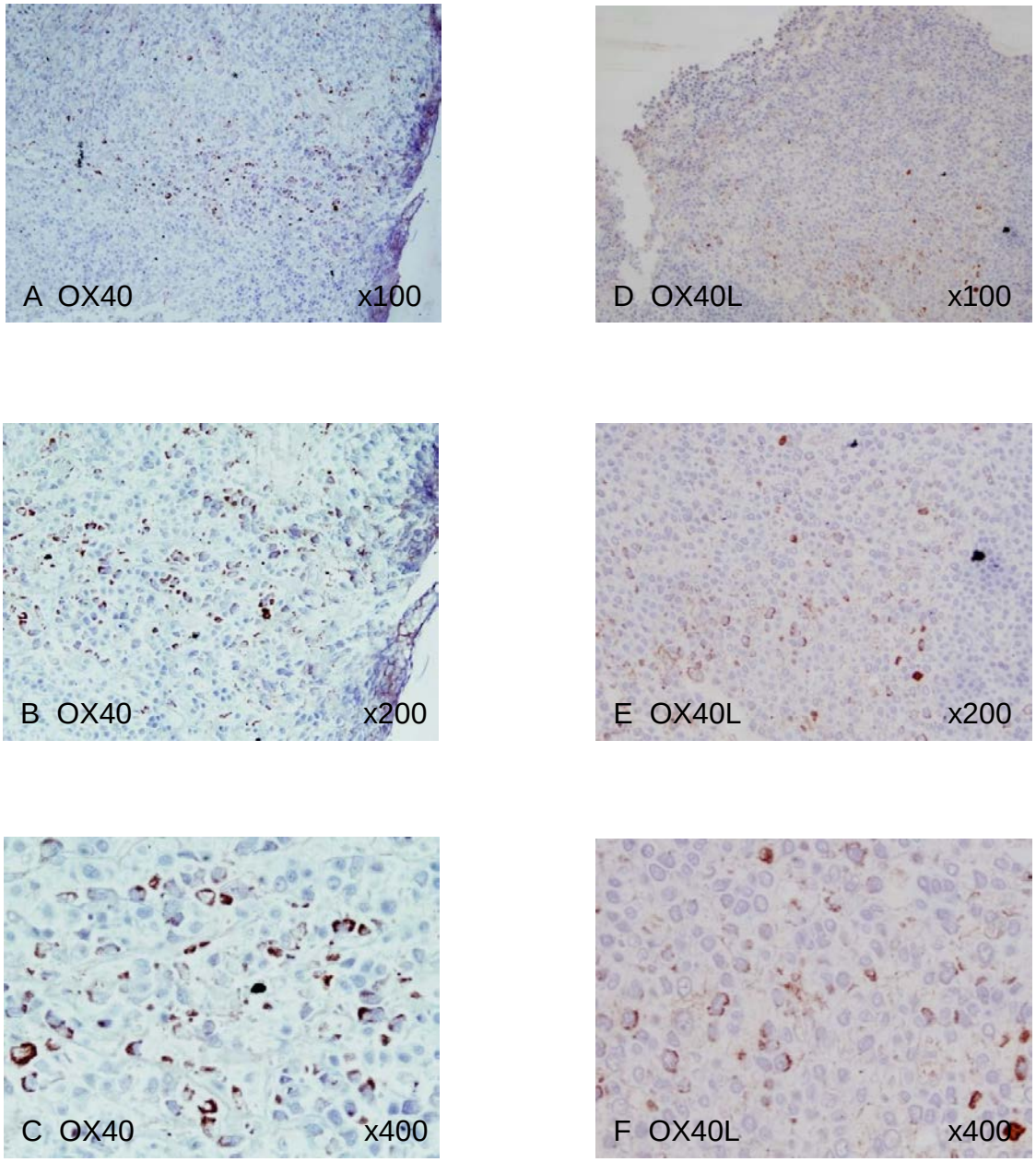


Figure 5.3 OX40 and OX40L validation in human control tonsil tissue

Immunostaining in tonsil tissue for A-C) OX40 and D-F) OX40L at X100, X200 and X400 magnification respectively.

5.4 OX40L and OX40 expression is sparse and is not elevated in asthmatic human airway tissue

In the airways, immunostaining for OX40L and OX40 was relatively sparse and data from all asthma groups is presented together with cell counts for CD1a and CD83 (Table 5.1). In spite of robust staining in tonsil, very sparse cellular staining for OX40 and OX40L was evident in the airways. There was also no significant difference between asthmatic patients and healthy control subjects (Table 5.1).

	Healthy control	**Asthma**
Epithelium cells/mm^2		
CD1a	1.3 [0.0-7.4]	0.0 [0.0-0.0]
CD83	0.0 [0.0-2.8]	2.1 [0.0-6.0]
OX40	0.0 [0.0-0.0]	0.0 [0.0-0.0]
OX40L	0.0 [0.0-0.0]	0.0 [0.0-1.0]
Lamina propria cells/mm^2		
CD1a	0.0 [0.0-1.2]	**0.6*** [0.0-1.2]
CD83	1.2 [0.6-2.2]	1.8 [0.8-3.1]
OX40	0.0 [0.0-0.8]	0.6 [0.4-0.7]
OX40L	0.1 [0.0-0.6]	0.4 [0.0-0.9]

Table 5.1 Summary of dendritic cell, OX40 and OX40L cell counts in the airway epithelium and lamina propria.

Median [interquartile range]. * p <0.05 compared to healthy control subjects

5.5 Discussion

T cells in particular Th2 cells can be induced to express OX40 and OX40L is inducible in dendritic cells as well as B cells and macrophages. Animal models propose that TSLP contributes to the immunopathology of asthma through the activation of the OX40L/OX40 pathway, leading to the generation of Th2 T cells. Seshasayee *et al*. (Seshasayee *et al.*, 2007) reported eosinophilic infiltration and IgE production can be inhibited by neutralising OX40L activity in mice and rhesus monkeys. Furthermore the inhibition of OX40L attenuated TSLP mediated Th2 inflammation. Another study reported OX40 knockout mice challenged with ovalbumin had significantly reduced lung inflammation, mucous secretion, eosinophilia, goblet cell hyperplasia and Th2 responses compared to wildtype mice (Jember *et al.*, 2001).

In contrast, the evidence for an OX40/OX40L pathway in humans is limited. Initial studies demonstrate peripheral blood derived CD11+ dendritic cells express OX40L mRNA and neutralising OX40L activity inhibits Th2 cytokine production in TSLP primed dendritic cells demonstrating OX40L expression on dendritic cells is critical for dendritic cells to initiate Th2 responses (Ito *et al.*, 2005). Airway smooth muscle cells have been reported to produce low levels of OX40L which is upregulated upon TNF-stimulation and bronchial epithelial cells reportedly express OX40L upon exposure to diesel exhaust fumes (Bleck *et al.*, 2010; Burgess *et al.*, 2004; Burgess *et al.*, 2005). There has therefore been great interest in targeting the OX40L/OX40 pathway in asthmatic patients (Rochman & Leonard, 2008).

However, despite clear OX40 and OX40L staining in human tonsil control tissue, staining for these cellular markers in the airways was notably sparse, and I could not find any evidence for increased OX40L or OX40 expression in asthmatic patients. Another study suggested that OX40 and OX40L expression was increased in patients with mild asthma compared with that seen in healthy subjects and patients with severe asthma, but the numbers of cells present were very small (Siddiqui *et al.*, 2010). Despite reports of OX40L expression by ASM (Burgess *et al.*, 2004) I did not observe OX40L expression in healthy controls or asthmatics. In parallel, no significant increase in the number of dendritic cells displaying the activation marker CD83 in asthmatic patients was found.

In summary OX40L and OX40 expression were minimal suggesting that OX40L and OX40 are unlikely to play significant roles in persistent airway inflammation in the bronchial mucosa. However, given the current understanding of the dynamics of dendritic cell–mediated T-cell stimulation, it remains possible that OX40L/OX40 interactions take place in secondary lymphoid organs, such as mediastinal lymph nodes, which unfortunately could not be sampled in this study.

CHAPTER 6.

Th2 cytokine expression in human asthmatic airway tissue

6.1 Th2 cytokines in the airways

There is substantial evidence that Th2 cytokines play an important role in the development of eosinophilic airway inflammation in asthma. TSLP expression and function has been associated with Th2 cytokine activity (see section 1.11.5.5 in introduction). To investigate whether IL-4 and IL-13 expression is related to TSLP expression in asthmatic bronchial tissue, immunostaining for IL-4 and IL-13 was undertaken.

6.2 IL-4 expression in human asthmatic airway tissue

The 3H4 mouse mAb against human IL-4 was used for immunostaining based on previous work by our group (Bradding *et al.*, 1994). The 3H4 clone probably binds to secreted IL-4 which can be seen as peripheral ring staining (Bradding *et al.*, 1994). The 3H4 monoclonal antibody stained more mast cells in the epithelium and submucosa of mild steroid-naïve asthmatics compared to healthy controls in previous studies (Bradding *et al.*, 1994).

Due to consumption of tissue, the numbers of donors available for studying the Th2 cytokines were reduced, particularly in the mild asthma group. Epithelial tissue for IL-4 analysis was available from 9 healthy subjects, and 3 mild, 6 moderate and 12 severe asthmatic subjects. Lamina propria tissue was available for analysis from 10 healthy and 4 mild, 11 moderate and 14 severe asthmatic subjects.

IL-4 immunostaining in the airway epithelium was sparse, similar in all subgroups and not different between asthma and healthy controls (Figure

6.1.B). The number of IL-4 positive cells in the airway lamina propria was elevated in asthmatic subjects as a whole compared to healthy controls although this did not quite reach statistical significance (p=0.0525). Subgroup analysis demonstrated that the increased expression was due to significantly increased levels in mild and not severe asthma as described previously (Figure 6.1.C) (Bradding *et al.*, 1994) (Vrugt *et al.*, 1999).

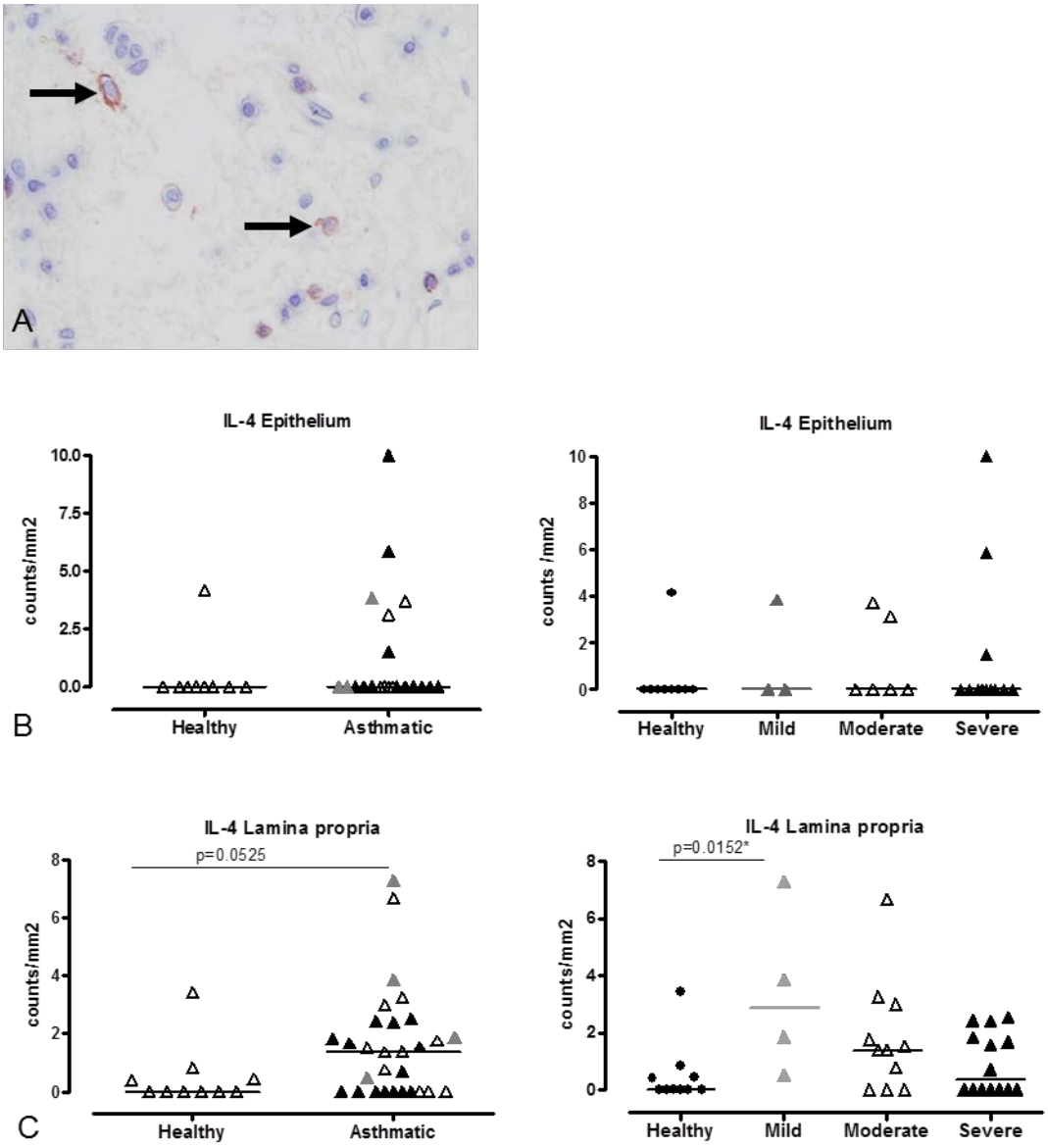


Figure 6.1 The expression of IL-4+ cells in bronchial biopsies

A) Representative photomicrographs of IHC showing IL-4 staining (black arrow) in human bronchial tissue X400 magnification. B) number of IL-4 positive cells within the epithelium and C) lamina propria in healthy controls and asthmatics as a whole and as subgroups. Lamina propria Kruskal-Wallis p=0.0478 Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

6.3 IL-13 expression in human asthmatic airway tissue

6.3.1 IL-13 validation for immunohistochemistry

IL-13 was detected in tissue using an anti-IL-13 antibody that was generated in-house at Genentech,Inc., San Francisco. The antibody was raised against formalin-fixed IL-13 protein. The antibody was titrated at concentrations ranging from 1 µg/mL to 20 µg/mL; 5 µg/mL was considered optimal. Rabbit IgG antibody isotype control sections were negative for immunostaining suggesting the staining was valid and not caused by non-specific immunoglobulin interactions.

Once the optimal concentration was determined the specificity of the antibody was investigated further. An absorption control assay prior to IHC was used to show that the antibody bound to specific antigens in the tissue. Recombinant IL-13 was pre-incubated with the IL-13 antibody at a 10:1 molar ratio to inactivate the antibody which inhibited immunostaining (Figure 6.2).

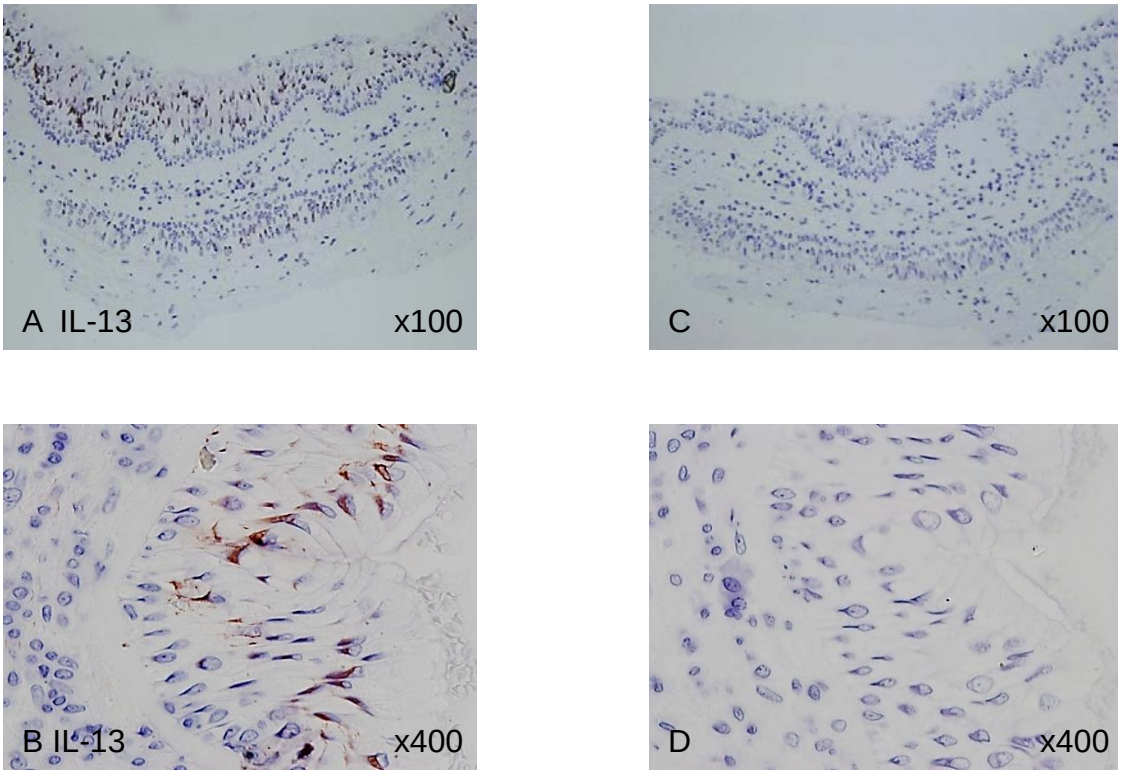


Figure 6.2 IL-13 immunoreactivity in the asthmatic airway.

A) and B) IL-13 immunostaining in the airway epithelium from a patient with asthma C) and D) which is inhibited by recombinant IL-13.

A) and C) X100 magnification

B) and D) X400 magnification.

6.3.2 Assessment and quantification of IL-13 immunostaining

Epithelial tissue for IL-13 analysis was available from 9 healthy subjects, and 2 mild, 6 moderate and 11 severe asthmatic subjects. Lamina propria tissue was available for analysis from 10 healthy and 4 mild, 11 moderate and 14 severe asthmatic subjects.

IL-13 positive cells were evident in both the airway epithelium and lamina propria (Figure 6.2.B and 6.3.A). Within the epithelium, both epithelial cells and non-epithelial cells were stained. The epithelial cell staining was discrete and intracellular. It was located predominantly in the differentiated pseudo-columnar cells (Figure 6.3.A). The non-epithelial cells expressing IL-13 within the epithelium and lamina propria were mononuclear, with a distinct cell-surface staining pattern accompanied by punctuate intracellular staining (Figure 6.3.A). Although it did not reach statistical significance there was an increased number of IL-13 positive cells that were classified as non-epithelial cells within the airway epithelium in the patients with asthma compared with that seen in the healthy control subjects and this increase was restricted to severe asthma subgroup $p=0.0327$. There was no difference in the extent of epithelial cell IL-13 immunoreactivity between asthmatic patients and healthy subjects (Figure 6.3.C).

Within the lamina propria, cells expressing both diffuse cytoplasmic staining and cell surface staining accompanied by punctuate intracellular staining were evident (Figure 6.3.A) however there were no differences across the study groups with respect to lamina propria IL-13 expression (Figure 6.4).

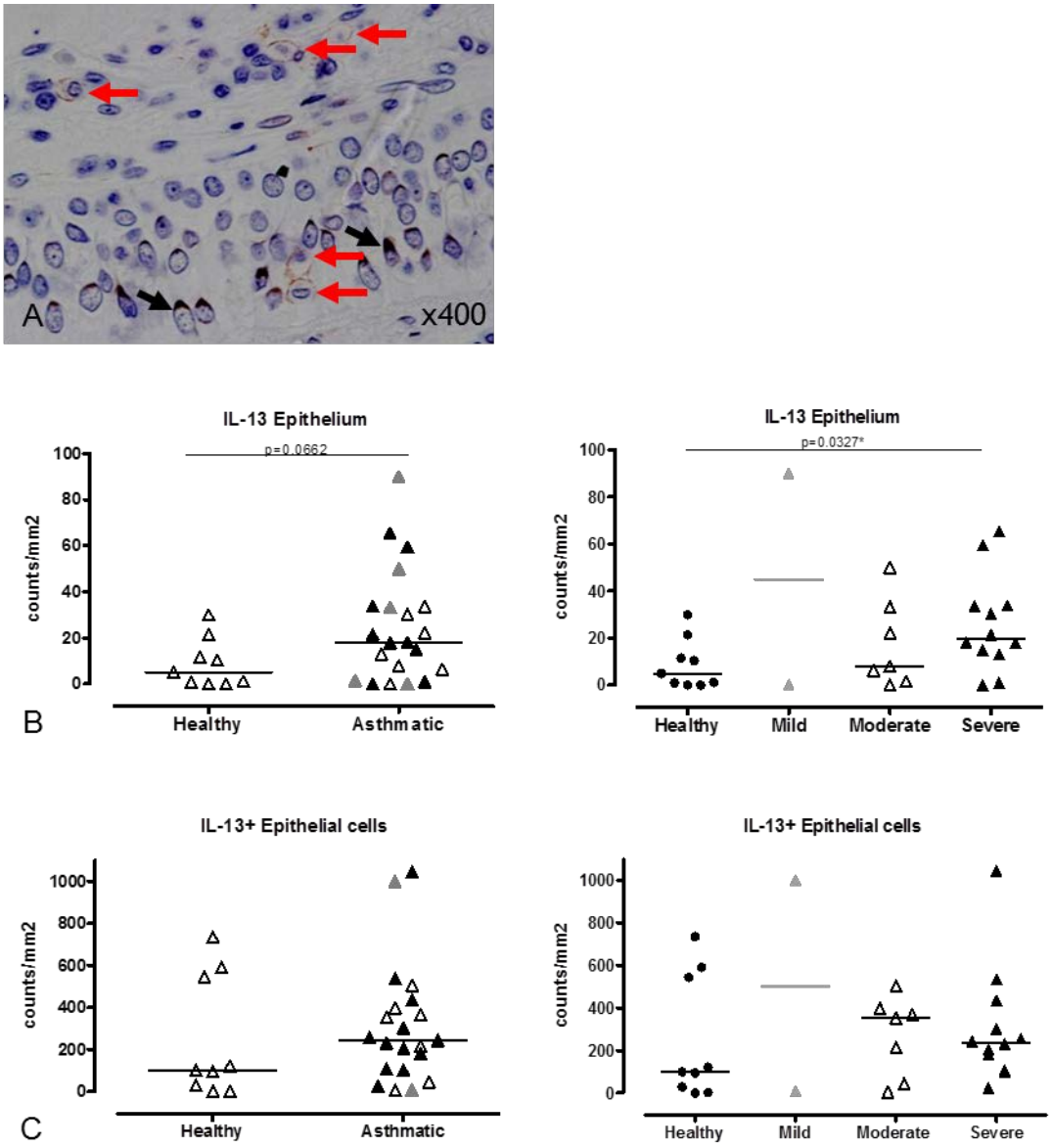


Figure 6.3 IL-13 expression in the airway epithelium

A) black arrows demonstrate IL-13+ pseudocolumnar epithelial cells and red arrows highlight IL-13+ non-epithelial cells. B) the density of IL-13+ non-epithelial cells within the airway epithelium in healthy controls and asthmatics as a whole and as subgroups p=0.033. C) the density of IL-13+ epithelial cells within the airway epithelium in healthy controls and asthmatics as a whole and as subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

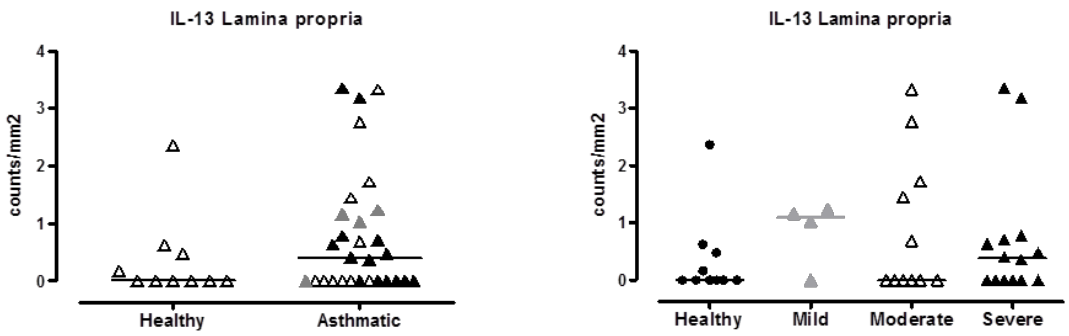


Figure 6.4 IL-13 immunostaining in airway lamina propria

The density of IL-13+ cells in the lamina propria of healthy controls and asthmatics as a A) whole and as B) subgroups. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma

6.4 IL-13 detection in sputum

IL-13 protein expression was elevated in non-epithelial cells within the epithelium in severe asthmatic bronchial tissue compared to healthy control subjects. Whether this expression extended to sputum and whether it correlated to bronchial expression was the next question to address. Sputum samples were available for the subjects that underwent bronchoscopy. IL-13 was measured using a commercial ELISA kit.

The IL-13 ELISA (Ebiosciences, UK) had previously been validated by our group (Berry *et al.*, 2004). They reported dithiothreitol (DTT) affected the recovery of IL-13 using this kit; however after dialysis they were able to recover 90% (Berry *et al.*, 2004). Our group has previously used 10kDa Spectra/Por DispoDialyzer which has been discontinued. They were replaced with Spectra/Por Float-a-lyzer G2. The effectiveness of the float-a-lyzer was compared with the dispodialyzer. The top IL-13 concentration was dialysed for 18 hours against PBS by using both dialysers. The recovery of the standards was compared with standards that were not dialysed (Figure 6.5).

The IL-13 standard was recovered after dialysis using the DispoDialyzer confirming previous published data. However the IL-13 standard could not be recovered using the replacement Float-a-lyzer G2 and any sputum measurements using this dialyser were unreliable. Therefore analysis of IL-13 in sputum could not be undertaken in this study.

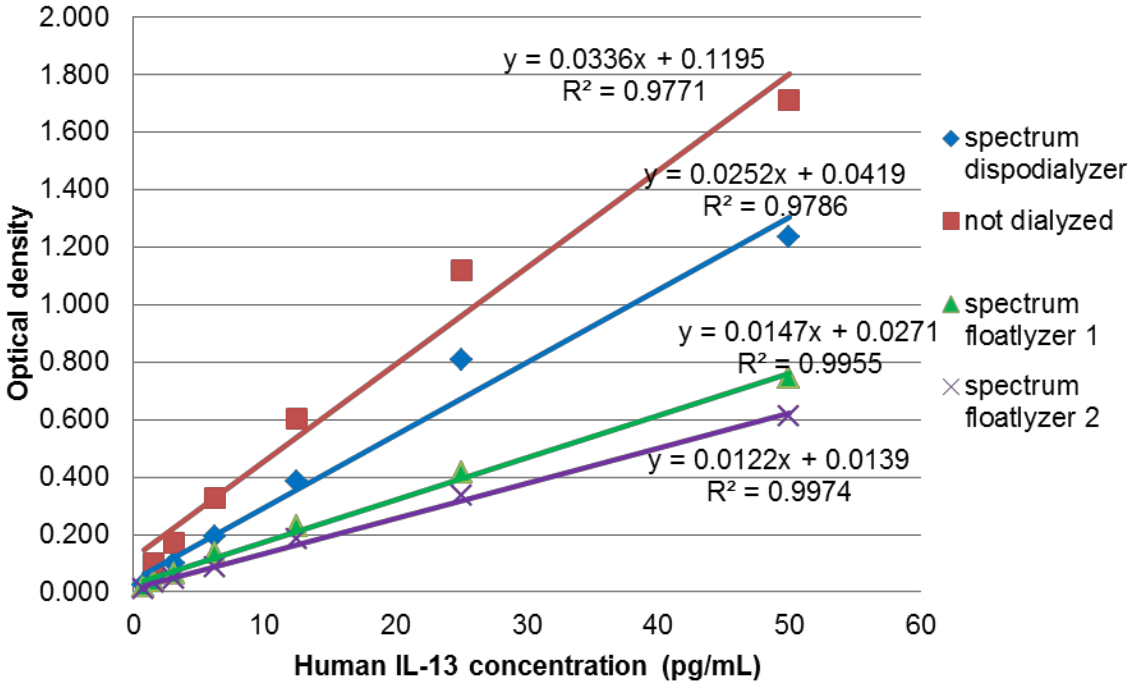


Figure 6.5 Effect of dialysis on the IL-13 standard using commercial dialysers

6.5 Relationships between TSLP and Th2 immunoreactivity

Taken continuously, staining patterns for IL-4, IL-13, and TSLP did not show any significant intercorrelations (data not shown). However, emerging evidence suggests that there might be discrete molecular and pathophysiological phenotypes of asthma. Examination of IL-4 and IL-13 immunostaining in the lamina propria (Figure 6.1.C and 6.4) revealed that these cytokines were detectable in only a subset of asthmatic patients in the study. Similarly, increased levels of TSLP staining in the lamina propria were observed only in a subset of patients with severe asthma (Figure 4.7). Therefore taken categorically as defined by the presence or absence of IL-13^{+} cells in the lamina propria, I found that the IL-13^{+} subjects show significant enhancement of both IL-4 (Figure 6.6.A) and TSLP (Figure 6.6.B) immunoreactivity in the lamina propria, suggesting that in terms of both TSLP and Th2 cytokine protein expression in bronchial tissue, there might be discrete “Th2-high” and “Th2-low” phenotypes of asthma.

