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Regulation of IL-4 mediated signalling in primary human bronchial fibroblasts by IL-13Rα2

by

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ABSTRACT

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REGULATION OF IL-4 MEDIATED SIGNALLING IN PRIMARY HUMAN BRONCHIAL FIBROBLASTS BY IL-13Rα2

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Fibroblasts are key effector cells involved in airway inflammation and remodelling in asthma. Interleukin (IL)-4 and IL-13 are important cytokines in the asthma phenotype which act on fibroblasts and other cell types. These cytokines exhibit overlapping functions through use of a common receptor, IL-4Rα:IL-13Rα1. Another receptor, IL-13 Receptor α 2 (IL-13Rα2), originally thought to be a decoy receptor for IL-13, has recently been shown to attenuate responses to IL-4 as well as IL-13, by an unknown mechanism. In this thesis, I tested the hypothesis that IL-13Rα2 is responsible for the regulation of IL-4 mediated signalling in bronchial fibroblasts and that regulation by IL-13Rα2 is altered in asthma.

The expression of IL-4 and IL-13 receptors on human bronchial fibroblasts (HBFs) was highly dynamic. IL-13Rα2 expression was significantly increased in response to both IL-4 and IL-13 over 24 hours, requiring de novo protein synthesis. A significant rapid reduction in IL-4Rα expression was also observed in response to either ligand, although levels rapidly returned to normal after removal of the stimulus. Use of a neutralizing antibody showed that induction of IL-13Rα2 suppressed STAT-6 activation and the pro-inflammatory effects of IL-4 and IL-13. No difference was observed in receptor expression levels or the regulatory effects of IL-13Rα2 between healthy and asthmatic subjects.

IL-13Rα2 was also up-regulated by a range of Th1 stimuli including IFNγ and IFNβ, as well as double stranded RNA (dsRNA), with no disease-related differences. The up-regulation of IL-13Rα2 in response to dsRNA hampered attempts to knock down surface expression of IL-13Rα2 using siRNA, but revealed a potential role for IL-13Rα2 in the anti-viral response due to its ability to down-regulate responses to IL-4 and IL-13.

An over-expression model of IL-13Rα2 identified the potential for IL-4 to cause activation of STAT3 mediated by IL-13Rα2. In HBFs naturally expressing high levels of IL-13Rα2, addition of IL-4, but not IL-13, significantly increased activation of STAT3, a transcription factor associated with cell survival.

Whilst IL-13Rα2 may have beneficial anti-inflammatory effects by suppressing STAT-6 mediated responses, further work is required to determine potential pro-fibrotic consequences of IL-4/IL-13Rα2 mediated STAT3 activation in HBFs. Since no difference was observed in IL-13Rα2 expression or in its anti-inflammatory efficacy in HBFs from normal or asthmatic donors, these data suggest that the atopic environment is more important than intrinsic differences in the ability of asthma-derived fibroblasts to respond to IL-4 and IL-13.

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DECLARATION OF AUTHORSHIP

I, Gemma Campbell-Harding, declare that the thesis entitled "Regulation of IL-4 mediated signalling in primary human bronchial fibroblasts by IL-13Rα2" and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published (see Appendix B for details).

Signed: Date: 31 October 2011

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Abbreviations

3′ UTR	3′ Untranslated Region
A2	Phospholipase A2
ADAM	A Disintigrin And Metalloprotease
AHR	Airway Hyperresponsiveness
αSMA	α Smooth Muscle Actin
ANOVA	Analysis Of Variance
AP-1	Activator Protein 1
APC	Allophycocyanin
APS	Ammonium Persulphate
ATS	American Thoracic Society
B2B	BEAS-2B Cells
B2B-FL	BEAS-2B Cells Transfected with Full Length IL-13Rα2
BAL	Bronchoalveolar Lavage
BEBM	Bronchial Epithelial Basal Medium
BEGM	Bronchial Epithelial Growth Medium
BSA	Bovine Serum Albumin
BTS	British Thoracic Society
CCL11	Chemokine (C-C motif) Ligand 11
CD4	Cluster of Differentiation 4

CD8	Cluster of Differentiation 8
CD23	Cluster of Differentiation 23
cDNA	Complimentary Deoxyribonucleic Acid
CHX	Cycloheximide
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
CT	Cycle Threshold
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
dNTP	Deoxyribonucleotide Triphosphate
DPBS	Dulbecco's Phosphate Buffered Saline
dsRNA	Double Stranded Ribonucleic Acid
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGR-1	Early Growth-Response Gene Product 1
ELISA	Enzyme Linked ImmunoSorbant Assay
EMTU	Epithelial Mesenchymal Trophic Unit
EPO	Eosinophil Peroxidase
EPX/EDN	Eosinophil Protein X/Eosinophil Derived Neurotoxin
FACS	Fluorescence Activated Cell Sorting

FAM	6-carboxyfluoroscein
FBS	Foetal Bovine Serum
FcεRI	High-Affinity IgE Receptor
FcεRII	Low-Affinity IgE Receptor
FEV_1	Forced Expiratory Volume in One Second
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead Box P3
FVC	Forced Vital Capacity
γc	Interleukin 2 Receptor γ Chain
GBM	Glioblastoma Multiforme
GCH	Goblet Cell Hyperplasia
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
H_2SO_4	Sulphuric Acid
HBFs	Human Bronchial Fibroblasts
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric Acid
HRP	Horseradish Peroxidase
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICS	Inhaled Corticosteroids
IFN	Interferon
IFNAR	Interferon α Receptor
IFNR	Interferon Receptor
IFN α/β R NAb	Interferon α/β Receptor Neutralising Antibody

Ig	Immunoglobulin
IGF-1	Insulin-Like Growth Factor 1
IL	Interleukin
IL-4Rα	Interleukin 4 Receptor α
IL-13Rα1	Interleukin 13 Receptor α 1
IL-13Rα2	Interleukin 13 Receptor α 2
IL-13Rα2 NAb	Interleukin 13 Receptor α 2 Neutralising Antibody
IPF	Idiopathic Pulmonary Fibrosis
IRS	Insulin Receptor Substrate
ITS	Insulin, Transferrin, Sodium Selenite
JAK	Janus Kinase
kDa	KiloDaltons
LABA	Long Acting β_2 Agonist
LPS	Lipopolysaccharide
LTC_4	Leukotriene C_4
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MBP	Major Basic Protein
MHC II	Major Histocompatibility Complex II
MIP	Macrophage Inflammatory Protein
MMLV	Murine Moloney Leukemia virus
MMP	Matrix Metalloprotease
mRNA	Messenger Ribonucleic Acid
NAb	Neutralising Antibody

NT	No Treatment
OD	Optical Density
PBECs	Primary Bronchial Epithelial Cells
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC_{20}	Provocation Concentration of Agonist Causing 20 % Decrease in FEV_1
PE	R-Phycoerythrin
PGD_2	Prostaglandin D_2
PI3K	Phosphoinositide 3-Kinase
PIAS	Protein Inhibitor of Activated STAT
Poly I:C	Polyinosinic:Polycytidylic Acid
PVDF	Polyvinylidene Difluoride
R130Q	Arginine to Glutamine Mutation at Position 130
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rs number	RefSNP Accession ID
RSV	Respiratory Syncytial Virus
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RV	Rhinovirus
SABA	Short Acting β_2 Agonist
sADAM33	Soluble ADAM33
SARP	Severe Asthma Research Program
SDS	Sodium Dodecyl Sulphate

SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SFM	Serum Free Medium
sIL-13Rα2	Soluble IL-13Rα2
sIL-4Rα	Soluble IL-4Rα
siRNA	Small Interfering RNA
SMFI	Specific Mean Fluorescent Intensity
SNP	Single Nucleotide Polymorphism
SOCS	Suppressor of Cytokine Signalling
STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TGFβ	Transforming Growth Factor β
TGS	Tris-Glycine-SDS buffer
Th0	Naïve T Helper Cell
Th1	T Helper Cell Type 1
Th2	T Helper Cell Type 2
TLR	Toll-Like Receptor
TMB	3-3′-5-5′-Tetramethylbenzidine
TNFα	Tumour Necrosis Factor α
Treg	Regulatory T Cell
Tris	Tris (Hydroxymethyl) Aminomethane
Tris-HCl	Tris (Hydroxymethyl) Aminomethane Hydrochloride
TSLP	Thymic Stromal Lymphopoietin
Tyk2	Tyrosine Kinase 2