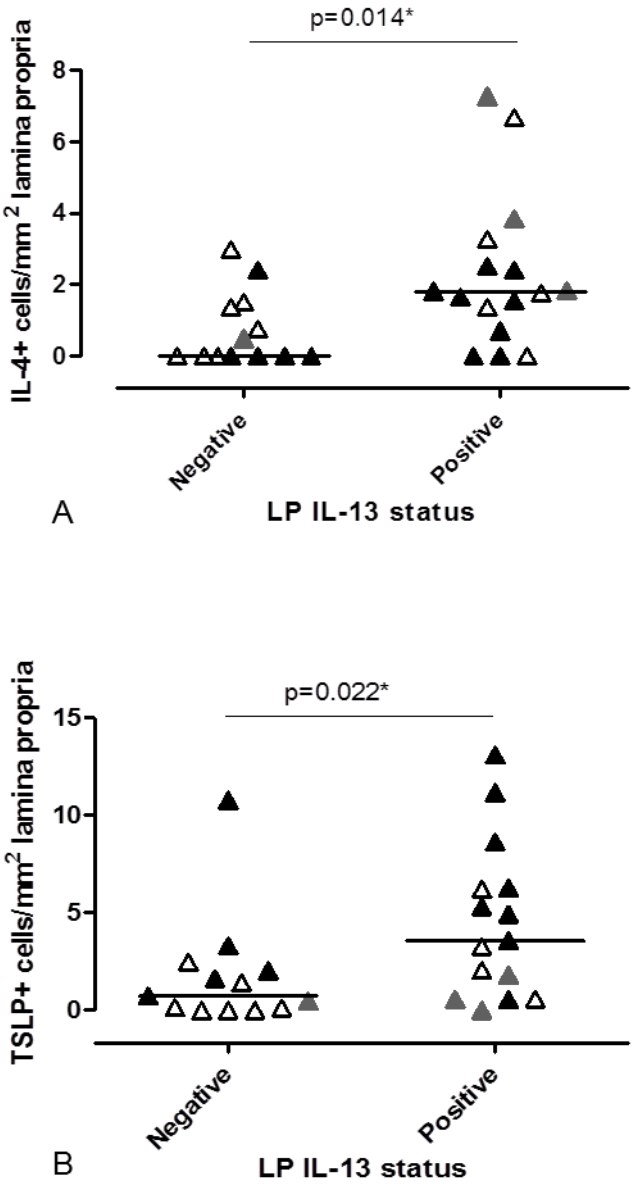


Figure 6.6 Relationships between IL-13, IL-4, and TSLP expression in the lamina propria in asthmatic patients

IL-13 immunoreactivity in the lamina propria was scored as absent or present; these categorical descriptions of IL-13 immunoreactivity were compared against A) IL-4 and B) TSLP immunoreactivity in the lamina propria. Grey symbols, mild asthma; white symbols, moderate asthma; black symbols, severe asthma. IL-4 p=0.014 and TSLP p=0.022.

6.6 Discussion

In keeping with previous work in asthmatic patients, IL-4 expression was suppressed in patients with severe asthma compared with that seen in patients with mild asthma (Vrugt *et al.*, 1999). Interestingly, IL-13 protein was detected in the airway epithelium in patients with severe asthma in cells that were not epithelial cells but was also detected in columnar epithelial cells. This latter finding is novel and was not described in previous studies of IL-13 expression in asthmatic patients using a different anti–IL-13 antibody (Saha *et al.*, 2008; Berry *et al.*, 2004).

The current study used an in-house–generated IL-13 rabbit polyclonal antibody. Specificity of immunostaining was confirmed by using isotype controls and immunoabsorption with recombinant human IL-13, suggesting that the staining is unlikely to be artifactual. Two recent studies have described the expression of IL-13 mRNA and secretion of IL-13 protein in submerged monolayers of primary human airway epithelial cells, suggesting that they might produce this cytokine (Allahverdian *et al.*, 2008; Semlali *et al.*, 2010). However in this study *in vitro* data (Chapter 8) shows that IL-13 could not be detected in bronchial epithelial cells. Therefore an alternate explanation for the immunostaining in this study is that IL-13 within airway epithelial cells represents IL-13 that has been taken up rather than synthesized. The cellular distribution of IL-13 in our current study was different than that described previously (Saha *et al.*, 2008) in that most nonepithelial IL-13 positive cells in the airway epithelium and lamina propria were not mast cells, T cells, or eosinophils.

Unfortunately due to experimental design difficulties sputum IL-13 could not be measured in this study. Sputum is processed using mucolytic DTT which has previously been shown to impair the detection of sputum IL-13 (Figure 6.7). Berry *et al.* (Berry *et al.*, 2004) assessed the effect of DTT on the IL-13 standards and were only able to recovery the standards after dialysis.

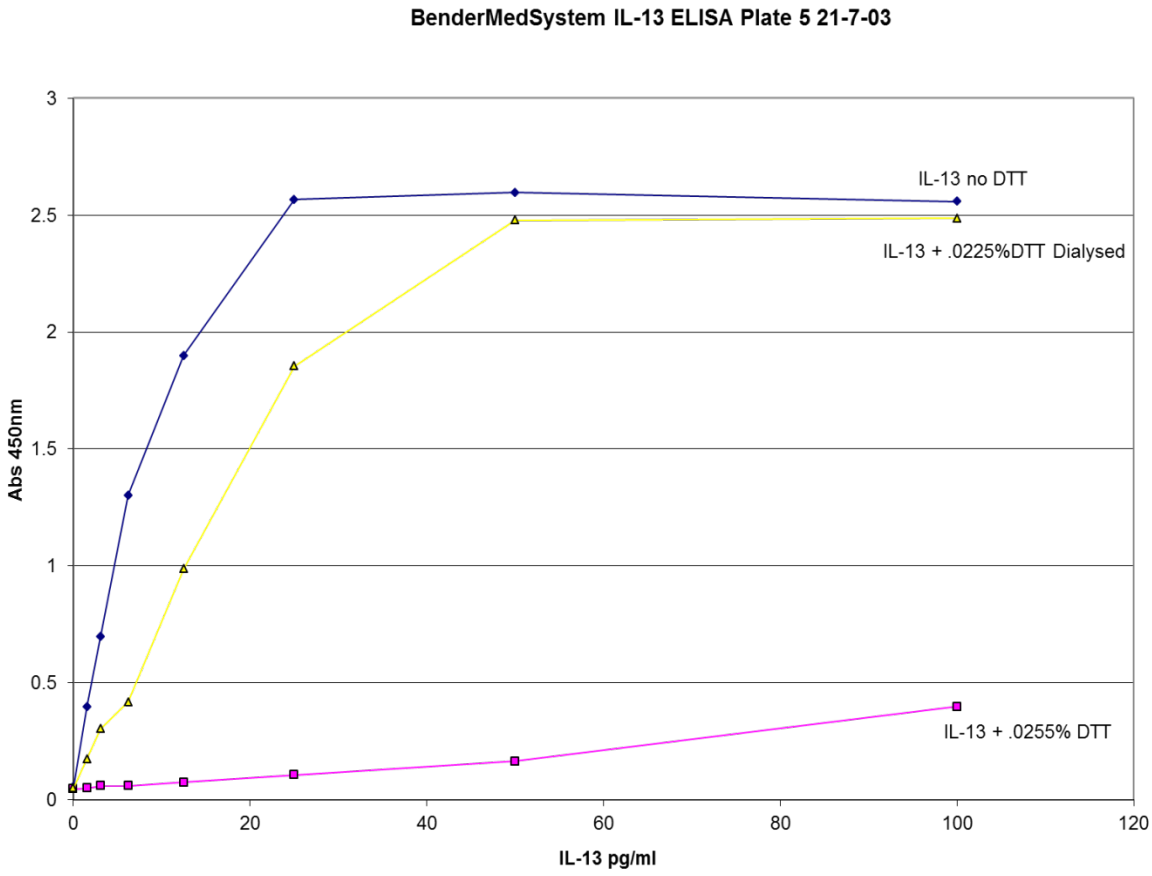


Figure 6.7 Effect of DTT on IL-13 standard

IL-13 standard is inhibited with the addition of 0.0255% DTT (concentration that is added to sputum). IL-13 standard is recovered after dialysing for a minimum of 12 h prior to measurement by ELISA using a 10kDa dispodialyzer (Spectra). Figure kindly provided by Berry *et al.* (Berry *et al.*, 2004).

There was considerable variation in the recovery of IL-13 using different dialysers and I was unable to recover IL-13 to a suitable level using the replacement 10kDa Spectropor dialyser. However increased IL-13 expression in the nonepithelial cells within the epithelial compartment in patients with severe asthma supports the previous observation that IL-13 protein concentrations in induced sputum are increased in a subgroup of patients with severe refractory asthma (Saha *et al.*, 2008). Interestingly, it is again evident that there is a subgroup of patients with severe asthma in whom IL-13 expression persists and a group in whom it is suppressed to levels similar to those seen in healthy subjects. This has important implications for studies examining the efficacy of anti–IL-13 therapy in patients with severe asthma. In the IL-13–low group, it is unlikely that IL-13 will be driving their disease, and anti–IL-13 therapy might be ineffective. Therefore it might be necessary to target this therapy to patients in whom there is evidence of ongoing IL-13 expression.

This study shows there was considerable heterogeneity in the levels of TSLP, IL-13, and IL-4 staining across the cohort of asthmatic patients examined, which is consistent with previous reports demonstrating subsets of asthmatic patients with variable levels of Th2 inflammation (Woodruff *et al.*, 2009). In general, subjects with increased IL-13 immunostaining in the lamina propria also had increased IL-4 and TSLP immunostaining. Bronchial biopsies obtained in this study were used to study TSLP gene expression in collaboration with Genentech Inc. and eosinophilic inflammation with Queens University Belfast. This collaboration revealed a significant correlation between TSLP gene expression, a Th2 gene expression signature, and eosinophilic inflammation in

bronchial biopsies obtained in this thesis (Shikotra *et al.*, 2012). Taken together with the established roles of TSLP in mediating both adaptive and innate IL-13–driven inflammation, these findings suggest that TSLP is indeed associated with the “Th2-high” sub-phenotype of asthma.

CHAPTER 7.

Innate Lymphoid Cells 2.

A candidate source of

IL-13 in bronchial

biopsies

7.1 Cellular sources of IL-13 in human asthmatic airway tissue

Non-epithelial cells within the epithelium and similar cells in the lamina propria expressed IL-13 but the origin of these cells in these biopsies was unclear.

In addition innate lymphoid cells 2 (ILC2) have recently been described as source of IL-13 (Neill *et al.*, 2010). ILC2 express a cocktail of cell surface markers including CD45, ICOS, IL-33 receptor T1/ST2, C-kit and IL-25 receptor IL-17BR (Neill *et al.*, 2010). They are termed linage negative as these and other surface marker combinations cannot assign these cells to hematopoietic cell lineages. IL-25 and IL-33 induce ILC2 to release IL-5 and IL-13 during helminth *nippostrongylus brasiliensis* infection in the gut (Neill *et al.*, 2010; Fallon *et al.*, 2006) and in response to various stimuli including TSLP in the lung (Halim *et al.*, 2012). Identifying the cellular sources of the IL-13 expression I have seen in bronchial biopsies is important and whether ILC2 have a role in asthma is unclear. Therefore IL-13 positive cells were co-localised to various cell types in bronchial biopsies.

7.2 IL-13 co localised to T cells, Mast cells and eosinophils

The expression of IL-13 was investigated by immunostaining 2 µm sequential sections for IL-13, T cells, mast cells and eosinophils from asthmatic bronchial biopsies (Figure 7.1). Co-localisation of cell markers demonstrated that IL-13 was localised to less than 2% of T cells and mast cells and that these accounted for less than 4% of the IL-13+ non-epithelial cells. IL-13 did not co-localise to eosinophils (Figure 7.1 and Figure 7.2).

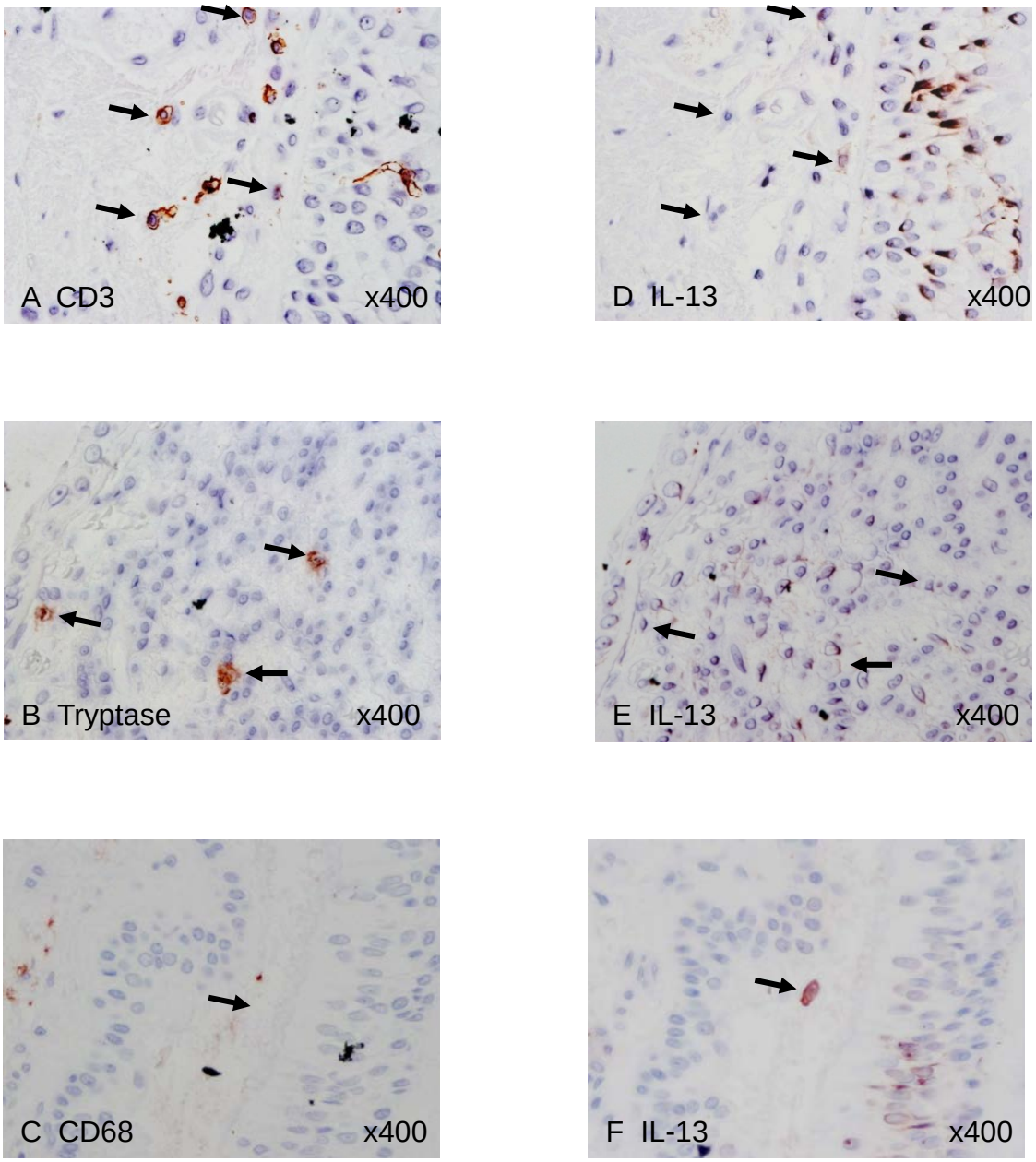


Figure 7.1 Photomicrographs of the cellular sources of IL-13 in the lamina propria of asthmatic airways

IL-13 colocalisation D-F) to A) T cells B) mast cells and C) macrophages within the lamina propria of bronchial biopsies (arrows X400 magnification)

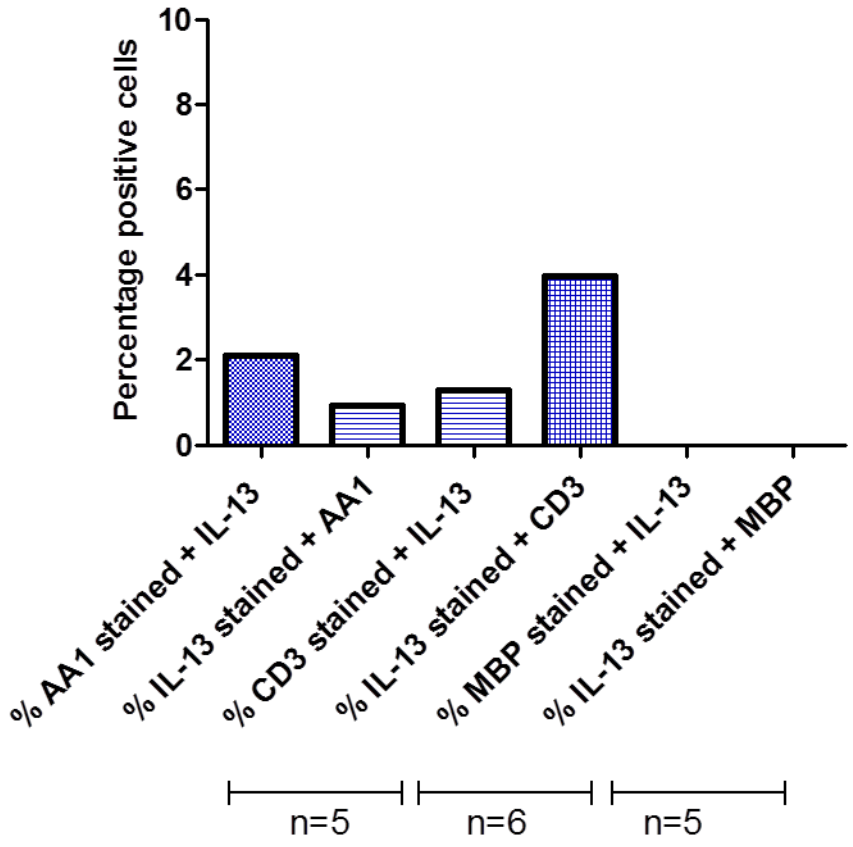


Figure 7.2 Cellular sources of IL-13 within the lamina propria of asthmatic airways

The percentage of T cells, mast cells and macrophages, and *vice versa* expressing IL-13 in a subset of severe asthmatic subjects.

7.3 IL-13 colocalises to CD45

To determine whether ILC2 were a viable source of IL-13 in human bronchial tissue, IL-13 sections were colocalised to CD45 (Figure 7.3.A and B). 70.2% ± 12.4% of the IL-13 positive non-epithelial cells in the airway epithelium were also CD45 positive cells. CD45 positive cells accounted for 36.4% ± 2.9% of the IL-13 positive cells in the lamina propria (Figure 7.3.C). This data suggested that these IL-13+ cells may be the human equivalent of murine ILC. To further examine the identity of IL-13 positive cells, additional ILC2 markers were sourced.

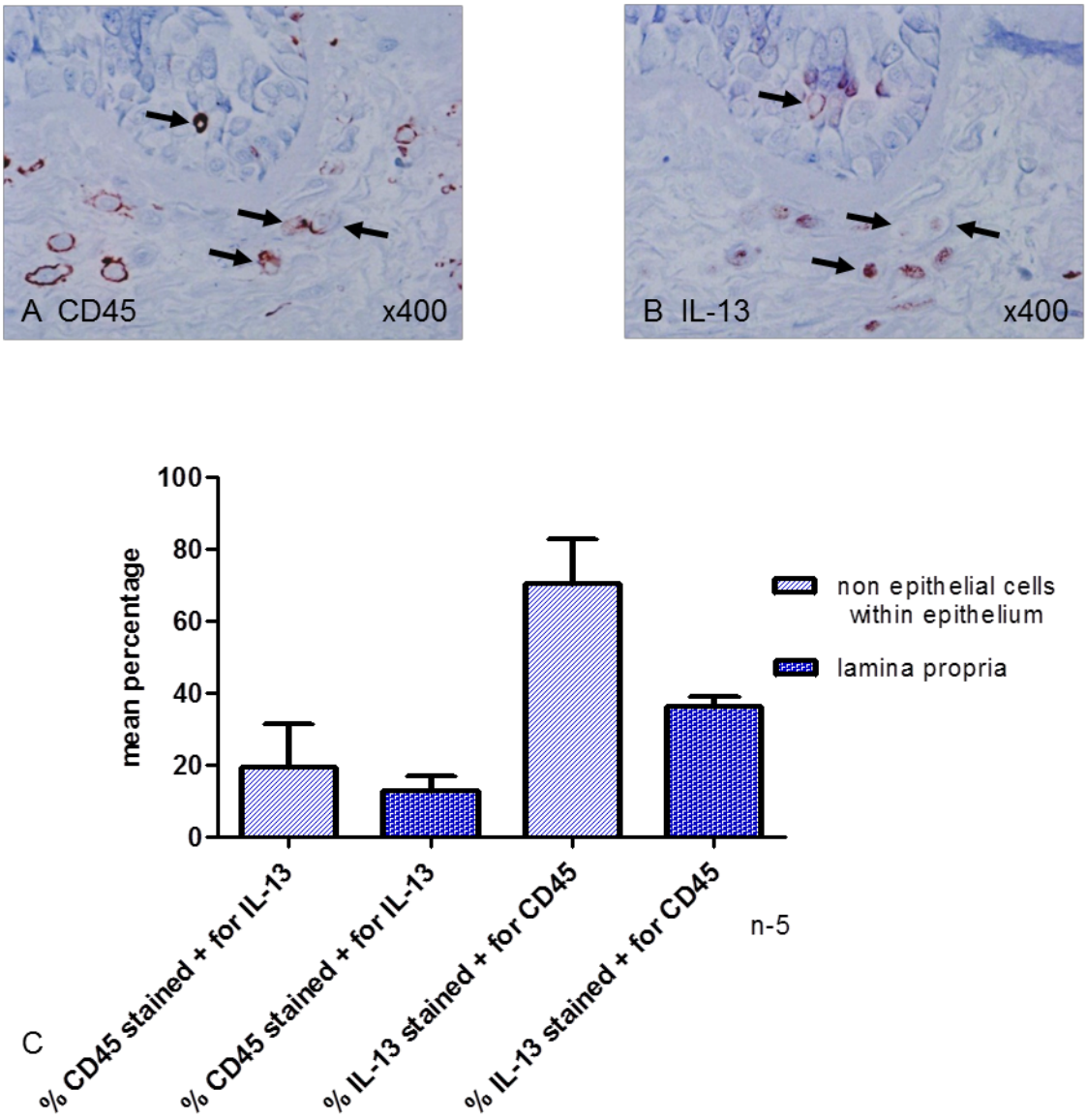


Figure 7.3 IL-13 colocalises to CD45 in asthmatic airways

A) B) Representative photomicrographs of IL-13 colocalisation to CD45 within the lamina propria and epithelium of bronchial biopsies. C) the percentage of CD45 positive cells, co-localised with IL-13 in a subset of 5 severe asthmatic subjects (arrows) X400 magnification.

7.3.1 Nuocyte marker validation for immunohistochemistry

Antibodies to ICOS, IL-33 receptor T1/ST2, Kit (CD117) and IL-25 receptor IL-17BR were used to identify ILC2 as a source of IL-13. Inducible T-cell Co-Stimulator (ICOS) belongs to the CD28 cell-surface receptor family. It is a co-stimulatory molecule that is rapidly induced upon T cell activation. ST2 is a member of the interleukin 1 receptor family which binds to IL-33. It is expressed by fibroblasts, mast cells, and Th2 cells. Th1 cells do not express ST2. Kit is the receptor for stem cell factor expressed on the surface of hematopoietic stem cells, melanocytes and mast cells. It binds to its ligand stem cell factor (SCF) which is a haemopoietic growth factor that supports the proliferation of multiple hematopoietic cell lines (Ashman, 1999; Vose & Armitage, 1995). Interleukin-17 receptor B (IL-17BR) is a receptor that specifically binds to proinflammatory cytokines IL17B and IL17E (IL-25) (Lee *et al.*, 2001a). Its expression has been colocalised to eosinophils, mast cells, endothelial cells and T cells (Corrigan *et al.*, 2011).

Nuocyte marker antibodies had not previously been used for immunostaining GMA-embedded tissue therefore the commercial antibodies were initially titrated to determine the optimal concentration. Tonsil tissue was used as a positive control and appropriate isotypes were used as a negative control. At optimal concentrations both bronchial and tonsil tissue exhibited immunoreactivity for each of these markers (Figure 7.4 to 7.7).