U-BIOPRED	Unbiased BIOmarkers in PREDiction of Respiratory Disease Outcomes
UBC	Ubiquitin C
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Introduction

1.1 Asthma

Asthma is a chronic respiratory disease classically characterised by reversible airflow obstruction, airway hyperresponsiveness (AHR) to various innocuous stimuli, significant airway remodelling and inflammation. [1] Asthma manifests as recurrent episodes of breathlessness, wheezing, coughing and chest-tightness due to widespread airflow obstruction, which is generally reversible, either spontaneously or with treatment. However there is much heterogeneity of symptoms between patients and with severe asthma, for example, the reversibility may be reduced to fixed airway obstruction. [2] The aetiology of asthma has not been clearly defined, although it is considered to be a multi-factoral disease involving a range of genetic and environmental triggers. [3]

Approximately 300 million people worldwide suffer from asthma, and figures released by Asthma UK suggest that currently 5.2 million asthmatics in the UK require treatment. Of these, 1.1 million (1 in 10) are children and 4.2 million (1 in 12) are adults. [4] The socio-economic impact of asthma is substantial. This is not only due to direct healthcare costs, such as medications and hospital admissions, and indirect burdens such as absence from work but also the reduction in quality of life, all of which affect not only the patient but also their family and friends. The costs of asthma increase with symptom and disease severity. This is particularly pronounced in more

severe cases, where the disease is poorly controlled and frequent emergency care is often required. [5–9] At present, 2.6 million people in the UK suffer from severe symptoms associated with asthma, of which 500,000 people are not effectively treated by the traditional therapy of steroids. [10] Recent attempts to develop novel therapeutics for asthma have been relatively unsuccessful with very few reaching clinical trials, therefore there is an unmet clinical need.

1.2 Symptoms of Asthma

Asthma is characterised by episodic airflow obstruction resulting in shortness of breath, wheezing, chest tightness or cough, with symptoms often occurring in the early morning hours, however the heterogeneity of asthma means the severity of symptoms experienced differs between patients.

As these symptoms are episodic, asthmatic patients experience cycles of stability with few to no symptoms interspersed with acute periods of symptom exacerbation. The severity of these exacerbations varies greatly between patients, ranging from slight dyspnoea, wheeze or cough to significant airflow reduction impacting on normal day to day tasks such as gentle exercise and speech. These symptoms may resolve on their own, but often require treatment with a β_2-agonist to relax the airway smooth muscle. Whilst asthmatic airflow obstruction is generally considered to be totally reversible, in more severe cases only partial reversibility with treatment may be observed.

Asthma exacerbations can be triggered by a variety of innocuous stimuli and asthma is often categorised as either extrinsic (allergic) or intrinsic (non-atopic) in origin. [11] With extrinsic asthma, allergens derived from pollen, house dust mites, mould and animal dander can trigger an exacerbation, whilst intrinsic asthma is more commonly associated with other trivial stimuli such as cold air and pollutants.

Extrinsic asthma generally becomes apparent during childhood and may only occur transiently, with few or no symptoms in adulthood. In allergic asthma allergen avoidance can reduce the requirement for medication. [12] Intrinsic asthma tends to have a later age of onset and is associated with no history of atopy. [11,13]

One of the main clinically defined signs of asthma exacerbation is a rapid decrease in FEV_1. Forced expiratory volume in one second (FEV_1) is a key measurement of lung function, which can also be used to determine the reversibility of the airflow obstruction by monitoring the FEV_1 both before and after administration of a bronchodilator. The ratio of the FEV_1 and the forced vital capacity (FVC), another measurement of lung function, (FEV_1/FVC) can be used to determine airflow limitation and is necessary for ruling out other respiratory conditions which can easily be confused with asthma, such as chronic obstructive pulmonary disease (COPD) or bronchitis. [14,15]

Asthmatic airway hyperresponsiveness (AHR) to innocuous stimuli can also be measured using provocation tests, where a known stimuli, such as methacholine, is administered and the patient is monitored for the drop in FEV_1. [16]

1.3 Airway Structure

The airway consists of several histologically distinct layers: the epithelium, submucosa and smooth muscle layer (Figure 1.1).