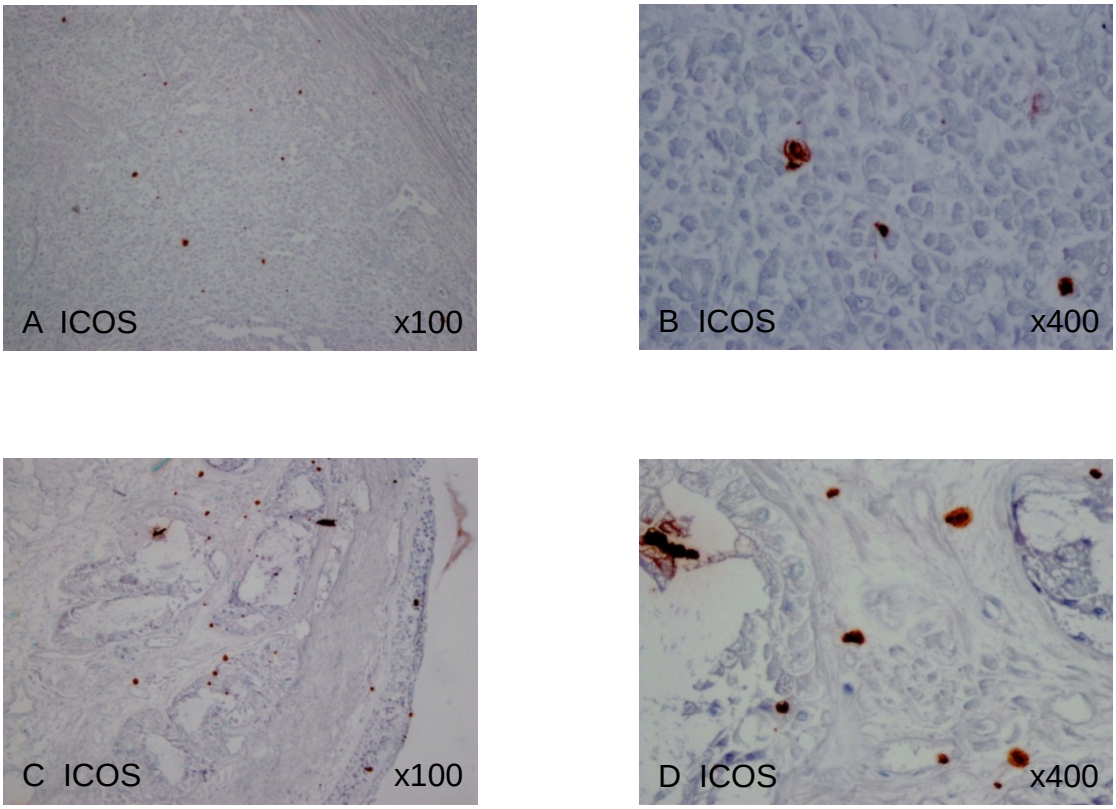


Figure 7.4 ICOS expression in human tonsil tissue

Representative photomicrographs of ICOS immunostaining at 15 µg/mL in A, B) tonsil and C, D) bronchial tissue. X100, X400 magnification respectively

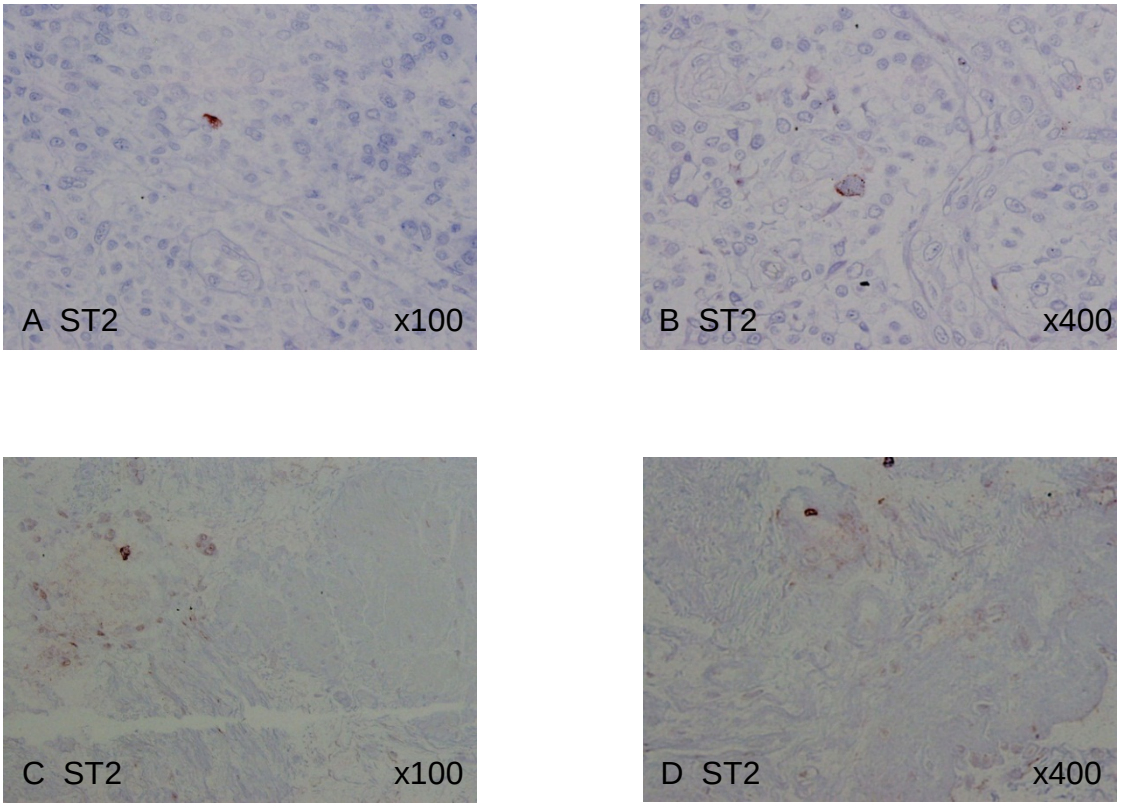


Figure 7.5 ST2 expression in human tonsil tissue

Representative photomicrographs of ST2 immunostaining at 15 µg/mL A, B) tonsil and C, D) bronchial tissue. X100, X400 magnification respectively

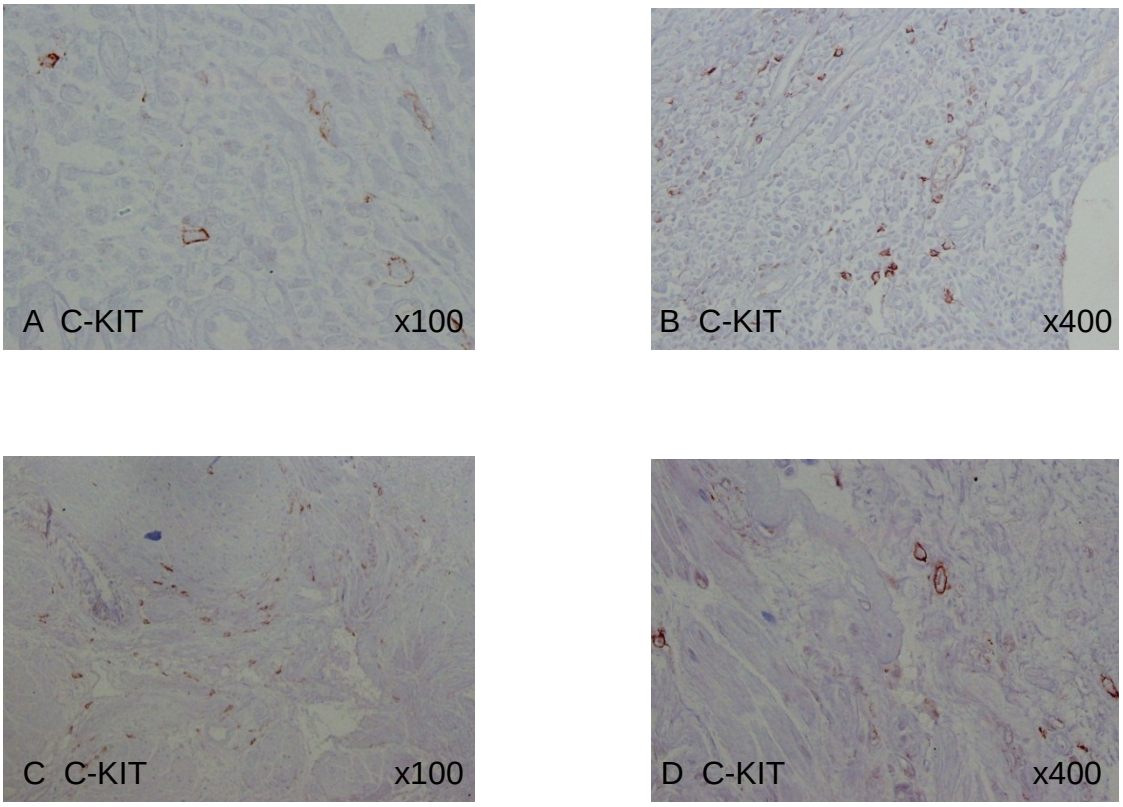


Figure 7.6 Kit expression in human tonsil tissue

Representative photomicrographs of Kit immunostaining at 5 µg/mL in A, B) tonsil and C, D) bronchial tissue. X100, X400 magnification respectively

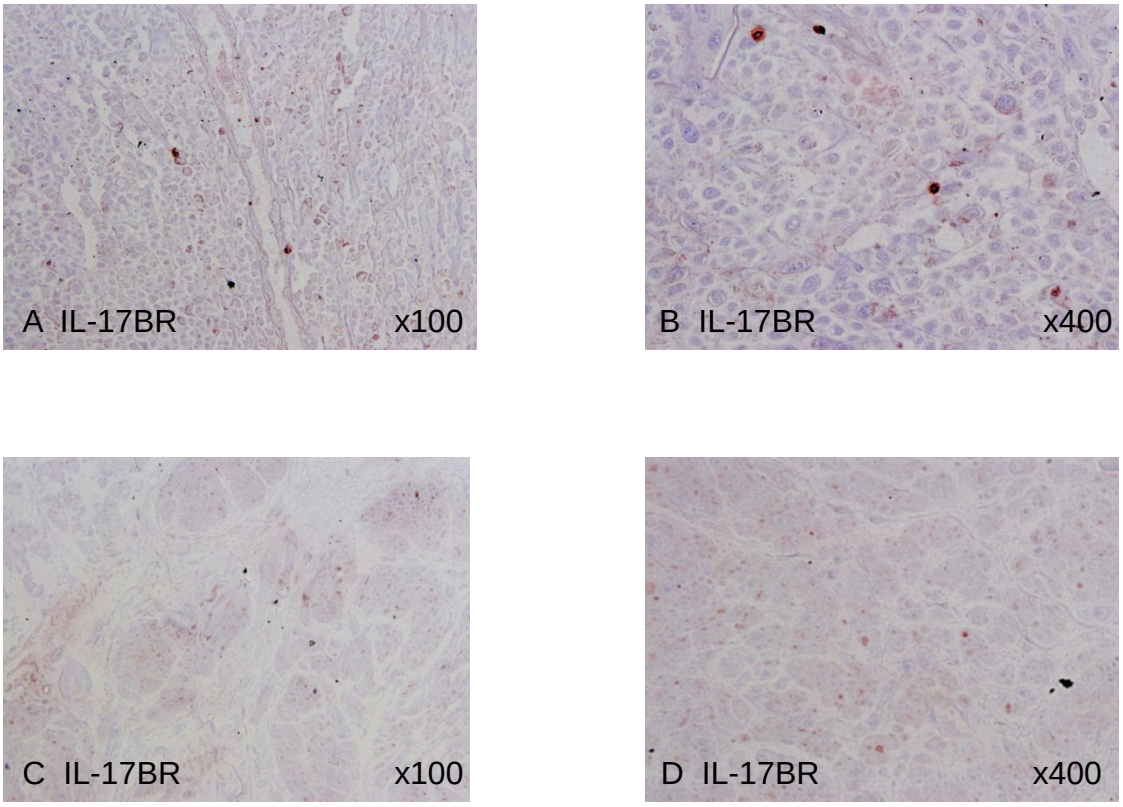


Figure 7.7 IL-17BR expression in human tonsil tissue

Representative photomicrographs of IL-17BR immunostaining at 5 µg/mL in A, B) tonsil and C, D) bronchial tissue. X100, X400 magnification respectively

ICOS monoclonal antibody gave good clean staining at 15 µg/mL and ICOS positive cells were clearly identifiable (BDBiosciences, UK). The mouse monoclonal ST2 antibody was optimised at 15 µg/mL and was clearly detectable in the control tonsil tissue. Two Kit clones were titrated clone E-1 (Santa Cruz, UK) and clone YB5.B8 (BDBiosciences, UK). Both gave good consistent staining but clone YB5.B8 was used further due to its potency (optimal at the lower concentration of 5 µg/mL). The mouse monoclonal IL-17BR antibody (R and D systems, UK) detected expression in the tonsil and in the bronchus. The antibody was optimal at 5 µg/mL. (Figure 7.4 to 7.7). The corresponding isotype controls were negative. These titrated antibodies were next used to co-localise the CD45/IL-13 positive cells previously identified.

7.3.2 IL-13 colocalises to nuocyte markers in bronchial airways

Figure 7.8 demonstrates the colocalising of nuocyte markers to IL-13 positive cells. Appropriate antibody isotype control sections were negative for IL-13 and ILC2 marker immunostaining. Co-localisation of cells in serial 2 µm sections from a subset of 6 severe asthmatic subjects showed that 29% ± 16% of ST2 positive cells and 10% ± 6% of Kit positive cells expressed IL-13. However only 3% ± 2% of IL-13 positive cells in the lamina propria were ST2 positive and 2% ± 1% were Kit positive. Although 15% ± 11% of ICOS positive cells expressed IL-13, less than 1% ± 0.2% of IL-13 positive cells expressed ICOS. In addition IL-17BR cells represented less than 2% of the total IL-13 positive cells in the lamina propria despite 25% ± 9% of the total number of IL-17BR cells expressing IL-13 (Figure 7.9 and 7.10).

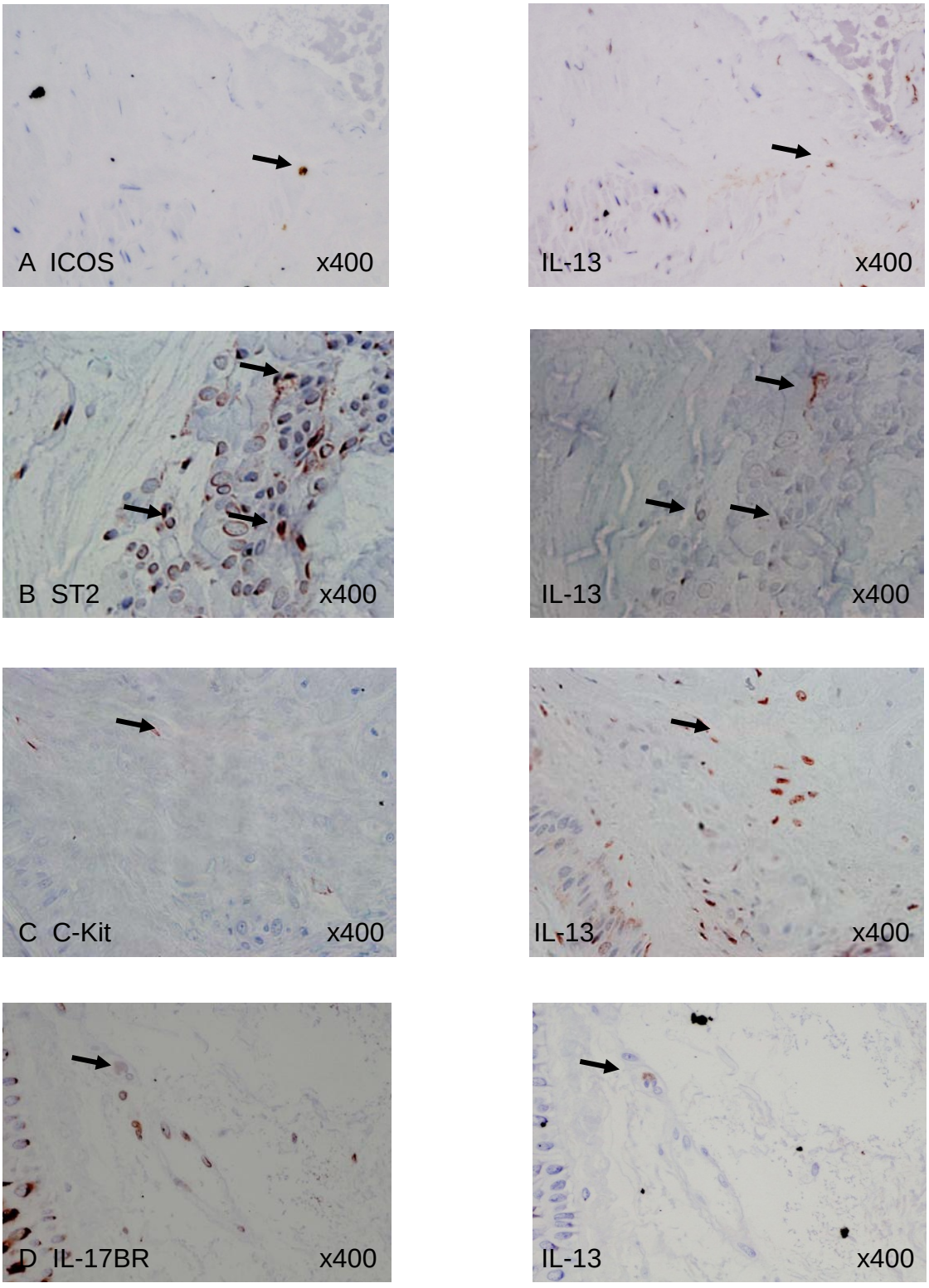


Figure 7.8 Photomicrographs of the IL-13 colocalised to nuocyte markers

Representative photomicrographs of IL-13 colocalisation to A) ICOS 15µg/mL B) ST2 15µg/mL C) C-Kit 5µg/mL and D) IL-17BR 5µg/mL within the lamina propria of bronchial biopsies. X400 magnification respectively.

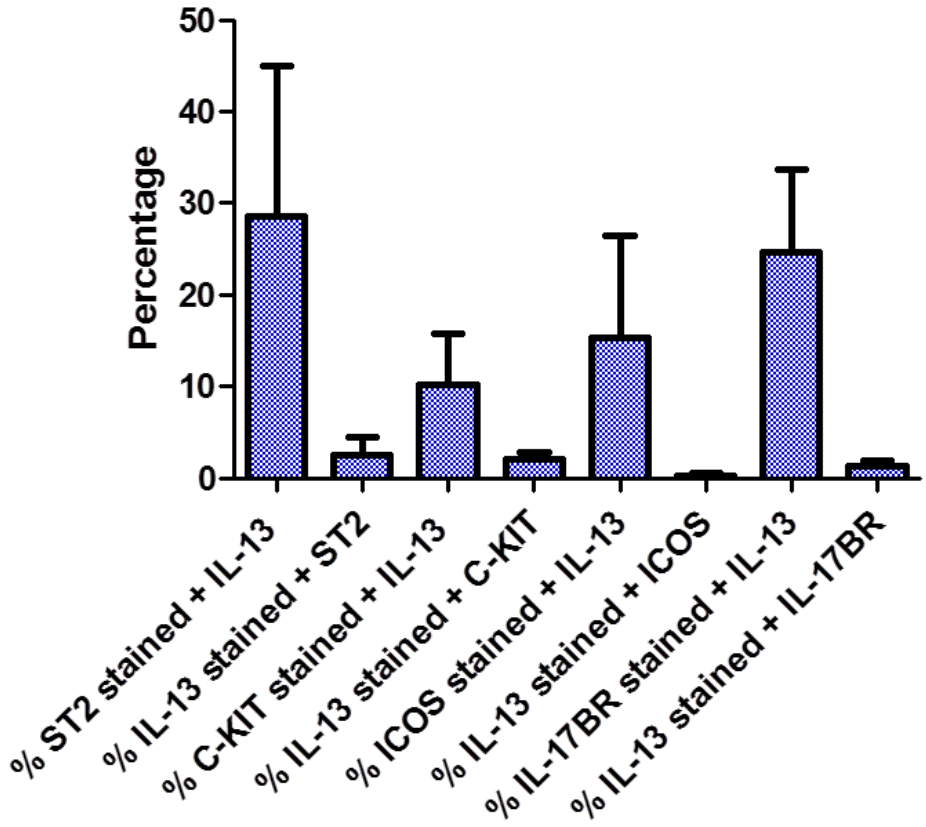


Figure 7.9 IL-13 colocalised to nuocyte markers within the lamina propria of asthmatic airways

The percentage of ST2+, C-Kit+, ICOS+ and IL-17BR+ cells, and vice versa expressing IL-13 in a subset of severe asthmatic subjects. (Mean ± SEM)

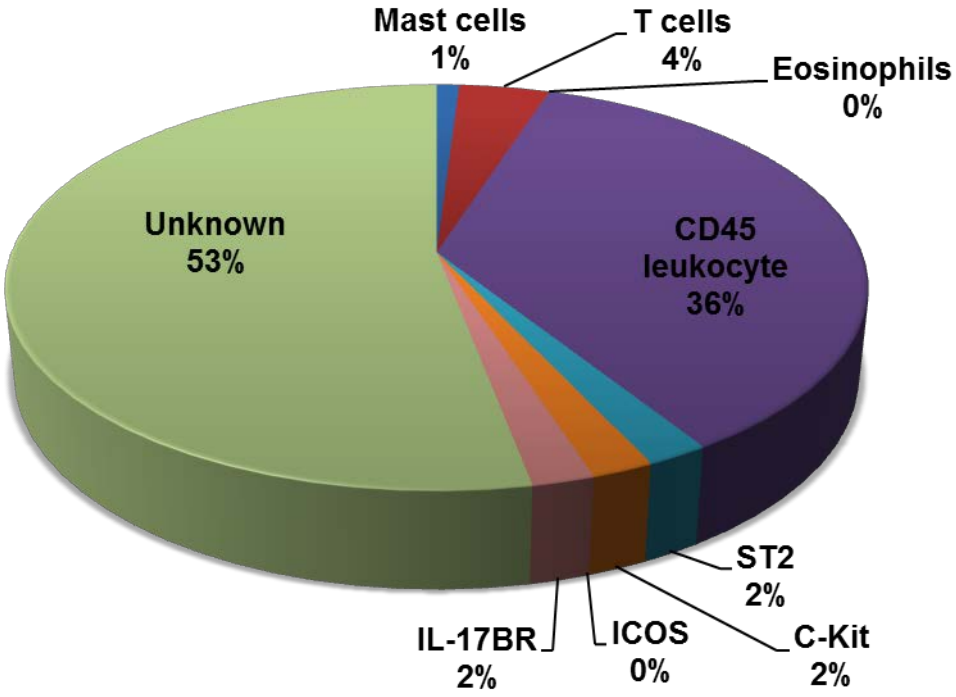


Figure 7.10 Summary of the cells in bronchial tissue expressing IL-13 as percentages of the total number of IL-13+ cells.

7.4 Discussion

The cellular distribution of IL-13 in our current study was different than that described previously (Saha *et al.*, 2008) (Berry *et al.*, 2004) in that most nonepithelial IL-13 positive cells in the airway epithelium and lamina propria were not mast cells, T cells, or eosinophils. Mast cells expressing IL-13 varied across all three studies ranging from 2, 8 and 22% (this study, (Berry *et al.*, 2004) and (Saha *et al.*, 2008)). It is possible that the variation in colocalisation may be due the IL-13 antibody itself. *Saha et al.* used a different antibody to the IL-13 antibody used in this study which may target a different epitope leading to variations in staining. Furthermore I colocalised only severe asthmatics whereas Saha *et al.* colocalised across the study which may reflect differences in expression especially between eosinophilic and non eosinophilic asthma.

The majority of the IL-13 positive cells were positive for CD45, suggesting that they maybe the human equivalent of the recently described CD45 positive murine nuocyte (Neill *et al.*, 2010). Four independent centres have described innate cell populations termed nuocytes, natural helper cells (NHC), innate type 2 helper helper cells (Ih2) and multi-potent progenitor type 2 (MPP^{type2}) cells which have been assigned the nomenclature ILC2 (Neill *et al.*, 2010) (Moro *et al.*, 2010) (Price *et al.*, 2010; Saenz *et al.*, 2010).

Whether these are all the same cell or different subsets is unclear however they all require IL-7 for their development and produce Th2 cytokines IL-13, IL-4 and IL-5 in response to stimulation with IL-33, IL-25 and TSLP. ILC2 proliferate and provide an early source of IL-13 during murine infection with helminth and

allergens (Neill *et al.*, 2010; Price *et al.*, 2010). ILC2 are lineage-negative innate immune cells as phenotypically they do not express the cell surface markers associated with hematopoietic linages. The surface marker expression varies depending on the cell type there were originally described as. Nuocytes are linage negative and in mice express CD45, IL-7R-α, ICOS, ST2, IL-17BR, C-Kit, CD25, Sca-1, Thy1 and CD127 (Neill *et al.*, 2010). Similarly Ih2 cells express Thy1, CD25, ST2, IL-17BR and ICOS. However in contrast to nuocytes they express only low levels of C-kit and are Sca-1-negative. The phenotype of natural helper cells in mice is similar to these cells in that they express ST2, Thy1, IL-7R-α, Thy1 and CD25. The expression of C-kit is heterogeneous and despite being IL-25 responsive IL-17BR expression has not been reported (Moro *et al.*, 2010). Furthermore although MPP^{type2} are linage negative they can differentiate into myeloid cells, and therefore can be regarded as a completely separate lineage.

After the completion the laboratory work in this study, ILCs have recently been described in humans (Mjosberg *et al.*, 2011; Monticelli *et al.*, 2011). Human lung ILCs were shown to express CD25, CD127, ST2 and prostaglandin D2 receptor CRTh2 (chemoattractant receptor-homologous molecule). However similar to murine ILC2, their Kit expression is heterogeneous (Mjosberg *et al.*, 2011).

In this study I have shown that the airway IL-13 expression colocalised to a low number of ILC2 cell markers when investigated in asthmatic bronchial tissue. It is likely that the expression of ILC2 is underestimated in this study due to the

variation in surface marker expression in this group of innate lymphoid cells. This gives rise to several questions and possibilities.

Firstly ILC have been grouped into Th1- and Th2-like subsets of innate lymphoid cells based on their phenotypical and functional characteristics. It is possible that the ILC2 cells in the bronchial tissue are in different stages of development and they express different markers during their lifespan. In addition the way of identifying ILCs may affect the variation in expression. For example nuocytes, Ih2 and MPPtype2 were identified based on their cell surface marker expression and Th2-like cytokine profile only after stimulation or with helminth infection whereas natural helper cells were identified in naïve mice. To account for the differences in surface marker expression I used antibodies to markers that would include cells across the group. However I used unstimulated bronchial tissue from asthmatic subjects to colocalise cells which may have reduced the detection of ILC2.

Alternatively the microenvironment may affect the phenotype of ILC2. Nuocytes and Ih2 are localised in mesenteric lymph nodes, the spleen and the lung after stimulation in mice (Neill *et al.*, 2010; Barlow *et al.*, 2012). Whereas natural helper cells are located in fat associated lymphoid clusters in the mesentery (Moro *et al.*, 2010). In humans, ILC2 are located in the gut, lung and BAL and tonsil. Interestingly their expression is not limited to these tissue barrier surfaces, ILC2 are detectable in peripheral blood suggesting they migrate to the target tissue when required (Mjosberg *et al.*, 2011).

Due to these limitations in this study only a proportion of IL-13 expressing cells were identified as ILC2. However ILC clearly have a role in model allergic airway disease. Following infection with influenza virus ILC have been shown to facilitate epithelial repair and promote tissue homeostasis (Monticelli *et al.*, 2011). ILC2 have been reported in nasal polys in chronic rhinitis and several studies have shown the presence of ILC2 in mouse models of airway inflammation (Barlow *et al.*, 2012; Bartemes *et al.*, 2012). The majority of studies that have described ILC2 have used acute artificial models and the results in this study may reflect a more chronic response whereby the ILC2 change phenotype depending on their microenvironment.

Taken together these novel cells might therefore contribute to the pathophysiology of chronic severe asthma and, through their presence within both the airway epithelium and lamina propria, contribute to the upregulation of TSLP expression.

CHAPTER 8.

Role of TSLP in mast cell-epithelial cell cross talk

8.1 HLMC and epithelial cell cocultures

There is increasing evidence indicating a close relationship between mast cells and the airway epithelium. Immunohistochemical analysis revealed TSLP is increased in asthmatic airway compared to healthy controls (Figure 4.4 and 4.7) and this expression is localised predominantly to the epithelium and to 20% of mast cells (Figure 4.9). Mast cells accumulate in the airway epithelium in asthmatic patients (Figure 3.2 and (Dougherty et al., 2010)) and epithelial cells have been shown to express the functional TSLP receptor (Semlali et al., 2010). Furthermore addition of IL-4/dsRNA stimulated epithelial cell supernatants to HLMC increases production of TSLP and IL-5 (Nagarkar et al., 2012). TSLP and IL-13 protein production was therefore investigated in freshly isolated HLMC and primary human airway epithelial cells.

8.2 Validation of ELISA kits

IL-13 and TSLP ELISA kits were validated using a calibration curve performance test. The IL-13 kit initially tested was the IL-13 module set (BMS231/3MST Ebiosciences, UK). The calibration curve performance was good as the standard concentrations observed were similar to the kits expected standards (Figure 8.1.A). A mean response of 80-120% is an acceptable range for a kit. The mean response of the measured concentrations was 93% compared to the expected standards. The product datasheet suggested the lower limit of detection was 1.56 pg/mL, however based on these validations the actual detection limit was 6.25 pg/mL as this was the point where the standard could not be distinguished from the reagent diluent alone.

The second IL-13 ELISA kit validated was human IL-13 DuoSet (DY213 R and D systems, UK). Again the standard concentrations observed were very similar to the expected concentrations (Figure 8.1.A). The mean response of the measured concentrations was 130% compared to the expected standards. The recombinant IL-13 readings were as expected. However the kit was less sensitive than the previous kit and the lower limit of detection was 100 pg/mL. As a result samples with IL-13 less than 100 pg/mL could not be effectively measured.