The epithelial layer lines the airway lumen. This layer includes a variety of distinct epithelial cell types, including ciliated epithelia, basal cells, clara cells and goblet cells, each with discrete functions and localisations. [17–19] These epithelial cells act as the first line of defence against allergens, viruses and pollutants as they form a highly protective structural barrier. [20] Goblet cells and mucus glands interspersed within this layer produce a viscous mucus which traps foreign particulates. This forms part of the mucociliary

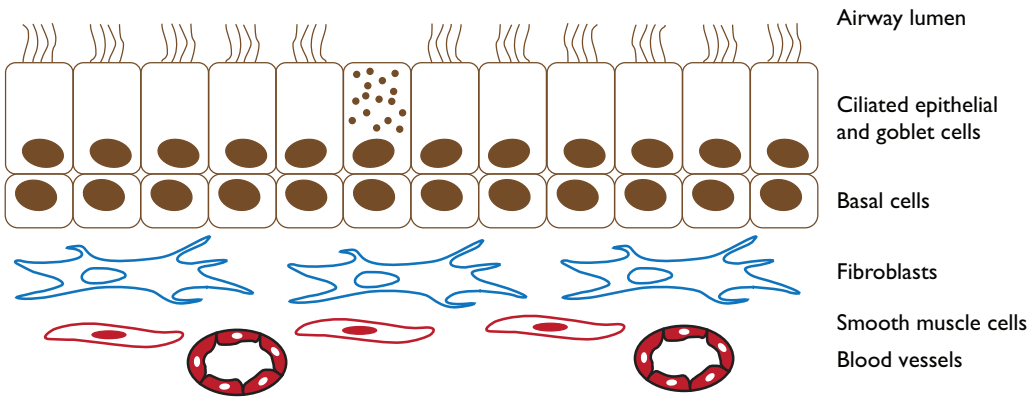

Figure 1.1: Normal structure of the inner airway wall. The airway consists of histologically distinct layers. The airway lumen is lined with epithelium, which consists of a layer of ciliated epithelial cells, which may be interspersed with mucus producing goblet cells, and subtended by basal cells, forming a physical, chemical and immunological barrier. Beneath this layer, lies the structural cells, in the form of fibroblasts and smooth muscle, and the vasculature. Adapted from [16]

escalator in conjunction with the cilia on the lumenal surface of the epithelia, which facilitates removal of foreign particles. As well as passive defence mechanisms, epithelial cells are also capable of mounting and modulating the immune response. Viruses and bacteria can be internalised, resulting in the release of cytotoxic and anti-microbial peptides. [20] Epithelial cells can also produce a series of cytokines and chemokines including Tumour Necrosis Factor (TNF) α, Interleukin (IL)-1β and macrophage inflammatory protein (MIP) 2, reactive oxygen species (ROS), adhesion molecules such as inter-cellular adhesion molecule 1 (ICAM-1), surfactant proteins, Toll-like receptors (TLRs) and growth factor receptors. [16,21,22]

Underneath the epithelial layer is the lamina propria, containing fibroblasts, myofibroblasts, extracellular matrix (ECM), nerves and capillaries. [19,22] Fibroblasts are mesenchymal cells, located directly below the basement membrane, that play an important role in wound repair. These are structural cells that produce ECM proteins, such as collagen, as well as matrix degrading proteases, such as matrix metalloproteases (MMPs), which are involved in

normal tissue turnover and repair after injury. [23,24] Fibroblasts also secrete an array of cytokines and are important for regulating host defence mechanisms as they recruit and activate leukocytes. [25–28] Upon stimulation with Transforming Growth Factor (TGF) β, fibroblasts can differentiate into myofibroblasts, which are contractile cells, important in wound repair, that secrete many cytokines. [29,30]

Beneath the lamina propria is the submucosa, containing smooth muscle cells, cartilage and connective tissue. This layer is of particular structural importance in the lung, with the smooth muscle bundles and cartilage providing support. [19]

1.4 Pathophysiology of Asthma

In the asthmatic airway there are many changes associated with the disease phenotype, involving a wide range of cell types. These phenotypic differences include significant airway inflammation as well as remodelling of the tissue, although the degree of each can vary substantially between patients.

1.4.1 Airway Inflammation

Asthma is considered an inflammatory disease, traditionally characterised by eosinophilia, mast cell activation and the predominance of type 2 T helper (Th2) cells. [31] These cells also induce the production of a range of inflammatory mediators by macrophages, epithelial cells and fibroblasts. However, as asthma covers a wide spectrum of clinical phenotypes there is variation observed between patients in the manifestation of this inflammation. For instance, many patients can be clustered as being either eosinophilic, neutrophilic, or mixed in their inflammation, whilst others are referred to as paucicellular, where limited numbers of both eosinophils and neutrophils are observed. [32]

Inflammation in the asthmatic lung, unlike the episodic nature of the symptoms, is persistent [33] and there has been debate regarding a link to the severity of the disease. [2,34–36]

There are many cells involved in the inflammatory response within the lung. These include eosinophils, T lymphocytes and mast cells.

Mast Cells

Mast cells store cytoplasmic granules containing a range of inflammatory mediators such as histamine, major basic protein (MBP), prostaglandins, leukotrienes and cytokines, many of which induce bronchocontriction. [37] Whilst classically viewed as being activated by allergens via high affinity immunoglobulin E (IgE) receptors, these cells can also degranulate due to changes in osmotic pressure which occur during exercise. Greater expression of these cells in airway smooth muscle has been observed in asthmatic patients and this has been linked to airway hyperresponsiveness. [38]

Eosinophils

Eosinophilia is often observed in asthma. [34] Whilst increased eosinophil numbers have been linked to disease severity, this does not appear to always be the case, with recent observations of both discordant eosinophilia in the absence of symptoms and clusters of non-eosinophilic patients with severe asthma symptoms. [2,32,39] In allergic and asthmatic individuals, eosinophils, however, have been found to exhibit increased migration, adhesiveness and degranulation. [40–42]

Like mast cells, eosinophils contain a range of toxic granule proteins, including eosinophilic cationic protein (ECP), MBP, eosinophil peroxidase (EPO) and eosinophil protein X/eosinophil derived neurotoxin (EPX/EDN), the release of which can cause serious tissue damage, and this is thought to contribute to remodelling in the asthmatic lung. These cells also produce a range of mediators and cytokines, such as leukotriene C_4 (LTC_4), eotaxin,

Th2 cytokines and TGFβ, all of which are involved in furthering the inflammatory and remodelling effects observed in asthma. [43]

T Cells

Naïve T lymphocytes can differentiate into either effector or regulatory T cells depending on the surrounding cytokine milieux. [44] Effector T cells induce clearance of pathogens, and can be further subdivided into Th1, Th2 or Th17 cells, each producing their own subset of cytokines, referred to as Th1, Th2 and Th17 cytokines. [16,44–46] The actions of these effector cells are controlled by regulatory T cells (Tregs), which prevent excessive clearance of commensal organisms and auto-immunity. [44]

The Th1 phenotype (driven by the presence of IL-12) is associated with an innate immune response to bacteria and viruses. [16] The Th2 humoral-immune phenotype (driven by IL-4), on the other hand, is generally associated with parasite clearance. [47–49] In many cases of asthma and allergy, this Th2 response appears to have been misdirected towards innocuous antigens. [16,46]

Differentiation of these effector T cells is not terminal and can be antagonised by the other Th phenotypes. For instance when low concentrations of IL-4 are present Th2 cells trans-differentiate into Th1, and the reverse occurs in low concentrations of Interferon-γ, thus enabling self-regulation of each of these phenotypes. [45,46]

Antigen presentation can also affect the differentiation pattern of naïve T cells. [45,46] Allergens presented by dendritic cells result in decreased levels of IL-12, leading to differentiation to Th2. These cells subsequently release IL-4, which has a positive feedback effect on the Th2 phenotype. These Th2 cells release a variety of mediators, which act on B cells, eosinophils and mast cells, triggering the release of further inflammatory cytokines and chemokines. [45]

In particular, the Th2 lymphocytes produce an array of cytokines including IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF (granulocyte-macrophage

colony-stimulating factor), which are important in the aetiology of the asthma phenotype. [50–56]

Fibroblasts

Fibroblasts are structural cells, which are important in airway remodelling, however they also play a role in the inflammation aspects of the disease, due to their ability to produce a wide range of inflammatory mediators. These include eotaxin, a chemoattractant for eosinophils, thymic stromal lymphopoietin (TSLP), which triggers dendritic cell-mediated Th2 inflammation and IL-6, which is a pro-inflammatory cytokine with broad ranging effects. [57–59] The production of these mediators occurs in response to the Th2 cytokines and affects a range of cell types, including eosinophils, T cells and mast cells, propagating the effects of the inflammation. [57,59]