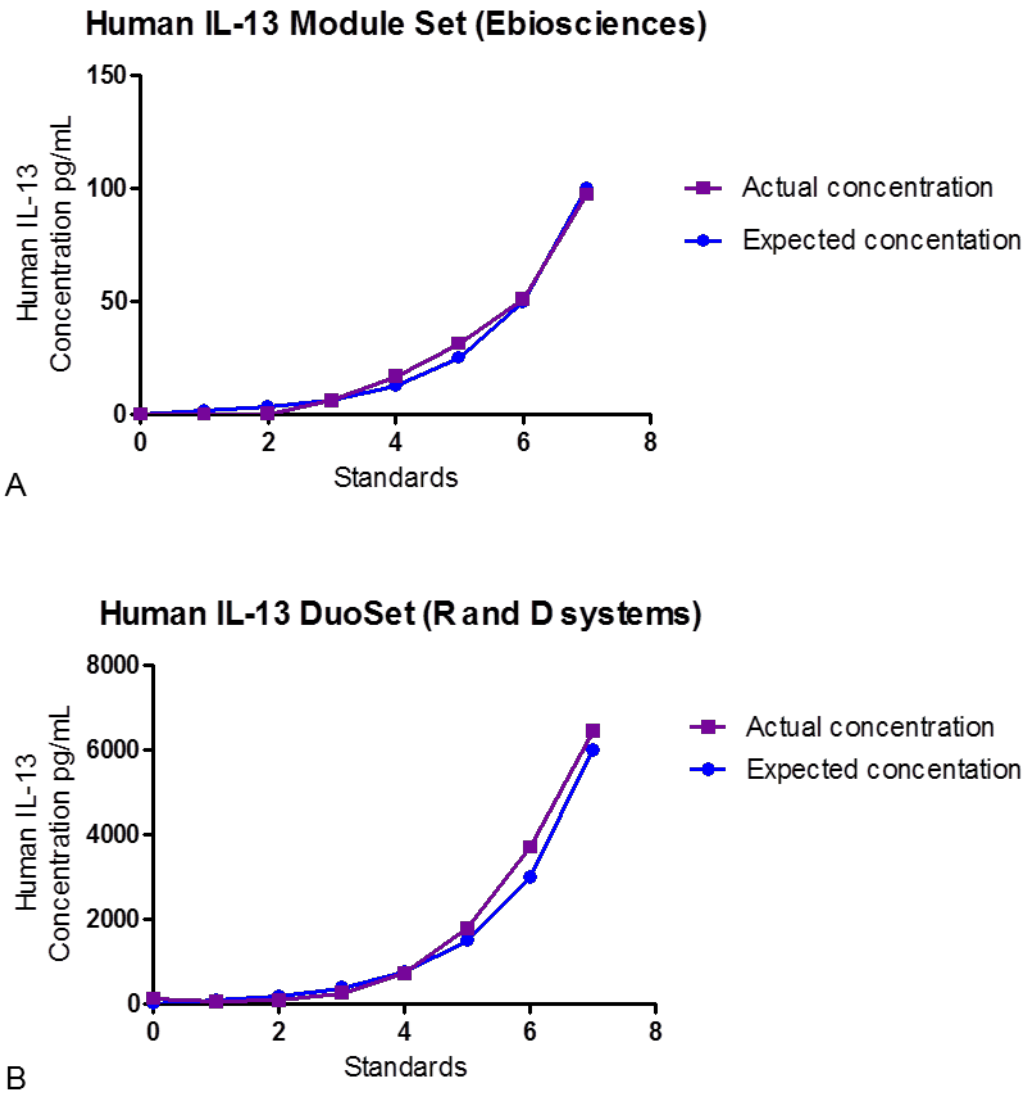


Figure 8.1 Human IL-13 ELISA kit validation

A) Human IL-13 module set Ebiosciences B) human IL-13 DuoSet R&D systems.

The Human TSLP DuoSet from R and D systems is commonly used to measure TSLP in samples using ELISA therefore TSLP was measured using this kit. The calibration curve performance was accurate as the standard concentrations observed were very similar to the expected concentrations (Figure 8.2) In addition the mean response of the measured concentrations was similar to the expected standards. The product datasheet suggested the lower limit of detection was 32 pg/mL, however based on these validations the actual detection limit was 15.5 pg/mL. In addition known concentrations of recombinant TSLP could be measured effectively using this kit.

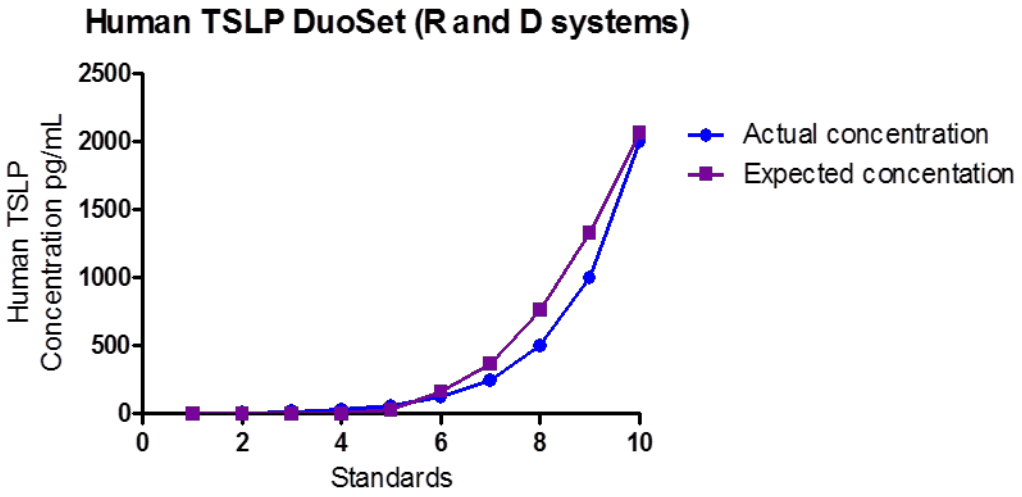


Figure 8.2 Human TSLP ELISA validation

Human TSLP DuoSet from R and D systems was validated for measuring TSLP in samples.

8.3 TSLP production in epithelial cells and mast cells

Epithelial cells and mast cells were co-cultured in 24 well plates containing 300 µL epithelial cell media. Epithelial cells alone and mast cells alone were used as controls and both healthy and asthmatic epithelial cells were assessed (n = 6 and 5 respectively). TSLP protein expression in healthy epithelial cells, HLMC alone and HLMC cultured with healthy epithelial cells was below the limit of detection (< 15.6 pg/mL). There was a significant increase in TSLP expression in asthmatic epithelial cells compared to epithelial cells from healthy controls ($p=0.0045$). Although TSLP protein levels were below the detection limit in HLMCs cocultured with healthy control epithelial cells, coculturing with asthmatic epithelial cells significantly increased the TSLP protein release ($p=0.0252$). There was a trend towards a reduction in TSLP in the asthmatic co-cultures compared to the asthmatic epithelial cells alone raising the possibility that HLMCs may degrade TSLP (Figure 8.3).

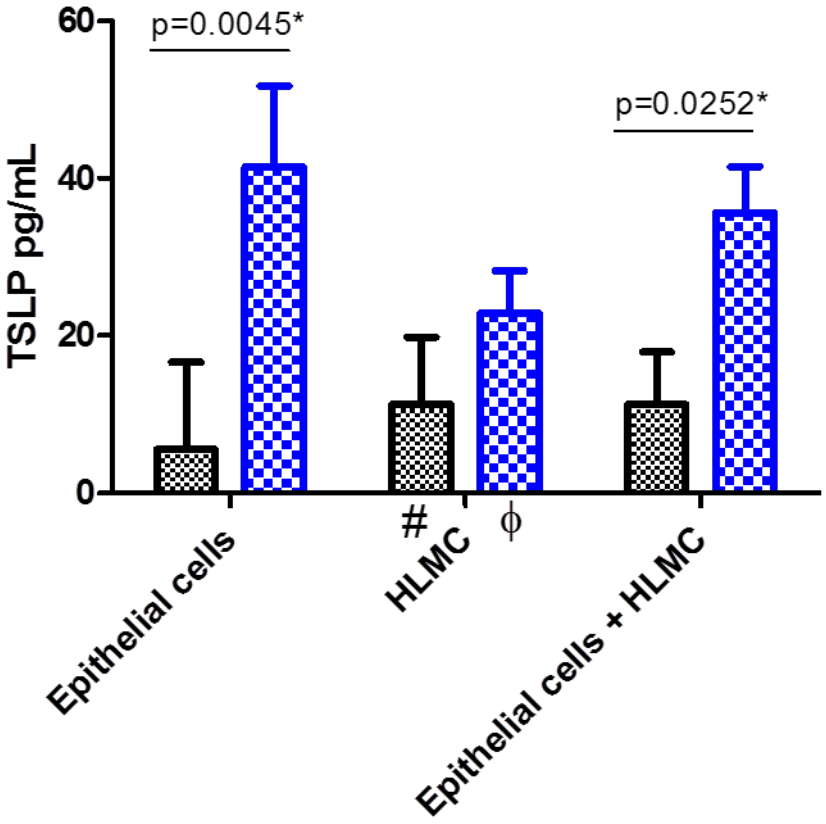


Figure 8.3 Induction of TSLP in asthmatic cocultures

Epithelial cells cocultured with mast cells and the effects on TSLP protein release. Epithelial cells from non-asthmatic donors n = 6 (black bars), epithelial cell from asthmatic donors (n = 5) (blue bars). # represents HLMC donors used in cocultures with non-asthmatic epithelial cells. Φ represents HLMC donors used in cocultures with asthmatic epithelial cells

IL-13 was measured in the co-culture supernatants however it was below the level of detection in all conditions. Furthermore IL-13 could not be measured in bronchial epithelial cells treated with various known IL-13 stimuli including poly:IC, Did:C, TNF-α and TSLP (data not shown).

8.4 Recombinant TSLP inhibits FcεRI activated mast cell degranulation and TSLP production

Mast cells have a unique ability to both produce and respond to TSLP. Although mast cells produce TSLP one study suggests they also breakdown TSLP (Okayama *et al.*, 2009). To assess whether TSLP could be recovered in HLMCs recombinant TSLP (rh.TSLP) was added to cells for 12 h in the presence or absence of FcεRI. FcεRI was used as activated mast cells have been reported to express TSLP mRNA (Soumelis *et al.*, 2002). The cells were cultured in mast cell media in the absence of FBS, SCF and recombinant cytokines. The recovery of TSLP was measured in the supernatants. Supernatants that had undetectable levels of TSLP were assigned the concentration of zero. In addition IL-13 was measured to assess the effects of TSLP on IL-13 production by mast cells.

TSLP was undetectable in unstimulated HLMCs without the addition of stem cell factor (SCF). In addition at 12 h rh.TSLP (100 pg/mL or 1000 pg/mL) could not be recovered in unstimulated HLMCs. Next the TSLP protein levels in the supernatants of HLMCs activated with FcεRI for 12 h were measured. Interestingly, after FcεRI aggregation, there was a significant increase in TSLP protein expression in the HLMCs cultured in the absence of SCF and cytokines

p=0.0017). Furthermore the addition of rh.TSLP significantly inhibited the TSLP release (p=0.0445 at 100 pg/mL) (Figure 8.4).

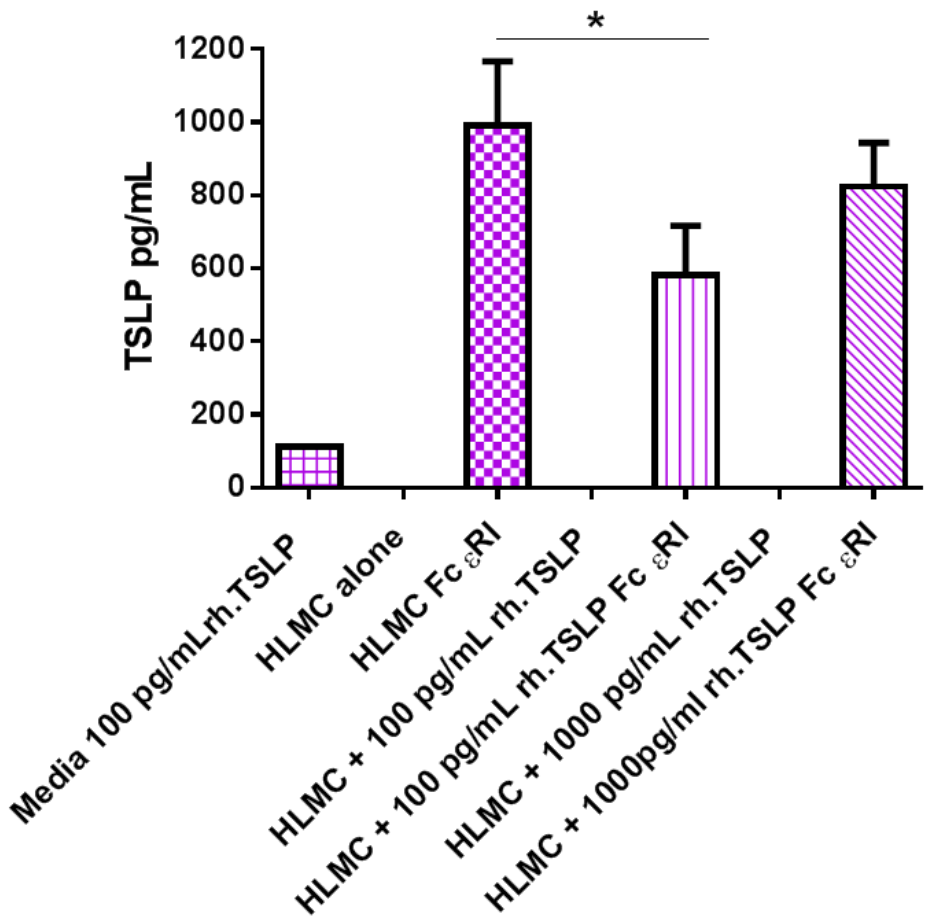


Figure 8.4 Recovery of recombinant TSLP in HLMC

Mast cells spiked with 100 pg/mL and 1000pg/mL in presence or absence of FcεRI for 12 hours. HLMC media spiked with 100pg/mL rh.TSLP was used was a control.

The effect of rh.TSLP was next assessed on mast cell degranulation. The amount of β-hexosaminidase released after stimulation is expressed as the fold change in β-hexosaminidase compared to the control (HLMCs in the absence of TSLP and FcεRI). Following FcεRI aggregation there was a significant increase in β-hexosaminidase release compared to mast cells alone ($p=<0.05$). There was also a significant increase in FcεRI activated HLMCs treated with rh.TSLP ($p=<0.01$). Interestingly addition of rh.TSLP to FcεRI activated cells inhibited β-hexosaminidase activity at both 100 pg/mL and to a lesser extent at 1000 pg/mL (Figure 8.5).

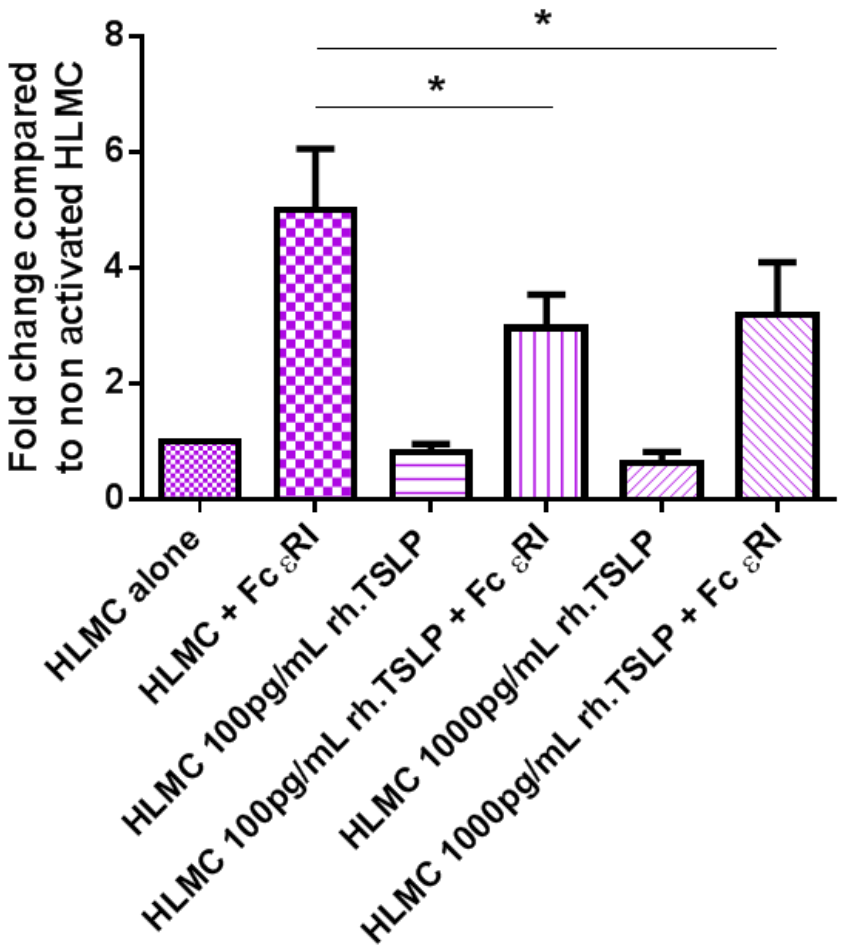


Figure 8.5 β-hexosaminadase release in HLMC

Fold change in β-hexosaminadase release in rh.TSLP treated and/or FcER1 activated HLMC compared to the unstimulated HLMC. One way analysis of variance p=0.0240 (* = p <0 0.05, Bonferroni post test). # = p=0.0313

Next the effect of FcER1 on HLMC and the effects of TSLP to IL-13 release were investigated. HLMC released significantly more IL-13 in response to FcεRI compared to unstimulated HLMC. The addition of TSLP had no effect on the levels of IL-13 produced by activated HLMC (Figure 8.6).

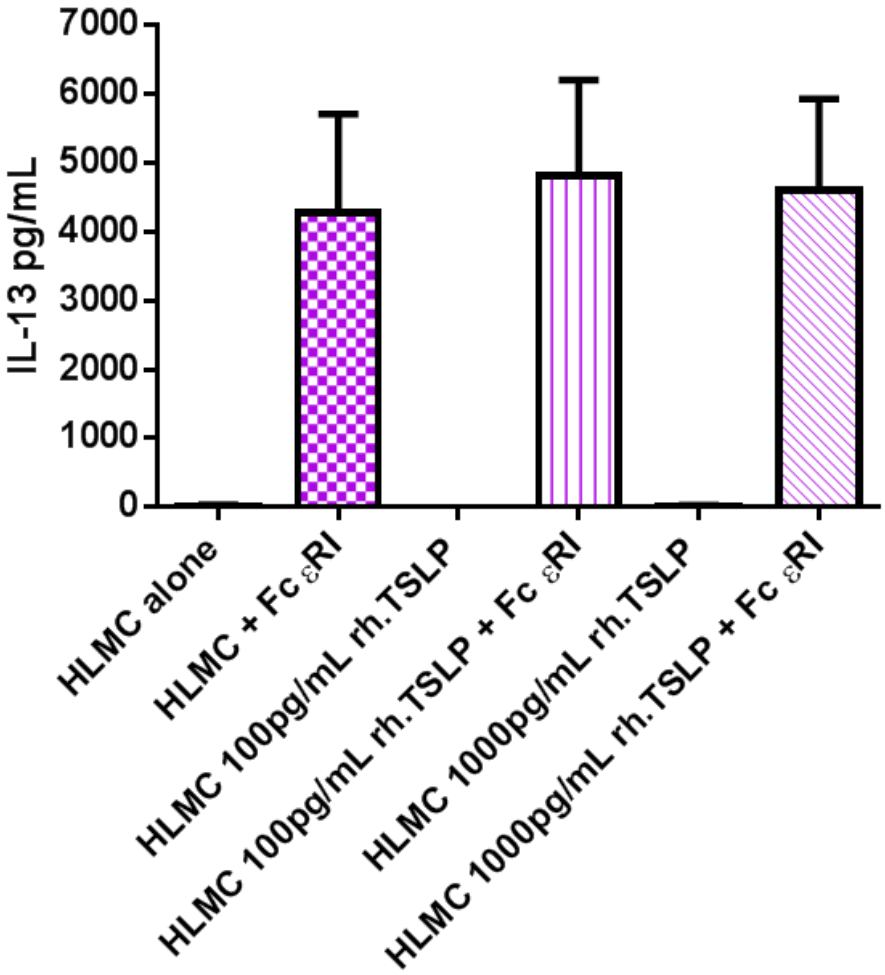


Figure 8.6 Effect of TSLP on IL-13 release by HLMC

Mast cells spiked with 100 pg/mL and 1000pg/mL in presence or absence of FcεRI for 12 hours. IL-13 measured in supernatants.

8.5 Discussion

Here I report that upregulation of TSLP by epithelial cells is a feature of asthma. Furthermore I have demonstrated that TSLP is expressed by human lung mast cells (HLMCs) upon FcεRI activation and shown for the first time that exogenous TSLP inhibits FcεRI activated TSLP release and mast cell degranulation suggesting TSLP exhibits it's effects in an autocrine or paracrine fashion.

IL-13 production by mast cells is inhibited by the blockage of TSLP release from activated epithelial cells suggesting an IL-13/TSLP pathway (Allakhverdi et al., 2007). Similarly, a recent study confirmed TSLP alone did not stimulate Th2 production by mast cells. However Th2 cytokine production from mast cells was elevated by the addition of epithelial cell supernatant pre-treated with IL-4 and dsRNA. This process was dependent on TSLP and IL-1 as this effect was supressed by neutralising antibodies to TSLP and IL-1. TSLP production by epithelial cells positively correlated with IL-5 production by mast cells. Therefore epithelial cell derived TSLP may activate mast cells to facilitate a Th2 microenvironment in response to viral infection (Nagarkar et al., 2012).

The presence of activated mast cells within and in close proximity to the bronchial epithelium in asthmatic patients (Bradding *et al.*, 1994) (Dougherty *et al.*, 2010) suggests that mast cells might regulate TSLP activity very closely in patients with this disease. Conversely, epithelial TSLP production in response to diverse innate stimuli might regulate mast cell IL-13 production. This would

be in keeping with recent hypotheses that a key factor driving chronic asthma is the activation of innate immune pathways (Anderson, 2008).

My study shows that primary asthmatic epithelial cells constitutively secrete more TSLP than cells from healthy control subjects. The addition of mast cells reduced the amounts of TSLP recovered in the cell supernatants. In contrast IL-13 release was undetectable in epithelial cell supernatants following activation with a number of stimuli, including TSLP. This is in contrast to the findings of Semlali *et al*. who reported that epithelial cells express the TSLP receptor and the addition of TSLP results in IL-13 release by epithelial cells (Semlali *et al.*, 2010). IL-13 was not produced by unstimulated HLMCs, either alone or in co-culture with epithelial cells. However, FcεRI-dependent activation of HLMCs increased both TSLP and IL-13 expression. The data here is in broad agreement with Allakhverdi *et al.* in that TSLP did not induce mast cell degranulation; in fact the data here shows that TSLP may inhibit degranulation (Allakhverdi *et al.*, 2007). Despite the epithelial IL-13 immunostaining in bronchial biopsies, IL-13 could not be detected in bronchial epithelial cells. A possibility is that IL-13 within airway epithelial cells represents IL-13 that has been taken up rather than synthesized.

In a mouse model of allergic rhinitis, the high affinity IgE receptor (FcεRI) in mast cells is required for TSLP production (Miyata *et al.*, 2008). The data here extends this in that exogenous delivery of TSLP to FcεRI activated mast cells inhibited TSLP production suggesting that similar to other cytokines, TSLP may

target mast cells in an autocrine or paracrine manner depending upon the microenvironment.

TSLP alone cannot stimulate mast cells but in the presence of IL-1 and TNFα can release IL-13 (Allakhverdi *et al.*, 2007). Importantly, in a murine model of allergic rhinitis, mast cell–dependent regulation of epithelial TSLP secretion was a key determinant of disease expression (Miyata *et al.*, 2008) . Activated mast cells reportedly increase TSLP mRNA expression (Soumelis *et al.*, 2002), however TSLP protein has been reported to be degraded by mast cell proteases (Okayama *et al.*, 2009). My study strengthens this work by demonstrating that HLMCs do not spontaneously release TSLP however this may be due to the possibility that they are rapidly degrading it. Interestingly following FcεRI aggregation TSLP protein release was greatly upregulated which was inhibited by addition of TSLP. The upregulation is in marked contrast to the data reported by Okayama *et al* whereby they reported a reduction in TSLP secretion after FcεRI activation. One possibility for the variation is that the data they showed was from only one donor who was representative of 3 donors, whereas in this study the data are shown as a mean of 6 donors. In addition the environment the cells were cultured in may affect TSLP degradation. Okayama *et al.* used HLMC suspended in IMDM containing recombinant SCF and IL-6 for all experiments, whereas in this study experiments with mast cells were conducted in mast cell media in the absence of SCF and IL-6.

CHAPTER 9.

Conclusion

9.1 Overview

In this thesis the expression and cellular provenance of immunoreactive TSLP and the downstream cellular networks associated with TSLP overexpression have been investigated in subjects with well-characterised asthma of varying severity. Specifically, the difference in inflammatory cell and Th2 cytokine expression has been investigated between healthy controls and asthmatic subjects. This has been achieved using immunohistochemistry on bronchial tissue from non-asthmatic subjects and asthmatic patients with varying disease severity. Secondly, the cellular provenance of TSLP in asthmatic airways was determined using colocalisation techniques. Thirdly, potential cross-talk between airway epithelial cells and HLMCs has been investigated in vitro using primary cells.

The key findings are that i) the expression of TSLP is increased across asthma severity in the airway epithelium, and increased in the airway lamina propria in severe asthma, ii) TSLP is expressed predominantly the airway epithelium and CD45+ lineage negative leukocytes, iii) increased TSLP expression is associated with increased Th2 pathway expression, iv) there is no evidence for a TSLP-OX40/OX40L pathway operating in asthmatic airways, but there are potential TSLP-mast cell and TSLP-ILC2 pathways at work, and v) there are potentially complex interactions between epithelial cells and mast cells in the regulation of TSLP biology, and mast cell function.

TSLP expression in both the airway epithelium and the lamina propria in asthmatic patients correlated with the severity of airflow obstruction. This

suggests that my observations are of functional relevance, although correlation does not prove causation. The finding of increased TSLP expression in patients with severe asthma suggests that TSLP is an attractive target for the development of novel anti-asthma therapies but its association with Th2 pathway activity suggests that targeting TSLP might only be efficacious in the subset of asthma characterised by increased TSLP expression and Th2 inflammation. Clinical trials are required to assess whether TSLP will have therapeutic advantages over anti-IL-5 or anti-IL-13 therapy. However, if there is a mechanistic link leading to fixed airflow obstruction, targeting TSLP in patients with severe asthma has the potential to inhibit both airway inflammation and remodelling, and might therefore offer a new approach with which to address the unmet clinical need in this group of patients.

9.2 Clinical viewpoint: TSLP pathway as a target in asthma

As mentioned there was no evidence of a TSLP–OX40L–dendritic cell axis in this study however this pathway has been associated with asthma in mouse models and a limited number of human in vitro studies. A clinical trial using human monoclonal (huMAb) OX40L to the prevent airway obstruction in patients with mild allergic asthma has recently been completed (January 2011). This study has been funded by Genentech and the outcomes of this trial are yet to be published (Genentech, Updated 15th April 2011). I would predict that this trial will be negative based on my results.

A phase 1b clinical trial of anti-TSLP therapy in mild atopic asthma has recently started (June 2011) using AMG 157 which is a human TSLP monoclonal

antibody that inhibits the interaction with the TSLP receptor. The clinical trial is collaboration between Amagen and Astrazeneca and is due to complete recruitment March 2013. The outcomes of this trial are eagerly awaited. However, some caution is required with respect to TSLP inhibition as it is an important component of the innate immune system. The baseline expression of TSLP in the lung, intestine and gut appears to be essential in the inhibition of Th1 type responses. If TSLP production is completely inhibited responses to basic infections may be attenuated at these sites.

9.3 Future Studies

TSLP biology is currently a fast moving area of research both within our department and internationally. The discovery of TSLP has furthered interest in the role of the epithelium in asthma and there are a number of lines of investigation that would advance the work presented in this thesis.

Firstly IL-13 expression was detected in the bronchial epithelium *in vivo* but could not be detected in cultured resting basal epithelial cells. Whether differentiated epithelial cells in air liquid interface (ALI) cultures have the potential to make IL-13 and release it will be explored by measuring IL-13 mRNA and protein expression. In addition the epithelial cell subtypes that express TSLP in the epithelium will be investigated. Preliminary work carried out will involve immunostaining ALI cultures in embedded in paraffin for TSLP and identifying cell subtypes based on IHC and morphology.

Secondly in this study, epithelial cells from both non-asthmatic and asthmatic patients were cocultured with unstimulated HLMCs. It is important to build on this data and establish the role of FcεRI activated HLMCs cocultured with epithelial cells on TSLP mRNA and protein production using quantitative PCR and ELISA. Unfortunately it was not possible to conduct these experiments in this study due to time. These studies are also likely to be complex, as it is known that healthy airway epithelium inhibits constitutive and FcεRI-dependent HLMC degranulation (Yang *et al.*, 2006b) (Martin *et al.*, 2012). However, with epithelial damage such as that present in asthma, our group hypothesise that this inhibitory effect of epithelium will be lost leading to important mast cell-epithelial cross-talk.

Furthermore, TSLP inhibits mast cell degranulation. As TSLP is released by the epithelium, it would be interesting to see if TSLP may be a contributing factor to this phenomenon, although recent work implicated a mediator working through a Gi-coupled GPCR (Martin *et al.*, 2012). To investigate this further, epithelial cells will be cocultured with FcεRI activated and non-activated freshly isolated HLMCs. The epithelial cells will be from both non-asthmatic and asthmatic patients. The role of TSLP will be investigated using a TSLP neutralising antibody. The supernatants and cell lysates will be collected and the extent of mast cell degranulation will be assessed by measuring histamine or β-hexosaminadase release.

At the onset of this study there was only a single published report on nuocytes. Since then there had only been several reports describing cells with similar

properties to nuocytes, and these are now described as innate lymphoid cells group 2 (ILC2) (Spits *et al.*, 2013). Mouse models suggest that these cells may have an important role in the development of allergic airway inflammation and given their cytokine profile may be the innate counterpart to Th2 cells (Barlow *et al.*, 2012) (Chang *et al.*, 2011). However to date only one group have described ILC2 in humans (Mjosberg *et al.*, 2011). Therefore data presented here is one of the earliest works looking at ILC2 in the human lung in relation to IL-13 expression and many avenues remain to be explored.

Finally, this report demonstrates TSLP is related to Th2 inflammation in asthma. Whether this extends to other disease states remains unclear. My preliminary data suggests that TSLP plays a role in idiopathic pulmonary fibrosis (IPF) which is also characterised by increased IL-13 expression ((Hancock *et al.*, 1998; Zhu *et al.*, 1999) Soumelis *et al*. (Soumelis *et al.*, 2002) reported high levels of TSLP mRNA expression in lung fibroblasts and two additional studies report a potential role for TSLP in relation to fibroblasts (Wu *et al.*, 2012) (Usategui *et al.*, 2013). Therefore whether there is evidence of a TSLP/Th2 cytokine interaction in IPF will be investigated. TSLP, IL-4 and IL-13 protein expression will be measured in resected lung tissue and in particular TSLP/TSLP-receptor expression in cultured human lung fibroblasts.

In summary, my work supports the view that TSLP is an attractive target for the treatment of patients with severe asthma, although careful disease stratification will be required to ensure it is administered to those patients most likely to respond.

9.4 Choice of fixation and embedding material

Chemical fixation preserves tissue from degradation and maintains cell structure by stabilising proteins, nucleic acids and mucosubstances. 10% neutral buffered formalin (4% formaldehyde in PBS) is the most common fixative used in which tissue morphology is preserved by protein crosslinking. The proteins form covalent chemical bonds which anchor soluble proteins to the cytoskeleton. Although the morphology is preserved the process can damage the biological function of proteins and denature them making it difficult to detect with IHC. Furthermore after the tissue is dehydrated and embedding in paraffin, antigen retrieval methods need to be used to unmask the antigens hidden by cross linking. It is this step after chemical fixation that destroys epitopes and delicate structures (lung tissue, bronchial tissue containing epithelium) which glycol methacrylate resin can overcome.

The tissue is preserved from decay and stabilises the tissue during fixation with acetone. The acetone disrupts hydrophobic bonds that give proteins their 3D structure. Acetone can cause shrinkage of tissue however this is overcome by adding iodoacetamide, a denaturant that can cause swelling. PMSF is also added during fixation as it deactivates protease degradation. GMA is water-miscible resin which can be cut at 2 μm and this polymerised resin supports the morphology of ultrathin sections. The cutting of ultrathin sections was vital in this thesis as it enabled me to obtain information from the same cell when cutting sequential sections from small biopsies. More importantly the availability of tissue from the bronchus is very limited therefore the cutting of thin sections enabled me to cut more sections compared to other embedding media. One

limitation in the cutting of thin sections is that staining maybe weak as there is less exposed antigen however in this study the immunohistochemical staining was quantified by counting the number of cells that were positive and the degree of staining was sufficient to analyse. If the intensity of staining is to be explored, the cutting of thicker sections using this resin maybe more advantageous.

9.5 Limitations

As with all studies there are limitations. In this study the orientation of the biopsy in the GMA resin affected the histology of the tissue sections. Often the epithelium and smooth muscle was difficult to analyse or access. To overcome this GMA resins was cut further into the tissue or other biopsies from the same donor with better histology were used. The orientation of the epithelium affected the angle at which the epithelium was cut so it is possible that there is variability in the staining based on the type of epithelial cells that are exposed to the antibody.

The human lung mast cells used for the in vitro work in chapter 8 were cells isolated from lung resections often from lung cancer patients. It’s is possible these cells may behave differently compared those in asthmatic patients. Furthermore the process of isolating mast cells from the lung is crude and many of the signalling processes maybe distorted.

Another limitation was to the inability to effectively measure IL-13 protein release by ELISA as DTT affected the protein and the dialysers were

ineffective. For future experiments removing the DTT after processing the sputum may overcome this limitation.

In this study many measures and multiple comparisons have been made in relation to the clinical and biological variables therefore the choice of statistics is important. Group comparisons were made using the Kruskal-Wallis test; non-parametric data comparisons between groups were made using the Mann–Whitney U test. However this does not factor in the probability of false discoveries. To overcome this limitation of multiple comparisons a Bonferroni correction would have been the simplest method to use. The Bonferroni adjusts the p-value for the number of hypothesis tests performed. Although the numbers of false positives are reduced often true positives are not recognised by this method. Thus a Bonferroni correction can reduce the power to detect true discoveries.

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Appendix

Full publications and abstracts arising from thesis

Increased expression of immunoreactive Thymic Stromal Lymphopoietin in Severe Asthma

Shikotra A[1], Hargadon B[1], Shelley M[1], Bennett J[1], Brightling CE[1], Austin CD[2], Choy DF, Wu LC[2], Arron JR[2], Bradding P[1]

Background: Thymic stromal lymphopoietin (TSLP) is a cytokine implicated in the pathophysiology of asthma through two pathways: a TSLP-OX40L-T cell axis and a TSLP-mast cell axis. Whether these pathways operate in human asthma is unknown.

Aims: To investigate whether mucosal TSLP protein expression relates to asthma severity, and distinct immunological pathways.

Methods: GMA-embedded bronchial biopsies from healthy subjects (n=12) and patients with mild (BTS step 1, n=8), moderate (BTS steps 2 and 3, n=12) and severe (BTS steps 4 and 5, n=16) asthma were immunostained for TSLP, OX40, OX40L, CD83, IL-13, and inflammatory cell markers. Extent of immunostaining was correlated with clinical data.

Results: Specific TSLP immunoreactivity was evident in both the airway epithelium and lamina propria of both healthy and asthmatic subjects. TSLP immunoreactivity was not present in airway smooth muscle. TSLP epithelial expression was significantly elevated in asthma as a whole compared to healthy controls (p=0.0005), particularly in mild and severe disease. The number of TSLP+ cells in the lamina propria was elevated in patients with severe asthma relative to other groups (p=0.0058). Co-localisation studies in 6 severe

asthmatic subjects showed that 5% of TSLP+ cells in the lamina propria were CD68+ macrophages and 20% were tryptase+ mast cells. There were significant inverse correlations between TSLP counts in both the asthmatic bronchial lamina propria and epithelium with the FEV_1/FVC ratio (r_s=-0.53, p=0.002 and r_s=-0.40, p= 0.037). Immunostaining for OX40, OX40L and CD83 in the airways was sparse, with no difference between asthmatic patients and normal control subjects. IL-13 staining was increased in non-epithelial cells within the airway epithelium in severe asthma (p=0.033).

Conclusions: TSLP expression is elevated in severe asthma despite high dose corticosteroid therapy. Although we did not detect activity of the TSLP-OX40L-T cell pathway within asthmatic bronchial mucosa, it is possible that this pathway operates in secondary lymphoid organs such as draining lymph nodes. The close approximation of airway stroma and mast cells suggests that the TSLP-mast cell axis, in which TSLP and IL-13 operate in a positive regulatory loop, is active in asthmatic bronchial mucosa and may be important in maintaining chronic airway inflammation.

The Hunt for Airway Nuocytes

A.Shikotra, P.Bradding

Background: Nuocytes are morphologically similar to lymphocytes but do not express the cell surface markers associated with immune cell linages. They have been likened to the innate counterparts of Th cells and the recent discovery of these cells has given rise to their new nomenclature - Innate lymphoid cell 2 (ILC2). They provide an early source of IL-13 in response to IL-25, IL-33 and TSLP. Although their functions have not been fully studied early studies implicate ILC2 in Th2 inflammation.

Aim: To investigate whether there is evidence of ILC2 in asthmatic airways.

Methods: GMA-embedded bronchial biopsies from patients with asthma were sequentially immunostained for IL-13, CD45, ICOS, IL-17BR, KIT, ST2 and inflammatory cell markers. Sections were colocalised with IL-13 expression.

Results: IL-13 staining was increased in non-epithelial cells within the airway epithelium in severe asthma of which lineage-negative CD45+ cells represented a substantial proportion.

Conclusion: The interaction of TSLP with CD45+ lineage negative innate lymphoid cells group 2 maybe the most important pathway contributing to increased IL-13 expression in a subset of patients with asthma, including those with severe disease. Targeting TSLP may only be efficacious in the subset of asthma characterised by increased Th2 inflammation.

Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma

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Background: Thymic stromal lymphopoietin (TSLP) is a cytokine implicated in the pathophysiology of asthma through 2 distinct pathways: a TSLP–OX40 ligand (OX40L)–T cell axis and a TSLP–mast cell axis. Whether these pathways are active in human asthma is unknown.
Objective: We sought to investigate whether mucosal TSLP protein expression relates to asthma severity and distinct immunologic pathways.
Methods: In healthy subjects and patients with mild-to-severe asthma, we immunostained bronchial biopsy specimens for TSLP, OX40, OX40L, T_H2 cytokines, and inflammatory cell markers. We examined gene expression using RNA microarrays and quantitative RT-PCR.
Results: There was considerable heterogeneity in the levels of TSLP, IL-13, and IL-4 immunostaining across the cohort of asthmatic patients examined. Overall, TSLP protein expression was significantly increased in airway epithelium and lamina propria of asthmatic patients, particularly in patients with severe asthma. TSLP immunostaining in both compartments correlated with the severity of airflow obstruction. The majority of leukocytes expressing IL-13 were possibly nuocytes. Accounting for intersubject variability, the 55% of asthmatic patients with increased IL-13 immunostaining in the lamina propria also had increased IL-4 and TSLP expression. This was further substantiated by significant correlations between TSLP gene expression, a T_H2 gene expression signature, and eosinophilic inflammation in bronchial biopsy specimens. Immunostaining for OX40, OX40L, and CD83 was sparse, with no difference between asthmatic patients and healthy subjects.
Conclusion: TSLP expression is increased in a subset of patients with severe asthma in spite of high-dose inhaled or oral corticosteroid therapy. Targeting TSLP might only be efficacious in the subset of asthma characterized by increased TSLP expression and T_H2 inflammation. (J Allergy Clin Immunol 2012;129:104-11.)

Key words: Thymic stromal lymphopoietin, asthma, mast cell, OX40, IL-13, T_H2, airway remodeling, nuocyte

Discuss this article on the JACI Journal Club blog: www.jaci-online.blogspot.com.

Asthma is an important cause of morbidity and mortality. Approximately 10% of asthmatic patients have poorly controlled disease using current treatments; in consequence, novel approaches to treatment are required urgently.[1]

The pathophysiology of severe asthma is poorly understood, and its study has been complicated by the recognition that asthma is a highly heterogeneous disease both clinically[2] and at the molecular level.[3] For many years, research has focused on the T_H2 cell hypothesis, whereby it is proposed that T cell–derived T_H2 cytokines, such as IL-4, IL-5, and IL-13, promote airway mucosal eosinophilia and disordered airway function.[4] These cytokines can contribute to disease pathogenesis in a proportion of patients, but a recent study of gene expression from asthmatic tissue suggests that up to 50% of patients do not exhibit evidence of T_H2-driven inflammation.[3]

Thymic stromal lymphopoietin (TSLP) is a cytokine released from airway epithelial cells in response to bacterial peptidoglycan, Toll-like receptor 3 stimulation, inflammatory cytokines, and IL-13.[5,6] Its overexpression in mice leads to asthma-type T_H2-driven airway changes, whereas its inhibition attenuates the airway changes seen in murine models of asthma.[7] The current paradigm for the role of TSLP is that it activates a dendritic cell–T_H2 T-cell axis through an interaction between OX40 ligand (OX40L) and OX40.[8-10] OX40L-OX40 interactions also contribute to the maintenance of ongoing T_H2 memory.[11]

However, T cell–independent pathways also contribute to TSLP-dependent allergic-type airway inflammation. In particular, the direct interaction of TSLP with mast cells is highly relevant. TSLP derived from human epithelial cells induces the release of numerous cytokines and chemokines from human mast cells, including IL-13.[6] Furthermore, in a murine model of

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Abbreviations used
FVC: Forced vital capacity
GMA: Glycol methacrylate
HSI: Hue saturation and intensity
OX40L: OX40 ligand
qPCR: Quantitative real-time PCR
TSLP: Thymic stromal lymphopoietin

rhinitis, mast cell activation was required for the increased TSLP production by airway epithelium.[12] Human mast cells are also able to produce TSLP.[13] Taken together, these studies suggest the presence of 2 distinct TSLP-driven pathways that might drive asthma pathophysiology: a TSLP–OX40L–T-cell axis and a TSLP–mast cell axis.

Whether these pathways are operating in human asthma is unknown. Increased expression of TSLP mRNA has been reported in both the airway epithelium and lamina propria in a small group of asthmatic patients with disease of varying severity, although expression in relation to severity was not presented.[14] A further study demonstrated increased concentrations of TSLP in bronchoalveolar lavage fluid from 13 patients with moderate-to-severe asthma compared with that seen in healthy control subjects.[15] Whether the increased expression of TSLP mRNA and bronchoalveolar lavage protein extends to mucosal protein expression is not known. However, one study demonstrated increased numbers of mast cells expressing immunoreactive TSLP in the airway mucosa of patients with predominantly mild-to-moderate asthma.[13]

Therefore there are few data regarding TSLP protein expression in asthmatic airways and in particular how TSLP expression relates to downstream effector pathways, disease severity, and phenotype. The aim of our study was to examine the expression and cellular provenance of immunoreactive TSLP and the downstream cellular networks associated with TSLP overexpression in subjects with well-characterized asthma of varying severity.

METHODS

Subjects

Leicester cohort. Asthmatic patients (n = 36) and healthy volunteers (n = 12) were recruited for the study of both gene expression and immunohistochemistry. Full details and the assessments undertaken are described in the Methods section in this article's Online Repository at www.jacionline.org. Asthma severity was defined by the "British guideline on the management of asthma" treatment steps (see the Methods section in this article's Online Repository).[16] Of the 16 patients with severe asthma at steps 4 and 5, 13 met the American Thoracic Society criteria for refractory asthma.[17]

Belfast cohort. For examination of airway gene expression and inflammatory cell infiltration, an additional cohort of subjects from Belfast was also studied. Participants with refractory asthma were recruited from the Belfast City Hospital, where systematic evaluation protocols ensure patients have well-characterized refractory asthma.[18] Of the 9 patients at steps 4 and 5, 7 met the American Thoracic Society criteria for refractory asthma.[17] Further details regarding patients with mild-to-moderate asthma are provided in the Methods section in this article's Online Repository.

Demographic details for each cohort are shown in Tables E1 and E2 in this article's Online Repository at www.jacionline.org. The study was approved by the research ethics committees of both institutions. Written informed consent was gained from all participants before their involvement.

Fiberoptic bronchoscopy

Subjects underwent bronchoscopy conducted according to British Thoracic Society guidelines.[19] Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae, fixed in acetone, and embedded in glycol methacrylate (GMA), as described previously.[20] Biopsy specimens were also placed immediately in RNA preservative (RNAlater; Ambion, Austin, Tex) and submitted for microarray and quantitative real-time PCR (qPCR) analysis.

Immunohistochemistry

GMA-embedded tissue was cut and immunostained, as described previously.[20] Primary antibodies were used against the following antigens: TSLP, mast cell tryptase, CD1a, CD83, IL-4, IL-13, CD68, CD3, CD4, CD8, neutrophil elastase, eosinophil major basic protein, OX40, and OX40L. Isotype controls were also performed. Full details of the antibodies used, including isotype, concentration, and source, are provided in the Methods section in this article's Online Repository. Tonsil tissue obtained from Scottish Biomedical (Glasgow, United Kingdom) was embedded in GMA and used as a positive control.

Assessment and quantification of immunohistochemical staining

Epithelial, submucosal, and smooth muscle bundle areas in sections were identified and measured by using a computer analysis system (analysis docu; Olympus, Hamburg, Germany). Numbers of positively stained nucleated cells in each compartment were counted blind. Cells staining in sequential sections were colocalized by using computer analysis.

Two methods were used for quantitative assessment of TSLP expression in the epithelium. First, a thresholding technique was developed based on the hue saturation and intensity (HSI) of TSLP staining. Further methodological details are provided in the Methods section in this article's Online Repository. We also used a semiquantitative score of staining (0, none; 1, weak intermittent; 2, strong intermittent or weak generalized; and 3, strong generalized). There was a good correlation between the 2 methods ($r_s = 0.837$, $P < .0001$).

Prism 5 software (GraphPad Software, Inc, La Jolla, Calif) was used for statistical analysis. Group comparisons were made with the Kruskal-Wallis test; nonparametric data comparisons between groups were made with the Mann-Whitney U test, and unpaired t tests were used for parametric data. The spearman rank correlation test was used for correlations. A P value of less than .05 was considered statistically significant.

Gene expression analyses

RNA was isolated from homogenized bronchial biopsy specimens, and qPCR was performed, as described previously.[21] TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif) were purchased and conducted per the manufacturer's instructions for TSLP (id: Hs00263639_m1). Further details are provided in the Methods section in this article's Online Repository. All gene expression analyses and plotting were performed with the R Project software package, version 2.10.1 (refer to http://www.R-project.org). The T_H2 score was calculated by using a generalized procedure[21] and is described in detail in the Methods section in this article's Online Repository.

RESULTS

Demographic data

The demographic data of the asthmatic patients from the Leicester and Belfast cohorts are shown in Tables E1 and E2.

Inflammatory cell infiltration (Leicester cohort)

Numbers of epithelial and lamina propria mast cells, macrophages, eosinophils, neutrophils, and T cells are shown in Table E3 and discussed further in the Results section in this article's Online Repository at www.jacionline.org.

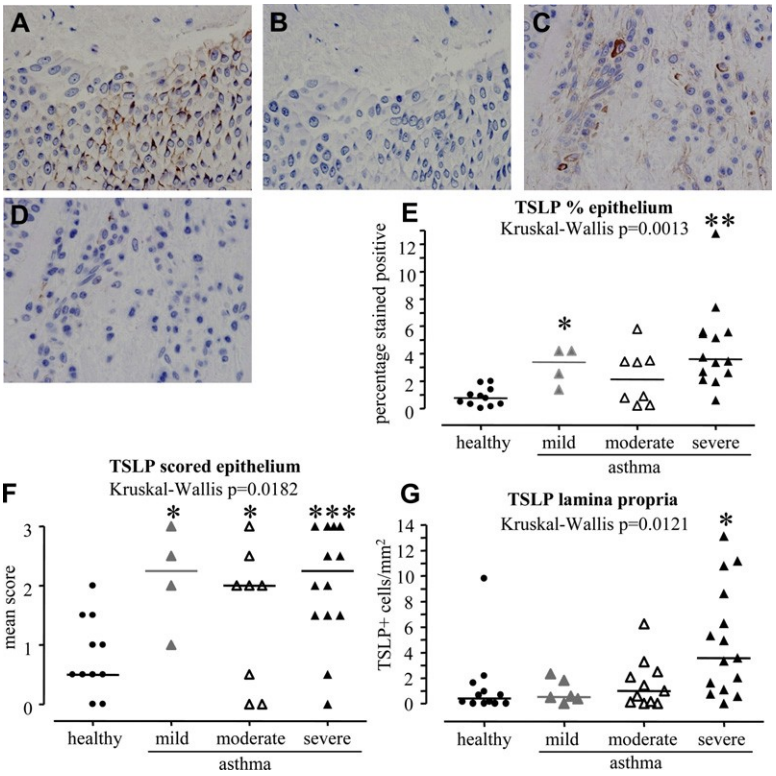


FIG 1. TSLP immunostaining is increased in asthmatic patients. A and B, TSLP immunostaining in the airway epithelium from a patient with asthma (Fig 1, A) and inhibition by recombinant TSLP (Fig 1, B). C and D, TSLP immunostaining in the lamina propria from an asthmatic subject (Fig 1, C) and inhibition by recombinant TSLP (Fig 1, D). E and F, Extent of TSLP immunostaining in airway epithelium assessed based on threshold measurements and scoring, respectively. *P < .05, **P 5 .0001, and ***P 5 .0014 compared with healthy control subjects. G, Extent of TSLP immunostaining in the lamina propria. *P 5 .0058 compared with healthy control subjects. Immunohistology is shown at 3400 magnification.

TSLP immunoreactivity is increased in both the airway epithelium and lamina propria in patients with severe asthma

Epithelial tissue for analysis was available from 11 healthy subjects and 5 patients with mild, 8 patients with moderate, and 14 patients with severe asthma. Lamina propria tissue was available for analysis from 12 healthy subjects and 6 patients with mild, 11 patients with moderate, and 15 patients with severe asthma. TSLP immunostaining in control human tonsil tissue showed the typical epithelial distribution described previously (see Fig E1, A, in this article's Online Repository at www.jacionline.org).[22] TSLP immunoreactivity was evident in both the airway epithelium and lamina propria of both healthy subjects and asthmatic patients, and this was inhibited by preincubating the TSLP antibody with recombinant human TSLP (Fig 1, A-D). TSLP immunostaining in the airway epithelium was localized predominantly to epithelial cells, although occasional mast cells, macrophages, and neutrophils were identified through colocalization in sequential sections. TSLP epithelial expression was significantly increased in asthmatic patients as a whole compared with that seen in healthy control subjects (P 5 .0005) whether measured semiquantitatively or based on the threshold intensity of staining. Subgroup analysis demonstrated significant increases in the epithelia of both patients with mild and patients with severe asthma compared with that seen in healthy control subjects by using the threshold assessment and in all asthmatic groups when scored semiquantitatively (Fig 1, E and F).

Numerous cells staining for TSLP were evident in the bronchial lamina propria (Fig 1, C). The number of $TSLP^1$ cells in the lamina propria was increased in the asthma group as a whole (P 5 .043), but interestingly, this was accounted for by a significant increase only in the group of patients with severe asthma (P 5 .0058 compared with healthy control subjects; Fig 1, G). Both endothelial cell and mononuclear cell staining in the lamina propria was evident (Fig 1, C). Colocalization of cells in serial 2-mm sections from a subset of 6 patients with severe asthma showed that approximately 5% of $TSLP^1$ cells in the lamina propria were $CD68^1$ macrophages and 20% were tryptase-positive mast cells (see Fig E2 in this article's Online Repository at www.jacionline.org). Endothelial cells identified based on morphology and location accounted for 42.0% 6 5.0% of $TSLP^1$ cells. Neutrophils accounted for only occasional $TSLP^1$ cells. There were significant inverse correlations between TSLP counts in both the asthmatic bronchial lamina propria and epithelium with the FEV_1/forced vital capacity (FVC) ratio (r_s 5 20.53, P 5 .002 and r_s 5 20.40, P 5 .037, respectively). There was also a significant correlation between lamina propria TSLP count and reliever use per week (r_s 5 0.40, P 5 .045). No other significant correlations between TSLP staining and clinical parameters were evident.

Appropriate antibody isotype control sections were negative for TSLP immunostaining (see Fig E1, B and C).

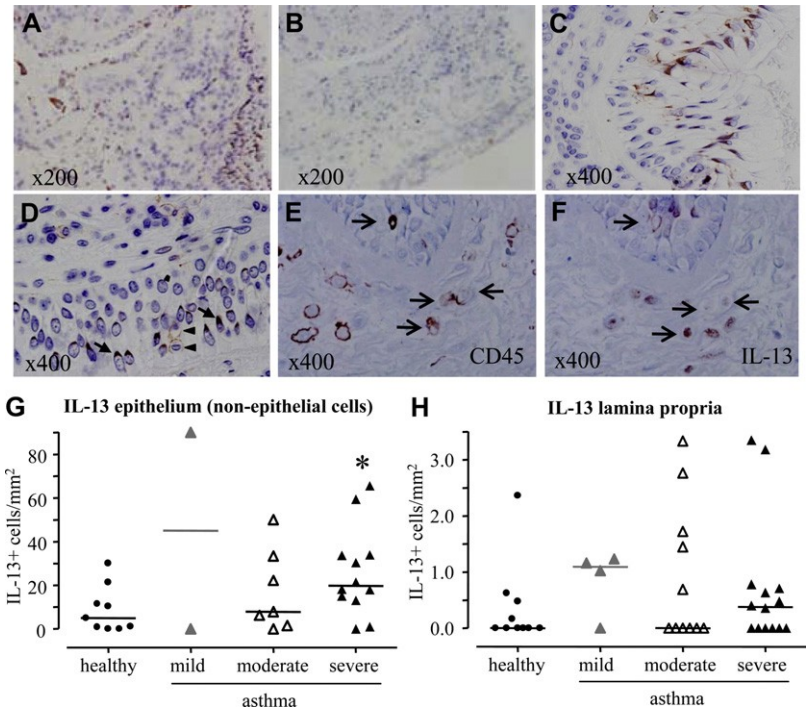


FIG 2. IL-13 expression in the airway epithelium and lamina propria. A, C, D, and F, IL-13 immunoreactivity in the airway epithelium and lamina propria from 4 patients with severe asthma. In Fig 2, D, arrows demonstrate IL-13^1 pseudocolumnar epithelial cells, and arrowheads highlight IL-13^1 nonepithelial cells. B, An immunoabsorption control with recombinant human IL-13 in a section adjacent to Fig 2, A. E and F, Sequential sections demonstrating colocalization of IL-13 to CD45^1 cells (arrows; 3400 magnification). G, The density of IL-13^1 nonepithelial cells within the airway epithelium. *P 5 .033. H, The density of IL-13^1 cells within the airway lamina propria.

The TSLP–dendritic cell–OX40L axis is not evident in human asthmatic airway tissue

For validation of CD83, OX40, and OX40L immunostaining, human tonsil tissue was first examined and shown to exhibit immunoreactivity for each of these markers (see Fig E1, D-F). In the airways immunostaining for each of these antigens was sparse, and data from all asthma groups are presented together (see Table E4 in this article's Online Repository at www.jacionline.org). There was a significant increase in the number of CD1a^1 dendritic cells in the lamina propria in the asthma group as a whole (see Fig E1, G, and Table E4). There were no significant differences for CD83, OX40, or OX40L.

IL-4 and IL-13 expression in human asthmatic airway tissue

Because of consumption of tissue, numbers of donors available for studying these 2 cytokines were reduced, particularly in the mild asthma group (see figure legends for details). The extent of IL-4 immunostaining in the airway epithelium was similar in all groups (see Fig E3, A, in this article's Online Repository at www.jacionline.org) but was increased in the lamina propria in patients with mild asthma but not those with severe asthma, as described previously (see Fig E3, B).[23,24]

IL-13^1 cells were evident in both the airway epithelium and lamina propria, and the staining was inhibited by preincubation of the antibody with recombinant human IL-13 (Fig 2, A-C). Within the epithelium, both epithelial cells and nonepithelial cells were stained (Fig 2, D-F). The epithelial cell staining was discrete, intracellular, and located predominantly in the differentiated pseudocolumnar cells (Fig 2, A, C, and D). There was no difference in the extent of epithelial cell IL-13 immunoreactivity between asthmatic patients and healthy subjects (see Fig E3, C). The nonepithelial cells expressing IL-13 within the epithelium and lamina propria were mononuclear, with a distinct cell-surface staining pattern accompanied by punctuate intracellular staining (Fig 2, D and F). There was an increased number of IL-13^1 cells that were classified as nonepithelial cells within the airway epithelium in the patients with severe asthma compared with that seen in the healthy control subjects (P 5 .033; Fig 2, G), but there were no differences across the study groups with respect to lamina propria IL-13 expression (Fig 2, H). Colocalization of cell markers in sequential 2-mm sections demonstrated that IL-13 was localized to less than 2% of mast cells and T cells and that these accounted for less than 4% of the IL-13^1 nonepithelial cells. No IL-13 was colocalized to eosinophils. However, 70.2% 6 12.4% of the IL-13^1 nonepithelial cells in the airway epithelium and 36.4% 6 2.9% of the IL-13^1 cells in the lamina propria were CD45^1 cells (Fig 2, E and F), suggesting they might represent the human equivalent of the recently described murine nuocyte.[25]

Relationships between TSLP and T_H2 cytokine immunoreactivity

Taken continuously, staining patterns for IL-4, IL-13, and TSLP did not show any significant intercorrelations (data not shown). However, emerging evidence suggests that there might be discrete molecular and pathophysiological phenotypes of asthma.