Early & Late Phase Response

In asthma this inflammatory response can be separated into an early and a late phase (Figure 1.2). [16]

The early phase (Figure 1.2), occurs within 30 minutes and resolves within 2 hours of exposure to an allergen or other trivial stimuli. [16,22] This results in a rapid decrease in the FEV_1 due to smooth muscle contraction caused by IgE-dependent degranulation of mast cells, [60,61] leading to the release of many inflammatory mediators, including leukotrienes, which are particularly problematic in asthma. [62,63] These lipids, also produced by leukocytes, eosinophils, monocytes and macrophages, promote localised inflammation at the site of tissue injury and cause bronchial smooth muscle contraction, eosinophil chemotaxis and oedema by opening up tight junctions between the cells. [63,64]

The late phase response occurs several hours after exposure to the allergen or trivial stimulus and generally resolves by 24 hours. [16,22] During this period the initial stimuli is no longer present, instead the response is caused by

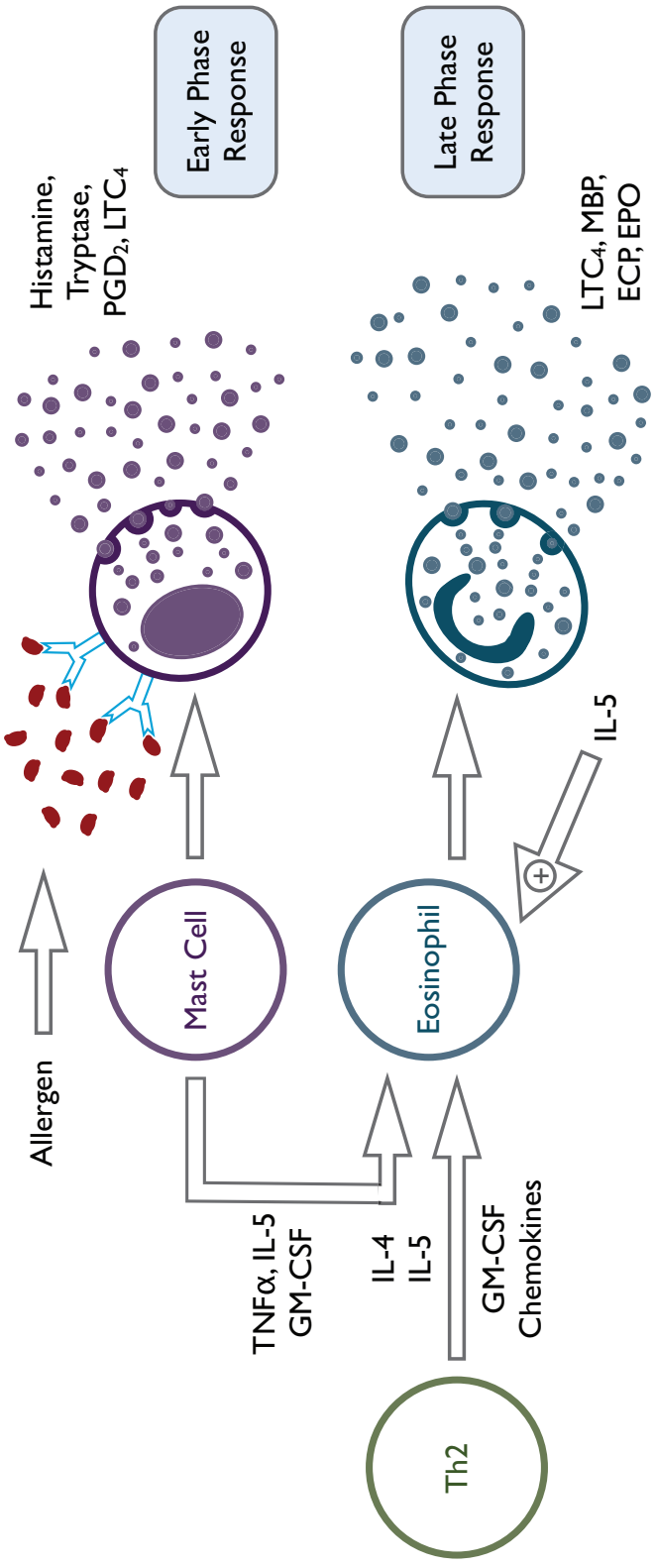

Figure 1.2: The early and late phase response. Asthmatic exacerbations can be split into early and late phase responses. The mediators released during the early phase cause the late phase response. ECP = Eosinophil Cationic Protein, EPO = Eosinophil Peroxidase, GM-CSF = Granulocyte-Macrophage Colony Stimulating Factor, IL = Interleukin, LTC_4 = Leukotriene C_4, MBP = Major Basic Protein, PGD_2 = Prostaglandin D_2, Th2 = T helper cell type II, TNFα = Tumour Necrosis Factor α Adapted from [16]