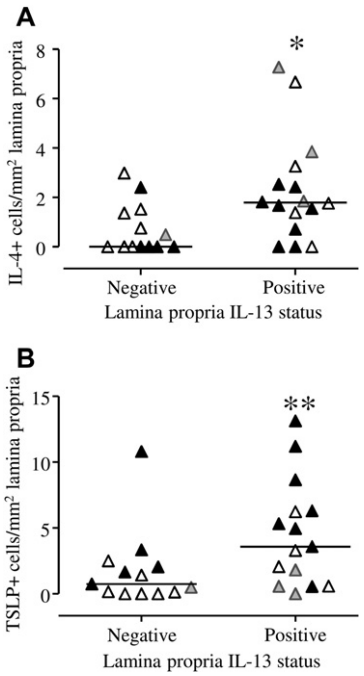


FIG 3. Relationships between IL-13, IL-4, and TSLP expression in the lamina propria in asthmatic patients. IL-13 immunoreactivity in the lamina propria was scored as absent or present; these categorical descriptions of IL-13 immunoreactivity were compared against IL-4 (A) and TSLP (B) immunoreactivity in the lamina propria. Gray symbols, Mild asthma; white symbols, moderate asthma; black symbols, severe asthma. *P 5 .014 and **P 5 .022.

Examination of IL-4 and IL-13 immunostaining in the lamina propria (see Fig E3, B, and Fig 2, H) revealed that these cytokines were detectable in only a subset of asthmatic patients in the study. Similarly, increased levels of TSLP staining in the lamina propria were observed only in a subset of patients with severe asthma. Therefore taken categorically as defined by the presence or absence of IL-13^{1} cells in the lamina propria, we find that the IL-13^{1} subjects show significant enhancement of both IL-4 (Fig 3, A) and TSLP (Fig 3, B) immunoreactivity in the lamina propria, suggesting that in terms of TSLP and T_H2 cytokine protein expression in bronchial tissue, there might be discrete "T_H2-high" and "T_H2-low" phenotypes of asthma.

TSLP gene expression relative to eosinophilic inflammation and T_H2 gene signature in biopsy tissue

To determine whether the expression of TSLP is related to independent molecular metrics of T_H2 inflammation, we performed gene expression microarray analyses of bronchial biopsy tissue. We assigned scores to individual subjects on the basis of a T_H2 inflammation gene signature, as we have previously described for a separate cohort of bronchial biopsy specimens from asthmatic patients.[21] There was a continuum of T_H2 signature intensity across the subjects in this cohort (Fig 4, A), although it was somewhat attenuated relative to the previous study, likely because of the variable effects of inhaled corticosteroid treatment on T_H2 cytokine–induced gene expression in asthmatic airways.[3] We performed qPCR to precisely measure the level of TSLP expression in the same biopsy samples. TSLP gene expression was significantly correlated with lamina propria TSLP protein expression (r 5 0.43, P 5 .026, n 5 27) and with the magnitude of the T_H2 signature and lamina propria eosinophil counts (Fig 4, B-D).

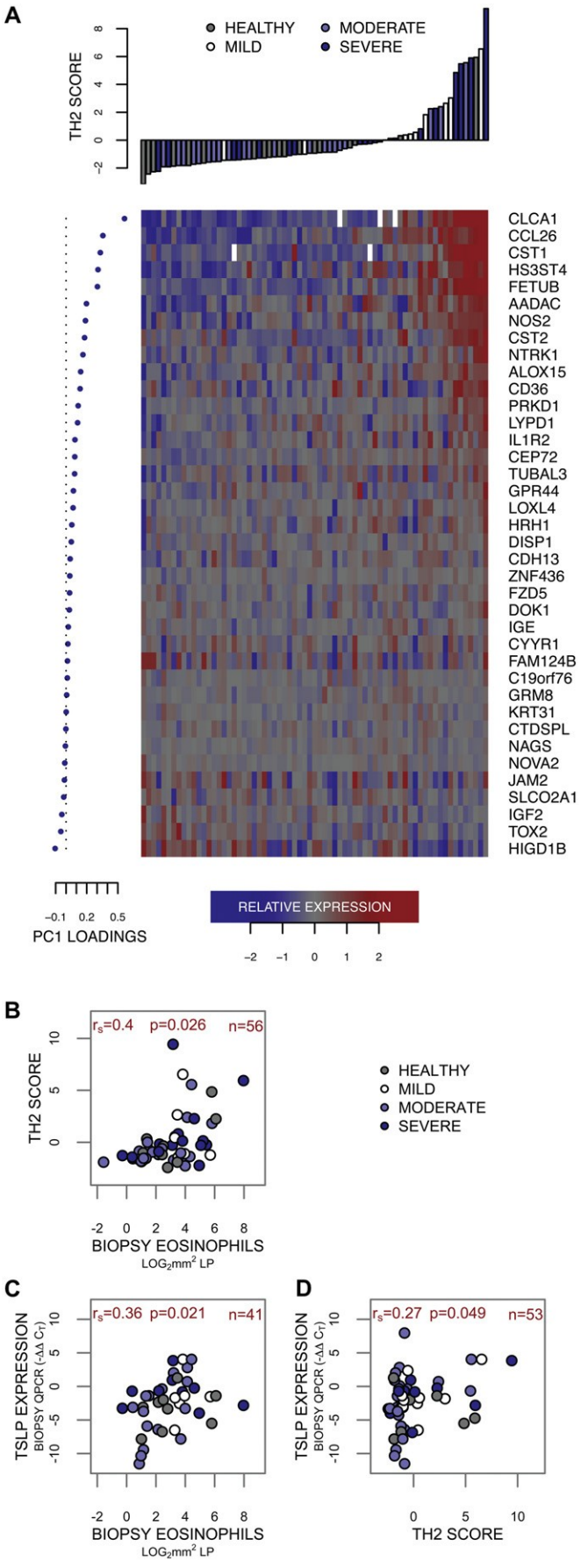
DISCUSSION

We have shown that expression of TSLP protein is upregulated in the airways of patients with asthma and that this persists in a cohort of patients with severe asthma despite high-dose corticosteroid treatment at steps 4 and 5 of the British asthma treatment guidelines. TSLP expression in the airway epithelium was increased across the spectrum of disease severity, although to a lesser extent in patients with relatively well-controlled moderate asthma. However, in the airway lamina propria, increased expression was confined to the group with severe disease. These findings suggest TSLP might be a key mediator of severe chronic asthma and that its anatomic site of expression might be related to asthma severity.

Our study extends previous work demonstrating increased TSLP mRNA expression in asthmatic airway mucosa and increased TSLP protein expression in the bronchoalveolar lavage fluid of 13 patients with moderate-to-severe asthma. However, our findings extend our understanding of the potential roles of TSLP in asthmatic patients through the coexamination of downstream TSLP-dependent cellular networks. Animal models propose that TSLP contributes to the immunopathology of asthma through the activation of the OX40L/OX40 pathway, leading to the generation of T_H2 T cells. There has therefore been great interest in targeting the OX40L/OX40 pathway in asthmatic patients.[26] However, despite clear OX40 and OX40L staining in human tonsil control tissue, staining for these cellular markers in the airways was notably sparse, and we could not find any evidence for increased OX40L or OX40 expression in asthmatic patients. Another study suggested that OX40 and OX40L expression was increased in patients with mild asthma compared with that seen in healthy subjects and patients with severe asthma, but the numbers of cells present were very small.[27] In parallel, we found no significant increase in the number of dendritic cells displaying the activation marker CD83 in asthmatic patients. Our data therefore suggest that OX40L and OX40 are unlikely to play significant roles in persistent airway inflammation in the bronchial mucosa. However, given the current understanding of the dynamics of dendritic cell–mediated T-cell stimulation, it remains possible that OX40L/OX40 interactions take place in secondary lymphoid organs, such as mediastinal lymph nodes, which could not be sampled in this study.

TSLP also activates mast cell IL-13 production,[6] and in turn, mast cell activation induces epithelial TSLP production.[12] Importantly, in a murine model of allergic rhinitis, mast cell–dependent regulation of epithelial TSLP secretion was a key determinant of disease expression.[12] The presence of activated mast cells within and in close proximity to the bronchial epithelium in asthmatic patients[23,28] suggests that mast cells might regulate TSLP activity very closely in patients with this disease. Conversely, epithelial TSLP production in response to diverse innate stimuli might regulate mast cell IL-13 production. This would be in keeping with recent hypotheses that a key factor driving chronic asthma is the activation of innate immune pathways.[4]

Mast cells are also a source of TSLP.[13,15] We have confirmed this and found that 5% of mast cells in patients with severe asthma express TSLP protein, which accounted for 20% of TSLP^{1} cells in the lamina propria. These findings are in broad agreement with the data of Ying et al[15] with respect to TSLP



mRNA expression but in marked disagreement with the data of Okayama et al,[13] who studied TSLP protein. Ying et al[15] also demonstrated TSLP mRNA expression in endothelial cells, neutrophils, CD68^1 macrophages, and CD3^1 T cells. We identified endothelial cell TSLP immunoreactivity by means of morphology and have confirmed the presence of TSLP protein within CD68^1 cells but have not investigated T-cell colocalization.

Ying et al[14] found a significant inverse correlation between TSLP mRNA expression in both the airway epithelium and lamina propria with FEV_1 percent predicted. We did not find a significant correlation with FEV_1 percent predicted but did find significant inverse correlations between TSLP protein expression in both the airway epithelium and lamina propria with the FEV_1/FVC ratio. Because there is wide variability between subjects in predicted FEV_1,[29] the FEV_1/FVC ratio provides a more accurate measure of airflow obstruction. This finding suggests that in addition to promoting airway inflammation, TSLP overproduction might play an important role in the remodeling of asthmatic airways and the subsequent development of fixed airflow obstruction.

In keeping with previous work in asthmatic patients, IL-4 expression was suppressed in patients with severe asthma compared with that seen in patients with mild asthma.[24] Interestingly, IL-13 protein was detected in the airway epithelium in patients with severe asthma in cells that were not epithelial cells but was also detected in columnar epithelial cells. This latter finding is novel and was not described in previous studies of IL-13 expression in asthmatic patients using a different anti–IL-13 antibody.[30,31] Our current study used an in-house–generated IL-13 rabbit polyclonal antibody. Specificity of immunostaining was confirmed by using isotype controls and immunoabsorption with recombinant human IL-13, suggesting that the staining is unlikely to be artifactual. Two recent studies have described the expression of IL-13 mRNA and secretion of IL-13 protein in submerged monolayers of primary human airway epithelial cells, suggesting that they might produce this cytokine.[32,33] An alternate explanation for our immunostaining is that IL-13 within airway epithelial cells represents IL-13 that has been taken up rather than synthesized. The cellular distribution of IL-13 in our current study was different than that described previously[31] in that most nonepithelial IL-13^1 cells in the airway epithelium and lamina propria were not mast cells, T cells, or eosinophils. However, the majority were CD45^1 cells, suggesting that they are the human equivalent of the recently described CD45^1 murine nuocyte.[25] Nuocytes are lineage-negative innate immune cells that provide an early source of IL-13 during murine infection with parasites. These cells might therefore contribute to the pathophysiology of chronic severe asthma and, through their presence within both the airway epithelium and lamina propria, contribute to the upregulation of TSLP expression.

FIG 4. Bronchial biopsy specimen gene expression of TSLP, T_H2 signature score, and eosinophil count are intercorrelated. A, A quantitative score for T_H2 signature score was calculated from gene expression microarrays (see the Methods section in this article's Online Repository). Relative expression is represented by means of heat map, where patient samples (columns) are organized left to right by increasing the T_H2 signature score, as indicated by the adjacent bar plot (top). Genes (rows) are organized by principal component analysis loadings as indicated by adjacent dot plots (left). B-D, All pairwise comparisons of bronchial biopsy TSLP expression, T_H2 signature scores, and eosinophil counts are represented by scatterplots (Spearman rank, all comparisons $P < .05$).

Increased IL-13 expression in the nonepithelial cells within the epithelial compartment in patients with severe asthma supports the previous observation that IL-13 protein concentrations in induced sputum are increased in a subgroup of patients with severe refractory asthma.[31] However, it is again evident that there is a subgroup of patients with severe asthma in whom IL-13 expression persists and a group in whom it is suppressed to levels similar to those seen in healthy subjects. This has important implications for studies examining the efficacy of anti–IL-13 therapy in patients with severe asthma. In the IL-13–low group, it is unlikely that IL-13 will be driving their disease, and anti–IL-13 therapy might be ineffective. Therefore it might be necessary to target this therapy to patients in whom there is evidence of ongoing IL-13 expression.

We observed considerable heterogeneity in the levels of TSLP, IL-13, and IL-4 staining across the cohort of asthmatic patients examined, which is consistent with previous reports demonstrating subsets of asthmatic patients with variable levels of T_H2 inflammation.[3] In general, subjects with increased IL-13 immunostaining in the lamina propria also had increased IL-4 and TSLP immunostaining. This observation is further substantiated by a significant correlation between TSLP gene expression, a T_H2 gene expression signature, and eosinophilic inflammation in bronchial biopsy specimens. Taken together with the established roles of TSLP in mediating both adaptive and innate IL-13–driven inflammation, these findings suggest that TSLP is indeed associated with the "T_H2-high" subphenotype of asthma.

In summary, our study demonstrates increased expression of TSLP in asthmatic patients, particularly in patients with severe disease, and this correlates with the severity of airflow obstruction. We cannot find evidence to support the presence of a TSLP–OX40L–dendritic cell axis in ongoing disease, but our results are compatible with the presence of a TSLP–mast cell and TSLP-nuocyte axis. The finding of increased TSLP expression in patients with severe asthma suggests that TSLP is an attractive target for the development of novel antiasthma therapies but that similar to other cytokine targets, such as IL-5 and IL-13, targeting TSLP might only be efficacious in the subset of asthma characterized by increased TSLP expression and T_H2 inflammation. Our data suggest that targeting TSLP in patients with severe asthma has the potential to inhibit both airway inflammation and remodeling and might therefore offer a new approach with which to address the unmet clinical need in this group of patients.

We thank Dr J. Bennett, Professor C. Brightling, and Dr T. J. Warke for assistance with bronchoscopy.

Key messages

- TSLP protein expression is increased in patients with severe asthma in spite of high-dose inhaled/oral corticosteroid use, but activity of the OX40-OX40L pathway is not detectable within the airways.
- IL-13^1 nuocytes might contribute to TSLP expression in patients with severe asthma.
- TSLP expression is heterogeneous and associated with T_H2 cytokine expression. Anti-TSLP treatment strategies might only be efficacious in patients with the subset of asthma characterized by increased TSLP expression and T_H2 inflammation.

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METHODS

Subjects

Leicester cohort. For the study of both gene expression and immunohistochemistry, asthmatic patients (n = 36) and healthy volunteers (n = 12) were recruited from respiratory clinics, including the Leicester Difficult Asthma Clinic; from staff at Glenfield Hospital; and from the general population through advertisement (Leicester cohort). Patients with asthma provided a suggestive history and had objective evidence of variable airflow obstruction, as indicated by 1 or more of the following: (1) methacholine airway hyperresponsiveness ($PC_{20}FEV_1$, <8 mg/mL), (2) greater than 15% improvement in FEV_1 10 minutes after 200 μg of inhaled salbutamol, and (3) peak expiratory flow (>20% maximum within-day amplitude from twice-daily peak expiratory flow measurements over a period of 14 days). Subjects underwent spirometry; allergen skin prick tests for Dermatophagoides pteronyssinus, dog, cat, grass pollen, and Aspergillus fumigatus; a methacholine inhalation test using the tidal breathing method; and sputum induction with incremental concentrations of nebulized hypertonic saline (ie, 3%, 4%, and 5%, each for 5 minutes).[E1] Patients with asthma also kept a diary card for 2 weeks before bronchoscopy, recording daytime and nighttime symptoms, daily short-acting β_2-agonist use, and twice-daily peak expiratory flow. Asthma severity was defined based on "British guideline on the management of asthma" treatment steps (mild = step 1, β_2-agonist only; moderate = steps 2 and 3, inhaled corticosteroid ≤800 mg of beclomethasone equivalent per day ± long-acting β_2-agonist; and severe = steps 4 and 5).[E2] Of the 16 patients with severe asthma at steps 4 and 5, 13 met the American Thoracic Society criteria for refractory asthma.[E3]

Belfast cohort. For examination of airway gene expression and inflammatory cell infiltration, an additional cohort of subjects from Belfast was also studied. Participants with refractory asthma were recruited from the Belfast City Hospital, where systematic evaluation protocols ensure patients have well-characterized refractory asthma.[E4] All participants had persisting symptoms despite treatment at steps 4 or 5 of the Global Initiative for Asthma guidelines. In addition, mild-to-moderate asthmatic and healthy participants were recruited either from hospital clinic or by advertisement. Subjects with asthma had a clinical diagnosis of asthma with a current history of recurrent wheezing and documented response to asthma medication. Healthy volunteers had no history of asthma or persistent respiratory symptoms and normal lung function.

In both cohorts all participants were current nonsmokers with no upper or lower respiratory tract infections in the 6 weeks before bronchoscopy. All asthmatic participants' symptoms were clinically stable on their usual medication at the time of bronchoscopy. Demographic details for each cohort are shown in Tables E1 and E2.

The study was approved by the Research Ethics Committee of both institutions (Leicester: Leicestershire, Northamptonshire, & Rutland Research Ethics Committee reference 04/Q2502/74; Belfast: Office of Research and Ethics Committee of Northern Ireland reference 06/NIR02/114). Written informed consent was gained from all participants before their involvement.

Fiberoptic bronchoscopy

Subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines.[E5] Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae, fixed in acetone, and embedded in GMA, as described previously.[E6] Biopsy specimens were also placed immediately in RNA preservative (RNAlater, Ambion) and submitted for microarray and qPCR analysis.

Immunohistochemistry

GMA-embedded tissue was cut and stained as described previously.[E6] The following primary antibodies were used (murine mAbs unless otherwise stated): sheep polyclonal anti-TSLP (catalogue no. AF1398, 7.5 μg/mL; R&D Systems [Minneapolis, Minn], Abingdon, United Kingdom), anti–mast cell tryptase clone AA1 (1:1000 dilution; Dako UK, Ely, United Kingdom), anti-CD1a clone 010 (26 μg/mL, Dako), anti-CD83 clone HB15e (1 μg/mL; AbD Serotec, Oxford, United Kingdom), anti–IL-4 clone 3H4 (12.5 μg/mL; a gift from Dr Chris Heusser, Novartis, Switzerland), rabbit polyclonal anti–IL-13 (5 μg/mL, in-house generated; Genentech, San Francisco, Calif), anti-CD68 clone PG-M1 (Dako), anti-CD3 clone UCHT1 (3 μg/mL; BD Biosciences, Oxford, United Kingdom), anti-CD4 clone MT310 (10 μg/mL, Dako), anti-CD8 clone DK25 (0.5 μg/mL, Dako), anti–neutrophil elastase clone NP57 (0.1 μg/mL, Dako), anti–eosinophil major basic protein clone BMK-13 (0.4 μg/mL; Monosan, Uden, The Netherlands), anti-OX40 clone ACT35 (5 μg/mL, BD Biosciences), anti-OX40L clone 159403 (10 μg/mL), and appropriate isotype controls (R&D, Dako, and BD Biosciences). The sections were counterstained with Mayer hematoxylin. Tonsil tissue obtained from Scottish Biomedical was embedded in GMA and used as a positive control.

Assessment and quantification of immunohistochemical staining

Epithelial, submucosal, and smooth muscle bundle areas in sections were identified and measured by using a computer analysis system (analysis docu). Numbers of positively stained nucleated cells in each compartment were counted and expressed per square millimeter. All counts were performed blind to clinical characteristics. Areas of less than 0.1 mm^2 were considered insufficient to quantify. Cells staining in sequential sections were colocalized by using the computer analysis system.

Two methods were used for quantitative assessment of TSLP expression in the epithelium. First, a thresholding technique was developed based on the HSI of TSLP staining. The HSI color system was defined by a scale of 0 to 255 for HSI. Sections were acquired at ×20 magnification and white balance corrected, and pixels of representative TSLP staining selected in the epithelium were measured. A minimum of 2 noncontiguous sections were measured for each patient. The median of the lower and upper limit of the HSI was then defined from 10 validation patients. The HSI was then selected as the final threshold to produce a threshold that appropriately captured highly saturated red light. All biopsy specimens were subsequently measured by using this final threshold. The mean percentage area of TSLP staining in 10 areas in the epithelium from 2 sections was taken as the final measurement. We also tested the final threshold measurement using comparison with a semiquantitative score of staining (0, none; 1, weak intermittent; 2, strong intermittent or weak generalized; and 3, strong generalized). We found there was a good correlation between the 2 methods (r_s = 0.837, $P < .0001$).

Prism 5 software was used for statistical analysis of immunohistochemical staining. Group comparisons were made by using the Kruskal-Wallis test; nonparametric data comparisons between groups were made with the Mann-Whitney U test, and unpaired t tests were used for parametric data. The spearman rank correlation test was used for correlations. A P value of less than .05 was considered statistically significant.

Gene expression analyses

RNA was isolated from homogenized bronchial biopsy specimens, and qPCR was performed as described previously.[E7] TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif) were purchased and conducted per the manufacturer's instructions for TSLP (id: Hs00263639_m1). RNA was amplified (Ambion) for Agilent (Santa Clara, Calif) 2-color Whole Human Genome 4x44k gene expression microarray analysis. Universal Human Reference RNA (Stratagene, La Jolla, Calif) was used for the reference channel. Probe intensities were transformed as $\log_2$ ratios of test and reference channels calculated by using the Agilent Feature Extraction software, protocol GE2-v5_95 (Agilent). Flagged outliers were not included in any subsequent analyses. All gene expression analyses and plotting were performed by using the R Project software package, version 2.10.1 (refer to http://www.R-project.org).

The T_H2 score was calculated by using a generalized procedure.[E7] Thirty-eight signature genes were selected based on array features corresponding to T_H2 signature genes with Entrez gene annotation that were previously observed to be upregulated in T_H2-high subjects were selected. If individual Entrez genes mapped to multiple probes, the probe with the greatest interquartile range was retained, and any others were removed. Gene expression values were mean centered. Missing T_H2 signature gene set values (5/2622 [0.19%]) were substituted by using mean replacement. Principal component

analysis was conducted on these expression values, and PC1 (which retained 39.5% of the variance of the signature gene set) was used as the T_H2 score.

qPCR values were calculated by using the DD method and are expressed as log_2-transformed values.

RESULTS

Inflammatory cell infiltration (Leicester cohort)

The number of epithelial and lamina propria tryptase-positive mast cells, CD68^1 macrophages, major basic protein–positive eosinophils, elastase-positive neutrophils, and T cells are shown in Table E3. Of note, mast cell numbers were increased significantly in the airway epithelium in patients with mild asthma, which is consistent with previous reports describing intraepithelial mast cells associated with T_H2 inflammation in patients with mild asthma.[E8,E9] They were also increased in the airway smooth muscle in asthmatic patients (median, 4.0; interquartile range, 0.8-9.0) compared with those seen in healthy control subjects (median, 0.0; interquartile range, 0-1.6; P 5 .029), but there was no difference across asthma severity. Eosinophil counts were increased in the airway lamina propria of asthmatic patients compared with those seen in healthy subjects, as accounted for by significant increases in the mild group (Table E3). However, 4 patients in each of the groups with moderate and severe asthma had lamina propria eosinophil counts higher than those in the mild asthma group, suggesting the presence of steroid-refractory eosinophilic inflammation. Lamina propria and epithelial eosinophil counts correlated significantly within the asthmatic population (r_s 5 0.45, P 5 .018), but consistent with previous reports,[E10] neither correlated with sputum eosinophil counts. CD68^1 macrophage density was increased in the airway epithelium of patients with mild asthma compared with that seen in healthy control subjects but not between asthmatic patients and healthy subjects as a whole (Table E3).

In keeping with previous studies of asthma,[E8] there was no significant difference in the number of epithelial or lamina propria CD3^1 or CD4^1 T cells in asthmatic patients compared with that seen in healthy subjects (Table E3). There was a significant increase in the number of CD8^1 T cells in the lamina propria of patients with mild asthma, which was of uncertain clinical significance (Table E3).

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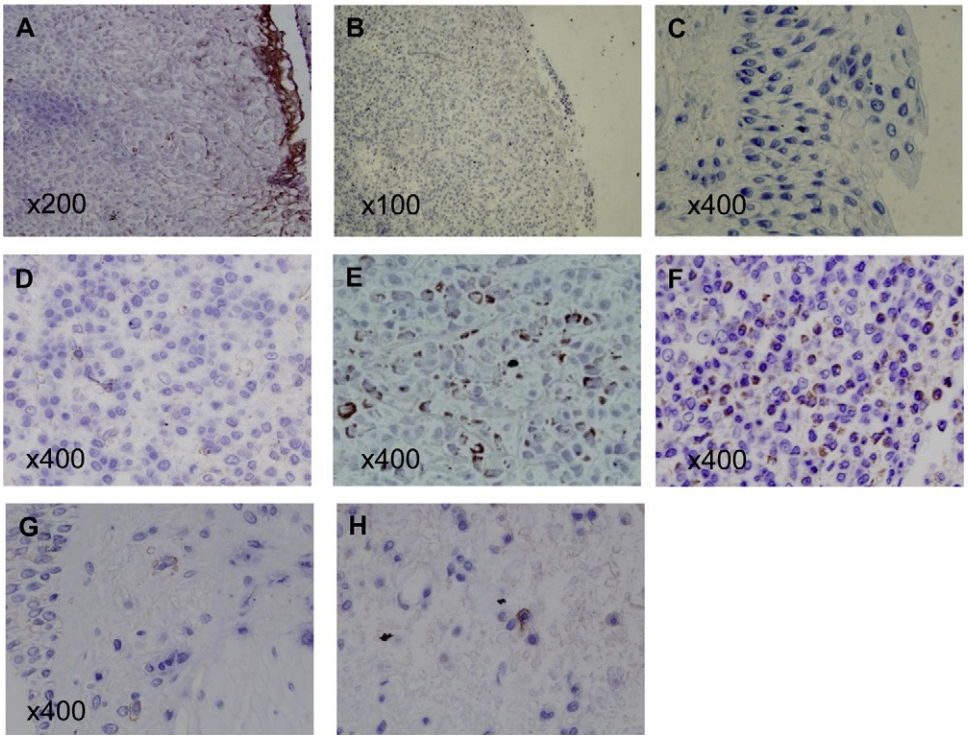

FIG E1. A and B, Positive control immunostaining for TSLP in human tonsil tissue (Fig E1, A) and negative immunostaining in tonsil tissue with sheep IgG isotype control (Fig E1, B; 7.5 mg/mL). C, Negative immunostaining with sheep IgG isotype control in a bronchial biopsy specimen from a patient with severe asthma. D-F, Immunostaining in tonsil tissue for CD83, OX40, and OX40L, respectively. G, Immunostaining for CD1a in normal bronchial epithelium and lamina propria. H, Immunostaining for CD83 in normal bronchial lamina propria.

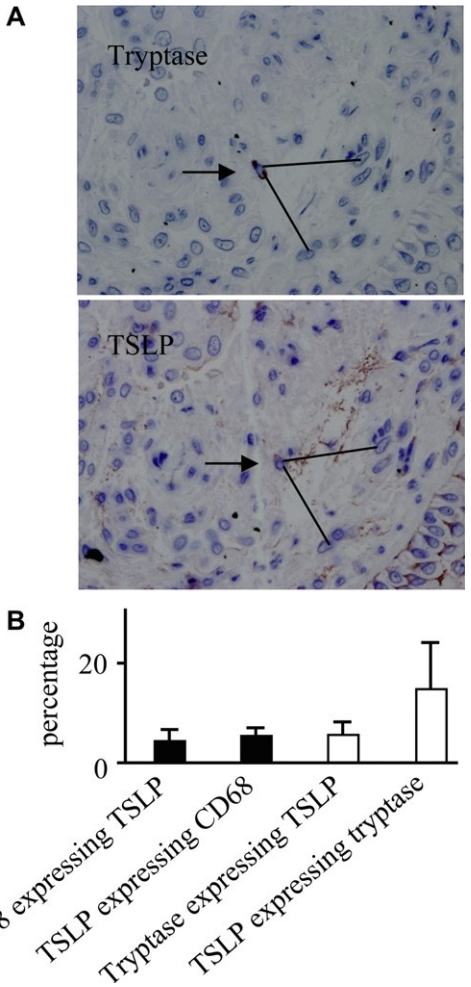

FIG E2. Colocalization of TSLP to mast cells and macrophages within the lamina propria. A, Two sequential 2-mm sections demonstrating colocalization of mast cell tryptase and TSLP (arrows). Lines demonstrate local landmarks for reference. B, The percentage of macrophages and mast cells expressing TSLP and vice versa in a subset of 6 patients with severe asthma. Immunohistology is shown at 3400 magnification.

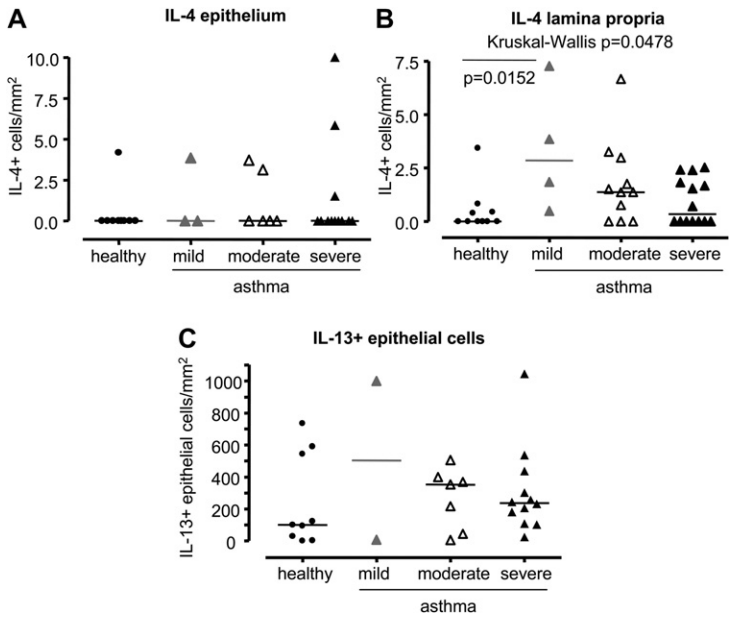

FIG E3. A and B, The density of IL-4[1] cells in the bronchial epithelium and lamina propria, respectively. C, The density of IL-13[1] epithelial cells within the airway epithelium.

TABLE E1. Demographic data of the Leicester asthmatic subjects according to disease severity

	Healthy control subjects	Patients with mild asthma (BTS step 1)	Patients with moderate asthma (BTS steps 2 and 3)	Patients with severe asthma* (BTS steps 4 and 5)	P valuey
No.	12	8	12	16	—
Age (y), mean 6 SEM	34.7 6 5.2	36.4 6 5.0	30.8 6 2.5	40.9 6 2.4	.056
Sex (M/F)	5/7	2/6	7/5	11/5	.123
Asthma duration (y), mean 6 SEM	NA	12.9 6 4.6	13.1 6 3.2	25.5 6 3.7	.017
Inhaled corticosteroid dose (mg), beclomethasone equivalents%	0	0	668 6 114	2325 6 277	.0001§
No. at BTS step 5	0	0	0	8	—
No. taking long-acting b-agonist	0	0	9	16	—
Exacerbations in last year, median (range)	NA	0 (0-2)	0 (0-3)	3 (0-12)	.001
Mean daytime symptom score, median (range)	NA	0.28 (0-1.25)	0.1 (0-1.14)	1.18 (0-3)	.091
Mean daily nighttime symptom score, median (range)	NA	0.07 (0-1.14)	0 (0-0.14)	0.32 (0-3)	.064
Reliever use/wk, median (range)	NA	2.0 (0-10)	0.5 (0-70)	30 (0-112)	.005
Sputum eosinophil count (%), geometric mean (95% CI)	0.39 (0.25-0.6)	2.77 (0.96-7.99)	3.1 (1.05-9.15)	2.85 (1.09-7.45)	
PEF amplitude (% of the mean), mean 6 SEM	NA	31.6 6 7.2	19.4 6 5.37	27.9 6 4.24	.296
FEV_1 (% predicted)	99.8 6 3.2	98.3 6 3.5	86.3 6 5.3	72.6 6 3.8	.002
FEV_1/FVC ratio (%)	81.7 6 3.6	77.0 6 2.0	73.5 6 3.58	64.5 6 3.1	.029
PC_{20} methacholine (mg/mL), geometric mean (95% CI)	>16	0.84 (0.10-7.11)	2.63 (0.87-7.97)	0.16 (0.05-0.53)	.004
Serum IgE (kU/L), geometric mean (95% CI)	32.5 (18.1-58.6)	68.6 (25.7-183.1)	228.8 (80.0-654.1)	200.5 (80.2-500.8)	.22
No. with positive skin prick test response	6	4	11	13 (n 5 15)	.051
No. with positive skin prick test response to Aspergillus fumigatus	0	1	1	4	.42

Significant values are shown in boldfaced text.

BTS, British Thoracic Society; NA, not applicable; PEF, peak expiratory flow.

*Thirteen of 16 meet the American Thoracic Society criteria for refractory asthma.

tStatistical analysis across asthma groups unless otherwise stated.

%Ratio for budesonide Turbuhaler calculated as 1.5.

§Moderate compared with severe asthma.

TABLE E2. Demographic data of the Belfast asthmatic subjects according to disease severity

	Healthy control subjects	Patients with mild-to-moderate asthma (BTS steps 1-3)	Patients with severe asthma* (BTS steps 4 and 5)	P valuey
No.	10	7	9	—
Age (y), mean 6 SEM	32.6 6 2.0	29.4 6 5.0	51.8 6 2.9	.001
Sex (M/F)	5/5	4/3	6/3	.93
Asthma duration (y), mean 6 SEM	NA	13.6 6 3.0	13.4 6 4.1	.98
Inhaled corticosteroid dose (mg), beclomethasone equivalents%	0	457 6 141§	1422 6 177.8	.0012
No. at BTS step 5	NA	0	7	—
No. taking long-acting b-agonist	NA	2	9	—
Exacerbations in last year, median (range)	NA	0 (0-2)	3 (0-6)	.006
Blood eosinophil count (3 10^9/L), geometric mean (95% CI)	0.09 (0.05-0.17)	0.17 (0.11-0.25)	0.50 (0.30-0.84)	.003
FEV_1 (% predicted)	108.8 6 4.0	100.5 6 4.3	86.1 6 5.1	.068
FEV_1/FVC ratio (%)	82.9 6 2.1	78.33 6 5.1	67.9 6 4.2	.14
Serum IgE (kU/L), geometric mean (95% CI)	22.2 (4.8-102.9)	81.1 (18.1-364.4)	363.0 (120.8-1090)	.42
No. with positive skin prick test response	ND	ND	6	—
No. with positive skin prick test response to Aspergillus fumigatus	ND	ND	0	—

BTS, British Thoracic Society; NA, not applicable; ND, not done.

*Seven of 9 meet the American Thoracic Society criteria for refractory asthma.

tStatistical analysis between asthma groups.

%Ratio for budesonide Turbuhaler calculated as 1.5.

§For the 6 subjects using inhaled corticosteroids.

TABLE E3. Inflammatory cell counts in the airway epithelium and lamina propria in patients from the Leicester cohort

	Healthy control subjects	Patients with mild asthma	Patients with moderate asthma	Patients with severe asthma
Epithelium: cells/mm^2				
Tryptase	2.8 (0-7.1)	31.7* (16.7-40.1)	9.2 (4.1-18.1)	3.2 (1.3-10.4)
MBP	0.0 (0-10.0)	13.5 (4.4-18.8)	1.7 (0.0-5.7)	3.2 (0.0-14.0)
CD68	0.0 (0-6.7)	10.0* (6.3-23.1)	2.4 (0.0-4.8)	15.3 (0.0-31.4)
Neutrophil elastase	2.8 (0-16.7)	0.0 (0.0-4.7)	3.3 (0.0-9.2)	5.6 (2.8-10.8)
CD3	33.3 (11.1-70.2)	60.0 (16.3-132.7)	31.1 (1.3-100.1)	44.7 (8.2-99.4)
CD4	3.3 (2.8-13.9)	10.0 (1.5-32.7)	5.0 (0.0-21.2)	9.8 (0.0-43.3)
CD8	14.7 (8.3-76.7)	30.2 (13.2-103.8)	9.2 (0.0-52.6)	29.1 (2.3-59.1)
Lamina propria: cells/mm^2				
Tryptase	14.5 (10.6-19.0)	12.5 (6.8-26.9)	15.1 (8.9-31.4)	8.4 (5.4-15.6)
MBP	2.4 (1.4-5.6)	11.2* (9.8-12.7)	4.4 (2.7-17.5)	5.3 (1.3-24.4)
CD68	4.0 (2.3-5.1)	4.7 (3.6-6.5)	2.7 (1.4-7.2)	4.5 (1.4-11.2)
Neutrophil elastase	9.4 (3.7-20.2)	3.4 (1.1-8.6)	8.4 (3.0-17.1)	8.9 (1.9-15.7)
CD3	31.0 (12.0-45.8)	41.0 (32.2-49.6)	19.9 (9.5-29.3)	30.0 (18.4-43.2)
CD4	9.7 (5.4-14.6)	11.5 (8.9-21.6)	7.22 (2.1-11.9)	11.7 (5.1-15.7)
CD8	20.3 (10.9-31.0)	30.2* (26.8-37.1)	11.3 (6.1-18.6)	13.3 (7.4-26.1)

Values are presented as medians (interquartile ranges).
MBP, Major basic protein.
*P < .05 compared with healthy control subjects

Individual subject database

Study number	Hospital no.	dob?	Age	Sex	Ethnicity	Diagnosis	Respiratory history	Length of asthma/EB history (yrs)
GENE 002	U4696982	08/05/1955	50	F	Caucasian	Asthma (mild)	Cough, mild exercise-induced asthma; "Chest Infections"	5
GENE 003	U4531863	20/08/1969	36	M	Caucasion	Asthma (severe)	Severe asthma	8
GENE 004	F490429	26/02/1976	29	M	Caucasian	Asthma (moderate)	asthma	3
GENE 006	u4703893	30/11/1986	19	M	Caucasian	Asthma (moderate)	asthma/ nut allergy	17
GENE 007	u4736950	16/07/1987	18	M	caucasian	normal	NIL	N/A
GENE 008	u4736947	26/12/1986	19	M	caucasian	normal	NIL	N/A
GENE 009	F076405	22/06/1941	64	F	caucasian	normal	NIL	N/A
GENE 011	U4731805	02/03/1978	28	F	caucasian	asthma (mild)	asthma	19
GENE 013	u4742745	23-Mar	56	F	caucasian	normal	NIL	N/A
GENE 014	F042892	11/09/1947	58	F	caucasian	normal	Nil	N/A
GENE 017	G382620	21/11/1983	22	M	caucasian	asthma (mild)	asthma	21
GENE 018	U4759873	08/11/1978	27	F	caucasian	asthma (moderate)	asthma	20
GENE 021	U4788623	06/02/1967	39	M	caucasian	asthma (moderate)	asthma	10
GENE 022	U4537339	03/12/1983	23	F	caucasian	normal	Nil	N/A
GENE 023	U4564457	02/05/1965	41	F	caucasian	normal	Nil	N/A
GENE 024	U4798884	01/09/1970	36	F	caucasian	asthma (moderate)	Asthma	2
GENE 026	F437287	09/04/1959	47	F	caucasian	asthma (severe)	Asthma	33
GENE 028	U4603856	16/10/1986	20	F	caucasian	asthma (moderate) with severe atopy	Asthma	17
GENE 030	U4816866	14/12/1984	22	M	caucasian	normal	Nil	N/A
GENE 031	U4610056	06/04/1986	21	F	caucasian	normal	Nil	N/A
GENE 032	U4817947	25/10/1985	21	F	caucasian	normal	Nil	N/A
GENE 034	U4812352	02/11/1978	28	F	caucasian	Asthma (severe)	Asthma	12
GENE 035	U4797235	06/01/1974	33	M	Middle Eastern	Asthma (moderate)	Asthma	1
GENE 036	U4837006	31/03/1955	52	M	caucasian	normal	Nil	N/A
GENE 037	U4802063	22/11/1966	40	F	caucasian	Asthma (moderate)	Asthma; rhinits	0.5
GENE 039	U4838215	08/05/1973	34	M	caucasian	Asthma (mild)	Asthma	21
GENE 041	U480685	19/05/1969	38	F	caucasian	Asthma (severe)	Asthma	25
GENE 044	U4830745	06/07/1972	35	F	Caucasian	Asthma (severe)	Asthma	32
GENE 045	F416168	12/07/1950	57	M	Caucasian	Asthma (severe)	Asthma	11
GENE 046	U4752499	14/08/1949	58	M	Caucasian	Asthma (severe)	Asthma	57
GENE 047	F494192	06/09/1973	34	F	Caucasian	Asthma (mild)	Asthma	0.5
GENE 048	U4653357	11/05/1969	38	M	Caucasian	Asthma (moderate)	Asthma	23
GENE 049	U4539962	09/10/1981	26	F	Caucasian	Asthma (mild)	cough on exertion 12 months; GP diagnosed possible asthma	1
GENE 051	U4816335	28/08/1978	29	M	Caucasian	Asthma (severe)	Asthma since childhood	25
GENE 053	U4618654	10/04/1985	23	F	Caucasian	Asthma (moderate)	Asthma	15
GENE 054	U4856784	11/04/1943	65	F	Caucasian	Asthma (mild)	Asthma	35
GENE 055	U4879081	18/05/1975	32	F	Caucasian	Asthma (mild)	Asthma	0.66
GENE 056	U4878332	02/04/1976	32	M	Caucassian	Asthma (severe)	Asthma	28
GENE 057	U4650980	03/07/1970	37	M	Caucasian	Asthma (severe)	Asthma	13
GENE 058	F037635	08/04/1976	32	M	Caucasian	Asthma (severe)	Asthma	25
GENE 059	F301045	13/10/1953	44	M	Caucasian	Asthma (moderate)	Asthma	38
GENE 060	U4896732	07/06/1987	21	M	Caucasian	Asthma (moderate)	Asthma	11
GENE 061	U4628761	24/01/1954	54	M	Caucasian	Asthma (severe)	Asthma	19
GENE 062	F355000	08/06/1960	48	F	Caucasian	Asthma (severe)	Asthma	45
GENE 063	F433120	25/02/1960	48	M	Caucasian	Asthma (severe)	Asthma	40
GENE 064	U4810226	10/12/1986	21	M	Asian	Normal	Nil	0
GENE 066	U4612354	18/06/1970	38	M	Asian	Asthma (severe)	Asthma	0.5
GENE 067	U4626601	27/02/1971	37	M	Caucasian	Asthma (severe)	Asthma	35

Current asthma treatment
None
seretide 500mcgs BD; Salbutamol 200mcgs PRN; theophylline 500mgs BD; methotrexate 12mgs weekly
Symbicort 100/6mcg OD; Salbutamol 200mcg PRN
symbicort 400/12 mcg bd
NIL
NIL
NIL
salbutamol 100mcg
NIL
nil
salbutamol 200mcg PRN
symbicort 400/12 BD; salbutamol 200mcg PRN
Beclazone 200mcg BD; salbutamol 200mcg PRN
nil
nil
Seretide 500mcg OD; salbutamol 200mcg PRN
Prednisolone 20mg OD PO; Symbicort 400/12 2 INH BD; Theophylline slow release 200mg BD PO; Monteleukast 10mg OD PO; Salbutamol 2.5mg NEB OD; Salbutamol 200mcg INH PRN
Budesonide 200mcg BD; Terbutaline PRN
Nil
Nil
Nil
Symbicort 400/12 BD; Salbutamol 200mcg PRN; Uniphylline 200mg BD PO
Symbicort 200/6 BD; Bricanyl 500mcg PRN
Nil
Pulmicort BD; salbutamol 200mcg PRN
Salbutamol 200mcg PRN
Symibort 600/18 BD & PRN; Bricanyl 500mcg PRN; Prednisolone 7.5mg PO OD; Uniphylline 200mg PO OD
Symbicort 400/12 BD & PRN; Salbutamol 2.5mg NRB PRN; Monteleukast 10mg PO OD; Aminophylliine 450mg/675mg alternate days PO; Kenalog 80 mg once a month IM; Salbutamol m/r tablets 8mg PO BD
Flixotide 500mcg BD; Salmeterol 100mcg BD; Salbutamol 200mcg PRN
Seretide 500mcg BD; Salbutamol 200mcg PRN
Salbutamol 200mcg PRN
Fluticasone 250mcg BD; Salmeterol 50mcg BD; Salbutamol 200mcg PRN
Salbutamol 200mcg PRN
Symbicort 400/12 BD; Combivent 20/200 2 puffs ODS and PRN; Prednisolone 7.5mg OD PO; Monteleukast 10mg OD PO
Seretide 100mcg OD; Salbutamol 200mcg PRN
Salbutamol 200mcg PRN
Salbutamol 200mcg PRN
Symbicort 400/12 BD and PRN; Combivent 2 puffs PRN; monteleukast 10mg OD; uniphylline 200mg PO PRN BD
Budesonide 1200mcg BD; oxis 12mcg BD; salbutamol 200mcg PRN
Prednisolone 40mg OD PO; theophylline 200mg TBS PO; Symbicort 800/24 BD; Salbutamol 200mcg INH PRN; Salbutamol 2.5mg Neb PRN
Beclomethasone 200mcg BD; Salmeterol 50 mcg BD; Salbutamol 200mcg PRN
Seretide 100mcg BD; Salbutamol 200mcg PRN
Symbicort 400/12 BD + PRN (8-10 puffs of 200/6 per day); Salbutamol 200mcg PRN; Prednisolone 35mg OD PO for 7 days each month
Symbicort 400/12 BD + PRN; Uniphylline 400mg OD PO; Atrovent 40mcg BD; salbutamol 2.5mg NEB PRN; monteleukast 10 mg OD; prednisolone 10mg OD
Symbicort 400/12 BD; Monteleukast 10mg OD; Theophylline SR 200mg BD; Salbutamol 200mcg PRN
Nil
Symbicort 400/12 BD; Monteleukast 10mg OD; Salbutamol 200mcg PRN
Symbicort 400/12 BD and PRN

Study number	Current symptoms	Severe excerbations
GENE 002	Cough	0
GENE 003	breathless, wheeze	1
GENE 004	ADC	0
GENE 006	None	0
GENE 007	None	N/A
GENE 008	None	N/A
GENE 009	None	N/A
GENE 011	ADC	2
GENE 013	None	N/A
GENE 014	None	N/A
GENE 017	None	0
GENE 018	None	2
GENE 021	None	0
GENE 022	None	N/A
GENE 023	None	N/A
GENE 024	Intermittent wheeze	0
GENE 026	Frequent cough, wheeze and dyspnoea	10
GENE 028	Exercise and cold induced wheeze	0
GENE 030	None	N/A
GENE 031	None	N/A
GENE 032	None	N/A
GENE 034	None	2
GENE 035	None	0
GENE 036	None	0
GENE 037	dry cough on exertion	0
GENE 039	None	0
GENE 041	Wheeze and dyspnoea on exertion	3
GENE 044	dyspnoea, wheeze & dry cough on exertion	12
GENE 045	cough on exertion	3
GENE 046	dyspnoea & wheeze on exertion	0
GENE 047	dyspnoea on exertion	0
GENE 048	None	0
GENE 049	cough on exertion	0
GENE 051	frequent cough and wheeze	1
GENE 053	cough and wheeze on exertion	0
GENE 054	None	0
GENE 055	cough and wheeze on exertion	0
GENE 056	daily dyspnoea and intermittent	3
GENE 057	frequent wheeze, cough and dyspnoea	4
GENE 058	cough and wheeze on exertion	12
GENE 059	daily dyspnoea on exertion and dry cough	3
GENE 060	dyspnoea and wheeze on exertion	0
GENE 061	dyspnoea and intermittent cough and wheeze	12
GENE 062	dyspnoea on exertion	0
GENE 063	dyspnoea and wheeze on exertion	0
GENE 064	nil	0
GENE 066	dry cough	0
GENE 067	dyspnoea and dry cough	3

Study number	Current non asthma treatment	Allergies
GENE 002	Sertraline	Nil
GENE 003	Nil	Aspirin
GENE 004	Nil	Horses
GENE 006	Neoclarityn 10mgs PRN; epipen 1mg / ml PRN	cat; dog; HDM; Grass; aspergillus; nuts
GENE 007	nil	nil
GENE 008	nil	nil
GENE 009	NIL	Grass
GENE 011	metformin	U/K
GENE 013	Premarin OD; Cod liver oil tablet	Nil
GENE 014	Prempack OD	NIL
GENE 017	nil	NIL
GENE 018	brevinor T OD; neoclarityn 5mg OD	Nuts
GENE 021	nil	nil
GENE 022	Combined Oral Contraceptive Pill	nil
GENE 023	Combined Oral Contraceptive Pill	Nil
GENE 024	Coal Tar Topical PRN	Nil
GENE 026	Risedronate 35mg/week; Nasonex Nasal OD	Nil
GENE 028	Azathioprine 100mg OD; Lamotragine 75mg BD	Nil
GENE 030	Nil	Nil
GENE 031	Nil	Nil
GENE 032	Nil	Nil
GENE 034	Fluoxetine 20mg OD	Nil
GENE 035		Nil
GENE 036	Nil	Nil
GENE 037	Nasonex x1 nasal OD; fluoxetine 20mg OD; ibuprofen PRN	Nil
GENE 039	Nil	Nil
GENE 041	Clarityn 10mg PO OD	Nil
GENE 044	omeprazole 40mg PO OD; Frusemide 20mg PO PRN	Nil
GENE 045		Nil
GENE 046	Penicillin 30mg PO BD	Nil
GENE 047	Nil	Nil
GENE 048	Flixonase 100mcg Nasal OD	NSAID anaphylaxis
GENE 049	Thyroxine 75mcg PO OD	Nil
GENE 051	methotrexate 5mg OD PO; folic acid 5mg/week PO; loratidine 10mg OD PO; ranitidine 150mg OD PO; adcal one tablet OD PO	Azathioprine; penicillin; erythromycin
GENE 053	Oral Contraceptive Pill OD	Nil
GENE 054	Cetirizine 10mg PO PRN; Epipen IM PRN	Latex
GENE 055	Nil	Nil
GENE 056	terfenadine 180mg OD	Nil
GENE 057	Nil	Nil
GENE 058	Mirtazepine 45mg OD	Penicillin
GENE 059	Loratidine 10mg OD PO; Triamcinolone 55mcg Nasal OD	Nil
GENE 060	Nil	Nil
GENE 061	Betnesol Nasal Spray BD	Nil
GENE 062	Esomeprazole 40mg OD	Nil
GENE 063	Mometasone x2 nasal OD	Erthromycin
GENE 064	Nil	Nil
GENE 066	Mometasone x2 nasal OD	Nil
GENE 067	Nil	Nil

Study number	Other history
GENE 002	Cholecystectomy; Pneumonia 14yr ago
GENE 003	Removal of left kidney; Nasal polypectomy
GENE 004	Eczema; Allergic rhinitis
GENE 006	Rhinitis ?polyps; Nut, banana, latex allergy
GENE 007	nil
GENE 008	pin in ankle
GENE 009	NIL
GENE 011	?food allergy/intolerance; polycystic ovaries
GENE 013	hysterectomy
GENE 014	arthritis
GENE 017	gastric ulcer secondary to NSAID's
GENE 018	cervical CIN grade I; migraine; nut allergy
GENE 021	nil
GENE 022	nil
GENE 023	Nil
GENE 024	Pericarditis; Psoriasis
GENE 026	Iron deficiency anaemia - normal Gastroscopy in the past
GENE 028	Eczema; Epilepsy
GENE 030	Nil
GENE 031	Nil
GENE 032	Nil
GENE 034	Depression
GENE 035	Nil
GENE 036	Nil
GENE 037	Depression; lactose intolerance
GENE 039	Nil
GENE 041	Nil
GENE 044	Intermittent odema; pregnancy associated cardiomyopathy 1998 - resolved post delivery; last echo in 2006 normal
GENE 045	Nil
GENE 046	Chronic right leg lymphoedema; Left leg DVT 1997
GENE 047	Nil
GENE 048	Rhinits; NSAID anaphylaxis but celecoxib tolerant
GENE 049	Hypothyroid
GENE 051	Severe eczema; alopecia totalis; vitiligo; seasonal rhino-conjunctivitis
GENE 053	Eczema
GENE 054	Oral allergy syndrome; latex allergy
GENE 055	Possible Crohn's Disease but currently no symptoms or treatment
GENE 056	Appendicectomy
GENE 057	Nil
GENE 058	Depression
GENE 059	Nasal Polyps; hayfever
GENE 060	Nil
GENE 061	Nasal polyps; umbilical hernia
GENE 062	Dog allergy; peanut allergy; oesophagitis
GENE 063	Post nasal drip
GENE 064	Nil
GENE 066	Hayfever; nasal polyps
GENE 067	Nil

Study number	Family history	Smoking history	Pets
GENE 002	Nil	Nil	Nil
GENE 003	father 'chesty'; no resp. prob diagnosed	10 pk /years	Cats x2
GENE 004	Nil	NIL	Incomplete
GENE 006	Nil	NIL	nil
GENE 007	Nil	nil	Nil at present; kept budgie from age 13 - 17 years
GENE 008	nil	nil	none
GENE 009	Son has asthma	nil	Cat; Dog
GENE 011	Incomplete	nil	Incomplete
GENE 013	Nil	3.7	dog
GENE 014	Nil	Nil	canary; budgie; owl
GENE 017	grandfather has asthma	2	Nil
GENE 018	Nil	1	2 cats
GENE 021	father may have asthma	Nil	2 dogs; kept sheep 4 years ago
GENE 022	grandmother had emphysema	Nil	Nil
GENE 023	Nil	0	1 cat
GENE 024	mother had childhood pleurisy, and breast cancer; sister had Hodgkins Lymphoma	1	1 cat
GENE 026	Father - Asthma	0	Nil
GENE 028	Paternal uncle - asthma	0	1 cat; 1 dog
GENE 030	Brother - Mild Asthma	0	Nil
GENE 031	Nil	0	Nil
GENE 032	Nil	0	Nil
GENE 034	Brother - asthma	0	dog
GENE 035	Nil	0	Nil
GENE 036	Nil	0	Nil
GENE 037	Nil	0	Nil
GENE 039	Grandmother - Eczema	0	Dog
GENE 041	Grandfather - asthma	0	2 Hamsters
GENE 044	Maternal aunt - asthma	0	Rabbit
GENE 045	Brother - asthma	5	Nil
GENE 046	Nil	0	Nil
GENE 047	Sister - asthma	0	cat
GENE 048	Mother - asthma	0	Nil
GENE 049	sister - eczema	0	
GENE 051	mother, sister and son - all asthma	0	Nil
GENE 053	Nil	0	Nil
GENE 054	father - asthma	0	Nil
GENE 055	mother - possible asthma	<1	dog and 2 goldfish
GENE 056	brother - asthma; sister - multiple allergies	<1	Nil
GENE 057	Nil	0	2 dogs
GENE 058	Grandfather, father and 2 sisters have asthma	<1	Nil
GENE 059	Grandfather - asthma	0	Nil
GENE 060	Nil	0	Nil
GENE 061	sister and daughter - asthma	0	Nil
GENE 062	Father and Brother - asthma	1	dog
GENE 063	Grandfather - asthma	2	Nil
GENE 064	Nil	0	Nil
GENE 066	Nil	0	Nil
GENE 067	Nil	<1	Dog