the mediators released by the mast cells in the early phase (Figure 1.2). These mediators cause a sustained decrease in FEV_1 and an infiltration of the airways with inflammatory cells. These cells include eosinophils, Th2 cells, neutrophils and monocytes, all of which release Th2-type cytokines.[65–69] These Th2 cytokines are pro-inflammatory, which cause further smooth muscle contraction,[70] epithelial damage,[71] increased mucus production[72] and an increase in adhesion molecules on the endothelium resulting in recruitment of leukocytes.[25,26]

Whilst these inflammatory mechanisms are essential for wound repair, in asthma this process appears to be misdirected. When inflammation becomes unregulated it can lead to long term airway damage in the form of remodelling.[1]

1.4.2 Airway Remodelling

Many cell types are dysregulated in the asthmatic lung, which leads to airway remodelling, resulting in loss of lung function (Figure 1.3). The alterations in the airway structure observed in asthma include disruption of the airway epithelium due to epithelial shedding and goblet cell hyperplasia, subepithelial fibrosis, in particular with thickening of the submucosa due to increased deposition of extracellular matrix proteins, as well as increased numbers of myofibroblasts and enhanced vascularisation.

Epithelial Barrier

Loss of barrier function caused by bronchial epithelial shedding and disruption of tight junction proteins is commonly observed in asthma, resulting in increased permeability, affecting the integrity of the underlying tissue.[73,74]

Under normal conditions, the tight junction proteins, such as occludin, zonula occludens 1–3, claudins, and junctional adhesion molecules, maintain the structural integrity and apical–basal polarity of the epithelial cell barrier. However, in asthma the tight junction is disrupted,[74] resulting in epithelial