Study number	Daytime symptom score	Night time symptom score	Reliever b2 agonist use/week	PEF amplitude	FEV1	FEV1 % pred	FEV1/FVC ratio (%)	reversibilty to b agonist (%)	PC20 (mg/ml)	Historical sputum eos (%)	Current neutro count
GENE 002	1.1	0.5	0	24%	2.75	92	83	0	4.6	3	69.5
GENE 003	3	3	35	18.75%	3	72	75	8	0.06	25	74.7
GENE 004	ADC	ADC	ADC	ADC	2.65	68	59	35	0.37	not done	34.5
GENE 006	0	0	0	6.30%	3.2	73	62	2	3.3	not done	90.95
GENE 007	N/A	N/A	N/A	N/A	3.38	75	60	11	>16	not done	80
GENE 008	N/A	N/A	N/A	N/A	5.15	101	87	4	>16	not done	24.1
GENE 009	N/A	N/A	N/A	N/A	1.95	101	68	0	>16	not done	48.75
GENE 011	ADC	ADC	ADC	ADC	3.6	106	74	20	2.8	not done	47.5
GENE 013	N/A	N/A	N/A	N/A	2.81	107	80	5	>16	not done	61.3
GENE 014	N/A	N/A	N/A	N/A	2.7	104	73	0	>16	not done	48.6
GENE 017	0	0.07	2	10.53%	4.6	94.3	77	4.6	1	N/A	8
GENE 018	0.07	0	0	12.35	2.72	83	83	3	0.22	N/A	N/A
GENE 021	0.43	0	3	6.8	3.8	95	75	2.6	11.5	N/A	78.2
GENE 022	N/A	N/A	N/A	N/A	3.71	107	97	1	>16	N/A	75.2
GENE 023	N/A	N/A	N/A	N/A	3.21	109	83	1	>16	N/A	74
GENE 024	0	0	0	22.2	3.4	96	88	1	0.5	N/A	67
GENE 026	1.7	1.78	35	67	1.38	58	50	8			41.
GENE 028	0.57	0	6	26.1	3.15	107	90	6	>16	N/A	25.75
GENE 030	N/A	N/A	N/A	N/A	5.1	101	100	0	>16	N/A	2.68
GENE 031	N/A	N/A	N/A	N/A	3.7	109	91	0	>16	N/A	87.1
GENE 032	N/A	N/A	N/A	N/A	3.65	107	91	0	>16	N/A	15.4
GENE 034	0.14	0.28	5	27.9	3.05	89	85	0	0.04	N/A	14.5
GENE 035	0	0	0	8.3	2.8	77	76	2	3	N/A	18.25
GENE 036	N/A	N/A	N/A	N/A	3.1	82	69	0	>16	N/A	no count
GENE 037	1.14	0	2	10.5	3.2	93	73	0	4.29	N/A	2.5
GENE 039	0.28	0	2	13.3	3.2	78	67	2	0.19	N/A	N/A
GENE 041	ADC	ADC	ADC	ADC	1.81	61	68	0	1.25	6.7; 2.9; 19; 6.7; 5.7	49.9
GENE 044	ADC	ADC	ADC	ADC	2.63	89	69	6	0.22	N/A	N/A
GENE 045	1.5	0	6	23.9	2.45	65	60	0	13	N/A	50
GENE 046	1.07	0	39	16.67	1.41	41	31	4	0.027	N/A	92.6
GENE 047	0.64	0.42	6	66.67	3.65	108	75	8	0.08	N/A	N/A
GENE 048	0	0	0	8.33	4	103	79	3	>16	15	N/A
GENE 049	0.21	0	0	41.76	3.41	101	81	7	8.4	N/A	97.25
GENE 051	ADC	ADC	ADC	ADC	2.17	61	55	35	0.059	N/A	87
GENE 053	0.14	0.07	1	33.33	3.33	102	81	4	10.91	N/A	26.3
GENE 054	0	0	0	35.05	1.62	106	75	7	0/saline	16.25	54
GENE 055	1.25	1.14	10	30.14	3.25	101	84	2	15.45	N/A	29
GENE 056	1.28	0.5	46	26.92	3.7	97	75	0	1	N/A	N/A
GENE 057	1.57	1.64	32	34.78	2.9	74	64	0	2.5	N/A	37.25
GENE 058	2.21	2.36	112	18.75	2.5	68	63	6	0.001	N/A	70
GENE 059	1.14	0.14	70	59.87	1.76	43	48	6	0.5	N/A	6
GENE 060	ADC	ADC	ADC	ADC	4.85	96	68	0	1	N/A	94.25
GENE 061	ADC	ADC	ADC	ADC	2.8	78	76	0	0.25	N/A	43.5
GENE 062	0.93	0.35	22	43.4	1.51	65	70	N/A	0.03	N/A	84.5
GENE 063	0.14	0	7	17.54	3.38	91	58	1	0.9	N/A	76.9
GENE 064	N/A	N/A	N/A	N/A	4.18	94	81	0	N/A	N/A	4.25
GENE 066	0	0	0	18.18	2.7	88	71	0	0.06	30	59.75
GENE 067	0	0	28	21.34	2.61	64	62	10	0.012	N/A	68

Study number	Current sputum eos (%)	blood eos x109/L	Exhaled NO (ppb)
GENE 002	2	0.4	39.3
GENE 003	0	1	20.1
GENE 004	33.5	0.52	33.1
GENE 006	3.1	0.81	4.9
GENE 007	0	0.1	23.7
GENE 008	0	0.08	24.9
GENE 009	1	0.1	10.5
GENE 011	0.75	0.08	11.1
GENE 013	1	0.2	15
GENE 014	0	0.06	10.9
GENE 017	5.5	0.15	34.3
GENE 018	1.4	0.2	5.4
GENE 021	8.06	0.18	N/A - broken
GENE 022	0.25	0.11	22
GENE 023	0	0.05	17
GENE 024	1.5	0.31	27
GENE 026	30.4		
GENE 028	14.75	0.45	60
GENE 030	0.25	0.1	20
GENE 031	0.5	0.12	65
GENE 032	0	0.11	12
GENE 034	2.5	0.44	20
GENE 035	0.5	0.09	27
GENE 036	0	0.09	23
GENE 037	0.5	0.08	37
GENE 039	4.1	0.48	N/A
GENE 041	9.9	0.29	16
GENE 044	N/A	0.02	N/A
GENE 045	4.5	0.83	N/A
GENE 046	2.4	0.39	N/A
GENE 047	7.48	0.52	99.5
GENE 048	0.25	0.18	17
GENE 049	0	0.44	56
GENE 051	0	0.49	
GENE 053	18.7	0.06	33.7
GENE 054	9.8	0.35	20.9
GENE 055	5.5	0.59	52.7
GENE 056	0.5	0.2	21.5
GENE 057	60.25	0.62	97
GENE 058	1.5	0.1	33.9
GENE 059	25	0.43	N/A
GENE 060	1	0.15	73.5
GENE 061	4	0.6	28.9
GENE 062	0.75	0.29	N/A
GENE 063	0.48	0.16	N/A
GENE 064	1.5	0.24	N/A
GENE 066	13	0.63	58
GENE 067	15.2	0.63	38

Skin tests
Histamine 6mm; others NAD
HDM 4mm; Histamine 4mm
Cat 4mm; Dog7mm; HDM 30mm; Histamine 5mm
dog 4mm; hdm 7mm; grass 5mm; aspergillus 4mm; cat4mm
Grass 9mm
Histamine 4mm; Others NAD
Histamine 6mm; Dog 3mm; HDM 10mm
histamine 8mm; cat 5mm; dog 10mm; Grass 10mm; HDM 8mm; Aspergillus 10mm
histamine 3mm; grass 8mm; HDM 1.5mm
Histamine 5mm
dog 5mm; HDM 10mm
Histamine 3mm; cat 5mm; dog; 3mm; grass; 8mm; 3 Tree 4mm; HDM 8mm; alternaria tenus 5mm; peanut 10mm; almond 5mm; brazil nut 11mm; hazel nut 6mm; cashew 3mm; walnut 3mm
positive control 3mm; dog 3mm
All negative
positive control 5mm; HDM 3mm; Timothy grass 4mm
positive control 4mm; dog 3mm; HDM 12mm
All negative
positive control 2mm; timothy grass 9mm; HDM 8mm; dog 5mm; cat 6mm
positive control 1mm; dog 1mm; HDM 2mm
positive control 6mm; HDM 10mm
positive control 2mm; HDM 2mm
positive control 3mm; HDM 6mm; Timothy Grass 7mm
positive control 6mm; cat 4mm; dog 3mm; HDM 10mm
positive control 5mm
positive control 4mm
positive control 3mm; cat 4mm; HDM 13mm; Timothy Grass 8mm
positive control 4mm; cat 3mm; dog 3mm; timothy grass 5mm
positive control 3mm
positive control 4mm; cat 11mm; HDM 11mm; aspergillus 4mm
positive control 7mm; cat 12mm; dog 4mm; HDM 15mm; timothy grass 8mm; aspergillus 10mm
positive control 3mm
positive control 5mm; negative control 2mm; cat 10mm; dog 5mm; HDM 13mm; timothy grass 9mm
positive control 3mm
N/A
positive 2mm; cat 7mm; HDM 5mm; timothy grass 3mm
positive control 4mm; HDM 3mm; birch 5mm; almond 2mm
positive control 3mm
positive control 4mm; cat 10mm; dog 4mm; grasses 7mm; three tree 5mm; HDM 4mm; aspergillus fum 2mm; cladasporium 2mm; penicillium 2mm
positive control 7mm; dog 6mm; HDM 7mm
positive control 4mm; dog 4mm; HDM 4mm; aspergillus 4mm
positive control 3mm; cat 3mm; HDM 4mm; timothy grass 9mm
positive control 4mm; cat 12mm; dog 5mm; HDM 8mm; timothy grass 10mm
positive control 4mm; cat 6mm; dog 4mm; HDM 6mm; timothy grass 6mm
positive control 3mm; cat 5mm; grasses 10mm; HDM 8mm
positive control 4mm; cat 6mm; dog 2mm; grasses 8mm; HDM 10mm
positive control 5mm
positive control 3mm; timothy grass 4mm
positive control 5mm; cat 5mm; dog 6mm; HDM 4mm; timothy grass 3mm; aspergillus 3mm

Study number	Total IgE kU/L	Mast cells/mm2 ep	Mast cells/mm2 lamina prop	Mast cells/mm2 smooth muscle	Eos/mm 2 ep	Eos/mm2 lp	CD3+ /mm2 ep	CD3+ /mm2 lp	CD4+/m m2 ep	CD4+/m m2lp	CD8+/m m2 ep	CD8+/m m2 lp	Neutrophils /mm2 ep	Neutrophils /mm2 lp
GENE 002	25.8													
GENE 003	19.3	1.72	6.36	0.00	2.33	11.43	9.30	47.14	10.47	96.43	30.23	26.07	27.91	15.7
GENE 004	3547	0.00	31.44	7.69	1.67	17.53	45.00	25.26	1.67	7.22	0.00	11.34	5.00	9.79
GENE 006	146	58.91	37.69		0.00	17.66	109.90	94.96	9.90	40.21	60.40	38.72	6.93	18.99
GENE 007	42	7.14	12.37		0.00	5.67	70.24	19.59	0.00	13.92	95.24	20.62	2.38	2.06
GENE 008	113	33.33	30.48	0.00	10.00	12.38	73.33	66.67	3.33	14.76	76.67	33.33	3.33	10.00
GENE 009	15	0.00	16.04		0.00	2.59	17.65	33.49	2.94	41.75	14.71	34.43	0.00	4.25
GENE 011	91.3		2.96			11.37		39.10		9.03		28.50		8.41
GENE 013	20.2	3.39	19.20	0.00	0.00	2.17	0.00	46.01	1.69	11.23	12.71	23.91	0.00	19.91
GENE 014	<2.0	2.78	20.97	0.00	0.00	1.21	11.11	48.39	13.89	37.10	4.17	36.69	2.78	20.56
GENE 017	364	33.33	16.22			14.22	60.00	42.94	10.00	12.21	84.62	30.79	3.33	3.24
GENE 018	201	20.83	34.82	0.75	2.08	2.68	0.00	0.00	25.00	11.90	8.33	11.01	0.00	4.46
GENE 021	119	10.00	5.48		0.00	4.41	5.00	19.92	0.00	8.56	0.00	11.90	0.00	11.1
GENE 022	9.5		10.05			5.37		6.31		3.27		8.64		18.93
GENE 023	29.8	4.17	12.08		8.33	2.01	33.33	9.40	41.67	2.01	8.33	8.05	16.67	8.05
GENE 024	25.7		4.68			3.39		9.52		2.10		6.13		4.03
GENE 026	25	14.29	22.73	17.14	11.90	24.43	14.29	36.36	0.00	2.84	2.38	7.39	4.76	6.25
GENE 028	>5000	10.00	11.76	2.50	0.00	21.49	0.00	18.55	0.00	10.41	10.00	11.99	0.00	1.8
GENE 030	14.7	2.08	9.63		2.08	0.93	66.67	45.03	12.50	6.68	8.33	17.70	18.75	20.34
GENE 031	61	0.00	15.91		0.00	0.97	11.11	28.57	2.78	8.44	5.56	20.45	2.78	3.57
GENE 032	34.8	30.00	18.35		13.33	4.48	41.67	26.89	3.33	10.92	25.00	20.17	16.67	8.82
GENE 034	87	0.00	2.81		0.00	1.34	329.63	43.20	46.30	15.38	129.63	29.59	3.70	1.92
GENE 035	141		15.08		3.06	1.82		23.62		3.27		10.55		1.26
GENE 036	21.6	0.00	3.90	0.00	0.00	1.77	28.95	8.51	7.89	4.96	15.79	6.74	0.00	1.42
GENE 037	17.8	4.69	8.91		0.00	1.98	17.19	8.42	0.00	0.00	0.00	0.50	1.56	2.97
GENE 039	76.4	42.31	8.77		11.54	9.88	165.38	45.36	26.92	12.25	123.08	31.46	0.00	1.1
GENE 041	92.3	30.00	10.00	8.97	20.00	0.00	5.00	30.00	0.00	11.67	0.00	8.33	0.00	1.67
GENE 044	61.2	blood clot thoughout block												
GENE 045	300	33.33	16.45		87.50	30.92	95.83	34.21	79.17	19.74	58.33	17.76	0.00	0.66
GENE 046	3086	4.76	10.66	3.45	72.62	43.65	45.24	18.44	13.10	5.12	35.71	17.83	8.33	10.86
GENE 047	8.9													
GENE 048	148													
GENE 049	235	12.24	25.24		2.04	9.71	32.65	35.92	3.06	10.68	16.33	29.61	6.12	9.22
GENE 051	>5000	2.34	11.17	8.33	0.00	4.74	131.31	79.80	55.14	71.33	110.23	53.72	11.21	10.6
GENE 053	375	3.85	22.00	9.38	25.00	14.40	200.00	103.60	140.00	59.20	70.00	62.00	10.00	8.40
GENE 054	74.8		8.01	4.17	15.38	12.15	100.00	62.43	38.46	49.45	73.08	54.14	0.00	3.59
GENE 055	47.6	30.00	32.00	4.00	20.00	11.00	0.00	21.00	0.00	8.50	10.00	21.50	0.00	1.00
GENE 056	349		5.56			0.34		12.29		5.22		10.94		5.05
GENE 057	336	8.97	5.38		1.28	8.97	110.26	21.03	42.31	12.05	61.54	13.33	5.13	4.36
GENE 058	48.9	0.00	15.57		0.00	0.82	48.48	31.97	7.58	9.02	21.21	22.13	156.06	36.89
GENE 059	439		10.71			2.86		29.29		5.00		18.57		17.1
GENE 060	211	8.33	19.74		8.33	9.21	70.83	14.47	8.33	1.32	29.17	3.95	29.17	19.08
GENE 061	127	4.10	5.00		4.10	37.05	44.26	44.55	9.02	7.95	27.87	4.09	10.66	8.86
GENE 062	128	2.00	8.42	13.64	8.00	8.66	0.00	10.40	0.00	0.74	2.00	10.40	8.00	17.33
GENE 063	36	1.47	7.38	2.68	5.15	5.33	51.47	22.95	28.68	12.30	16.18	6.15	5.15	29.51
GENE 064	158	0.00	13.18	3.13	15.00	6.96	435.00	41.77	135.00	8.23	255.00	18.35	170.00	20.89
GENE 066	802	0.93	3.05	0.00	0.00	2.64	31.48	27.44	7.41	15.65	43.52	29.67	6.02	13.0
GENE 067	2303	9.09	16.67	0.00	0.00	2.22	0.00	3.70	0.00	0.37	0.00	7.41	0.00	1.85

Study number	Macrophages /mm2 ep	Macrophages /mm2 lp	OX40/mm 2 ep	OX40/ mm2 lp	OX40L/ mm2ep	OX40L/mm2 lp	CD83/mm2 ep	CD83/ mm2 lp	CD1a/mm2 ep	CD1a/m m2 lp	IL-4 3H4/mm2 ep	IL-4 3H4/mm2 lp	IL-13 fixed/mm2 ep epi cells
GENE 002													
GENE 003	20.93	3.21	0.00	0.53	1.16	0.71	4.60	2.83	17.24	1.59	1.52	1.82	101.5
GENE 004	1.67	7.22	3.33	0.52	0.00	0.52	6.67	3.09	0.00	1.03	0.00	3.26	504.1
GENE 006	59.90	37.54	0	1.93	0.99	0.30	0.99	1.78	0.00	0.89	0.00	6.67	352.5
GENE 007	0.00	5.15	0.00	0.00	0.00	0.00	0.00	0.00	9.52	7.46	0.00	0.00	734.6
GENE 008	6.67	12.86	0.00	0.95	0.00	0.00	10.00	4.29	36.67	2.38	4.17	3.44	543.7
GENE 009	0.00	0.71	0.00	0.00	0.00	0.94	2.94	1.18	0.00	1.18	0.00	0.00	28.9
GENE 011		2.34		0.47									
GENE 013	0.85	4.35	0.00	2.17	0.00	1.45	0.00	2.17	0.00	0.00	0.00	0.45	95.0
GENE 014	0.00	3.63	0	0.81	4.17	0.40	0.00	1.61	0.00	0.81		0.39	
GENE 017	3.33	4.39	0.00	0.76	0.00	0.38	0.00	1.34		0.00		3.85	
GENE 018	4.17	2.68	0.00	0.60	0.00	0.89	10.42	3.87	0.00	0.00	3.13	0.00	215.6
GENE 021	5.00	1.07	0.00	0.67	0.00	0.40	2.50	1.20	0.00	0.43	0.00	2.99	368.1
GENE 022		2.34		0.00		0.00		0.70		0.00			
GENE 023	0.00	2.68	0.00	0.00	0.00	0.67	poor quality tissue		poor quality tissue				
GENE 024		1.13		0.65		1.61		0.81		0.48		0.00	
GENE 026	0.00	3.41				1.14	0.00	5.68	0.00	0.61	0.00	0.00	180.0
GENE 028	0.00	2.49	0.00	0.00	5.00	1.13	0.00	1.80	0.00	1.10	0.00	0.00	44.7
GENE 030	8.33	4.97	0.00	0.00	0.00	0.16	0.00	0.62	0.00	0.00	0.00	0.00	100.0
GENE 031	0.00	2.27	2.80	0.30	0.00	0.32	2.80	2.30	2.80	0.00	0.00	0.00	0.0
GENE 032	3.33	5.18	0.00	0.00	0.00	0.00	0.00	1.12	0.00	0.00	0.00	0.83	3.4
GENE 034	7.41	1.33	0	0.15	0.00	0.44	3.70	2.07	0.00	0.00	0.00	0.00	243.4
GENE 035		5.03		0.25		0.25		1.51		1.51		1.76	4.5
GENE 036	0.00	0.71			0.00	0.00	2.60	1.77	2.60	0.00	0.00	0.00	122.7
GENE 037	3.13	1.98			0.00	0.99	0.00	0.50	0.00	0.00			
GENE 039	19.23	4.97			0.00	0.00	3.85	2.98	0.00	0.33	3.85	1.85	7.6
GENE 041	0.00	0.56			0.00	0.00	25.00	3.89	0.00	0.00		poor tissue none left	
GENE 044													
GENE 045	54.17	11.18	0.00	0.70		0.00	33.30	17.80	0.00	1.30		0.00	
GENE 046	10.71	4.51	0	0.4	0.00	1.43	6.00	4.70	2.40	1.20	0.00	2.42	23.4
GENE 047													
GENE 048						0.00						0.76	
GENE 049	9.18	6.31	1.02	0.97	2.04	1.46		0.00		0.00	0.00	0.49	
GENE 051	31.31	15.80			2.80	0.34	7.94	5.98	0.93	0.90	0.00	1.67	435.9
GENE 053	0.00	12.40			0.00	0.00	0.00	0.00	0.00	1.60		1.52	
GENE 054	26.92	6.91			0.00	0.91	8.33	2.27	0.00	0.00	0.00	7.27	1000.0
GENE 055	10.00	4.00			0.00	1.00	0.00	5.47					
GENE 056		1.35				0.51	0.00	0.00	0.00	0.62		2.53	
GENE 057	38.46	10.26			0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00	230.7
GENE 058	31.82	18.03			0.00	0.00	0.00	0.82	0.00	0.00	0.00	0.00	301.6
GENE 059		1.43						0.00		0.00		1.37	
GENE 060	0.00	5.92			0.00	0.00	2.08	0.90	0.00	1.35	3.70	1.38	398.1
GENE 061	27.05	16.36					3.28	0.23	0.82	0.00	0.00	0.00	257.1
GENE 062	0.00	2.97			0.00	0.25	0.00	0.89	0.00	1.12	0.00	0.71	1043.7
GENE 063	19.85	5.74			0.00	0.00	2.17	2.50	1.09	3.06	0.00	0.00	535.7
GENE 064	90.00	4.43				0.00	0.00	0.51	6.67	0.00	0.00	0.00	590.0
GENE 066	7.41	6.10			0.00	0.20	0.00	1.22	0.00	0.41	5.85	1.55	107.4
GENE 067	0.00	1.11			0.00	0.00	0.00	1.85	0.00	1.48	10.00	2.40	205.0

Study number	IL-13 fixed/mm2 ep other cells	IL-13 fixed/mm2 lp	TSLP % /ep	TSLP SCORED /ep	TSLP /mm2 lp
GENE 002					
GENE 003	18.18	0.78	0.61	0.50	3.57
GENE 004	33.33	1.45	0.78	0.50	2.06
GENE 006	50.00	3.33	3.38	2.00	6.23
GENE 007	11.54	0.48	1.37	1.00	9.79
GENE 008	10.42	0.63	1.92	1.50	0.00
GENE 009	0.88	0.00	0.75	0.50	0.94
GENE 011					2.34
GENE 013	5.00	0.00	1.00	0.00	2.17
GENE 014		2.36	0.32	0.50	1.6
GENE 017		1.03	4.20	2.50	0.57
GENE 018	6.25	1.72	0.89	2.00	0.60
GENE 021	0.00	0.00	3.49	2.50	0.13
GENE 022					0.00
GENE 023			0.14	0.50	0.67
GENE 024		0.00	5.80	3.00	0.16
GENE 026	0.00	0.00	7.39	3.00	10.80
GENE 028	7.89	0.00	0.21	0.00	2.49
GENE 030	21.43	0.00	1.99	1.00	0.16
GENE 031	0.00	0.00	0.31	0.50	0.65
GENE 032	1.15	0.17	0.03	2.00	0.14
GENE 034	13.04	0.00	2.60	0.00	0.00
GENE 035	1.53	2.76			
GENE 036	0.00	0.00	0.49	0.00	0.00
GENE 037					0.99
GENE 039	0.00	1.23	2.54	2.00	0.00
GENE 041					1.1
GENE 044					
GENE 045		3.18	3.74	3.00	11.1
GENE 046	33.67	0.40	12.78	3.00	5.33
GENE 047					
GENE 048		0.00			0.00
GENE 049		0.00	4.21	3.00	0.49
GENE 051	65.49	0.48	5.15	2.50	0.56
GENE 053		0.00	0.24	0.00	0.00
GENE 054	90.00	1.16	1.37	1.00	1.82
GENE 055					0.39
GENE 056		0.63	3.45	1.50	8.64
GENE 057	17.95	0.00	5.58	3.00	3.33
GENE 058	33.87	0.00	3.35	2.00	1.64
GENE 059		0.00			1.43
GENE 060	22.22	0.69	3.43	2.00	3.29
GENE 061	21.43	0.00	2.67	1.50	2.05
GENE 062	59.38	0.71	2.10	2.00	4.95
GENE 063	30.36	0.36	5.45	3.00	13.1
GENE 064	30.00	0.00	0.90	1.50	0.00
GENE 066	1.06	3.35	5.60	2.50	6.30
GENE 067	15.00	0.00	1.95	1.50	0.74

Letter of ethical approval

9620-04.

Leicestershire Local Research
Ethics Committee Two
Lakeside House
4 Smith Way
Grove Park
Enderby
Leicester
LE19 1SS

21 October 2004

Dr P Bradding
Reader/Honorary Consultant
University of Leicester/ UHL NHS Trust
c/o R&D Office
LGH

Dear Dr Bradding,

Full title of study: Gene expression profiling in mild and severe asthma, eosinophilic bronchitis and normal controls
REC reference number: 04/Q2502/74
Protocol number: 2, 04-Q2502-74rp040827.doc

Thank you for your letter of 07 October 2004, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation.

The favourable opinion applies to the following research site:

Site: UHL NHS Trust; Genentech, USA
Principal Investigator: Dr P Bradding

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type: Application
Version: 1, 04-Q2502-74af040827.ofd
Dated: 01/09/2004
Date Received: 06/09/2004

Document Type: Investigator CV
Version: 1, 04-Q2502-74cv040827.doc
Dated: 27/08/2004
Date Received: 27/08/2004

Document Type: Protocol
Version: 2, 04-Q2502-74rp040827.doc
Dated: 24/08/2004
Date Received: 27/08/2004

Document Type: Peer Review
Version: 1
Dated: 09/07/2004
Date Received: 06/09/2004

Document Type: Copies of Advertisements
Version: 2, 04-Q2502-74ra-p041018.ppt
Dated: 18/10/2004
Date Received: 18/10/2004

Document Type: Letters of Invitation to Participants
Version: 1, 04-Q2502-74il-p040827.doc
Dated: 28/06/2004
Date Received: 27/08/2004

Document Type: GP/Consultant Information Sheets
Version: 1, 04-Q2502-74is-dr040827.doc
Dated: 28/06/2004
Date Received: 27/08/2004

Document Type: Participant Information Sheet
Version: 2, 04-Q2502-74is-p(unmarked)041014.doc
Dated: 14/10/2004
Date Received: 14/10/2004

Document Type: Participant Information Sheet
Version: 2, 04-Q2502-74is-p041014.doc
Dated: 14/10/2004
Date Received: 14/10/2004

Document Type: Participant Consent Form
Version: 2, 04-Q2502-74cf-p040723.doc
Dated: 23/07/2004
Date Received: 27/08/2004

Document Type: Participant Consent Form
Version: 2, 04-Q2502-74cf-p041014.doc
Dated: 14/10/2004
Date Received: 14/10/2004

Document Type: Response to Request for Further Information
Version: 1, Response to ethics.doc
Dated: 07/10/2004
Date Received: 18/10/2004

Management approval

The study may not commence until final management approval has been confirmed by the organisation hosting the research.

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH AND DEVELOPMENT

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05 October 2004

Dr Peter Bradding,
Department of Respiratory Medicine and Thoracic Surgery
Glenfield Hospital
Leicester

Dear Dr Bradding,

ID: 09620 **Gene expression profiling in mild and severe asthma, eosinophilic bronchitis and normal controls**

LREC Ref: 04-Q2502-74 **MREC Ref:**

We have now been notified by the Ethical Committee that the above titled project has been given favourable ethical opinion subject to receiving a complete response to the requested information listed in the Ethics letter dated 28-09-04.

In order to secure full Trust approval, please send one hard copy of the amended paperwork together with an electronic copy to the Research Office as soon as possible. We will pass this on to the Ethics Committee on your behalf.

Since all other aspects of your R+D Notification have been completed, I anticipate that there will be no further barrier to the approval of your project by the Trust as soon as full Ethical authorisation has been granted.

However, please note that the Trust has NOT yet given approval to this project, nor indemnified it, until you receive the full, formal approval letter from this office.

I look forward to hearing from you.

Yours sincerely

Chris Cannaby
R&D Programme Board Co-ordinator