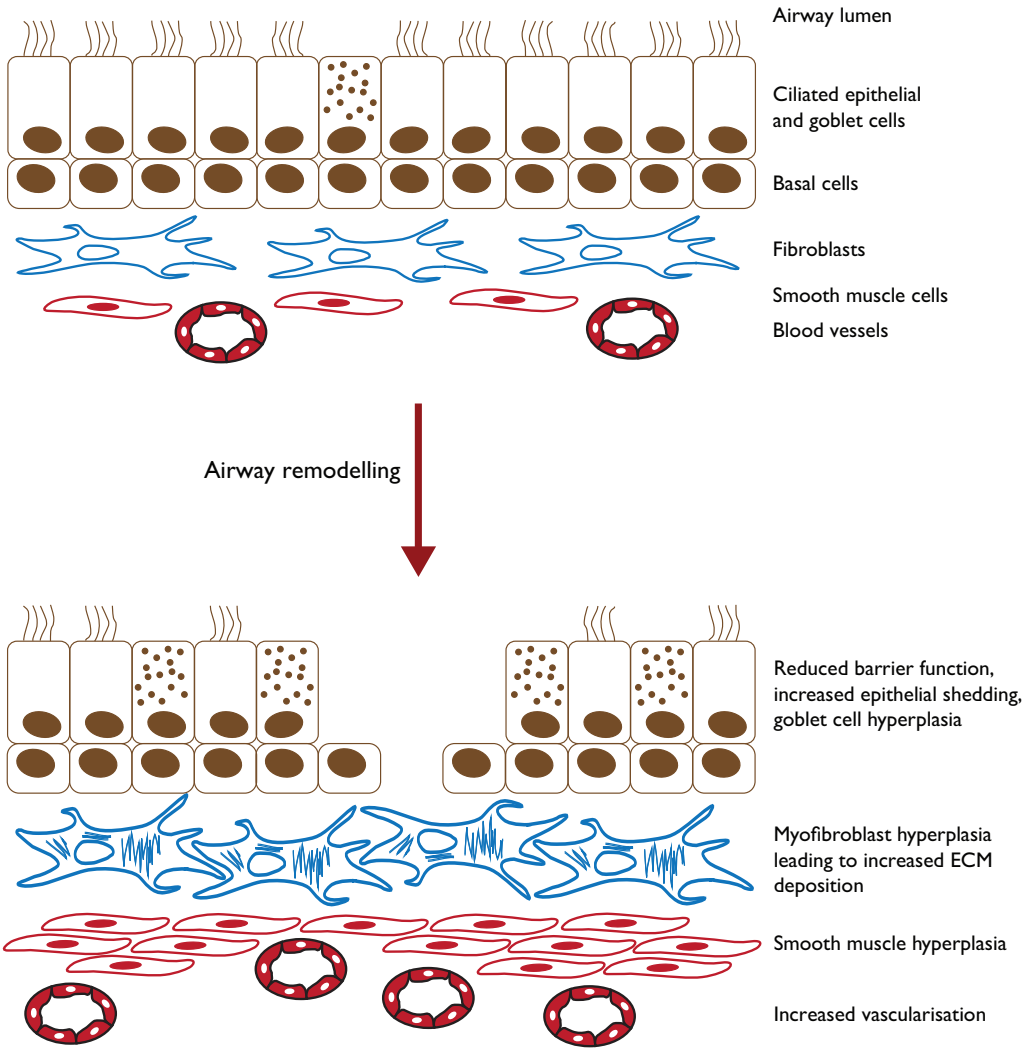

Figure 1.3: Airway remodelling in asthma. Significant changes to the airway architecture are observed in asthmatics, including epithelial shedding, goblet cell hyperplasia and increase production of mucus. Due to this damage there is an increase in the number of myofibroblasts present, resulting in increased extracellular matrix deposition leading to subepithelial fibrosis. These cells also produce Th2 cytokines resulting in a perpetuation of the inflammatory immune response at allergens, pollutants and other innocuous stimuli. Adapted from [16]

desquamation and reducing the effectiveness of this layer as a physical, chemical and immunological barrier.[20] This loss of the epithelial barrier increases susceptibility to viral and bacterial infections and allows infiltration of leukocytes, which combined with excessive production of inflammatory mediators causes damage resulting in airway remodelling.[71,75]

In asthma, these epithelial cells demonstrate increased expression of growth factor receptors, such as fibroblast growth factor, keratinocyte growth factor, and epidermal growth factor receptor, and their ligands.[20,73,76] These growth factors play a role in wound repair, but this process may be dysregulated in asthma resulting in aberrant airway remodelling.[77] In addition, in response to this damage the epithelium becomes stressed, leading to increased activation of NF-κB and thus the increased production of a range of inflammatory mediators, such as IL-6, IL-8 and TSLP, a known chemoattractant for, and activator of, dendritic cells.[77,78]

Within the epithelial layer, goblet cell hyperplasia has been attributed to the predominance of Th2 cytokines, in the asthmatic lung.[79–83] The increased number of goblet cells and their hypersecretion of mucus,[31] reduces airflow and results in luminal occlusion, which combined with airway oedema and bronchoconstriction results in chest tightness and breathlessness.[82]

Angiogenesis

Increased vasculature within the asthmatic airway is thought to be an important aspect of asthma disease pathogenesis, with increased expression of angiogenic mediators and receptors correlating to disease severity and acceleration of lung function decline.[84–86]

For instance, vascular endothelial growth factor (VEGF), angiogenin and angiopoietin-1 are potent proangiogenic factors, which promote proliferation and migration of endothelial cells as well as tubule formation, and are elevated in asthmatic subjects.[87] Also, the eosinophil-derived MBP, which causes generalised tissue damage, has recently been found to induce angiogenesis *in vitro*.[88]

Fibroblasts & Myofibroblasts

Fibroblasts are important structural cells in the airways, however in asthma they are the cause of many of the aspects of remodelling, due to their production and deposition of collagen and proteoglycans and their ability to differentiate into myofibroblasts.

Myofibroblasts are highly contractile cells which secrete larger quantities of extracellular matrix proteins than their fibroblast counterparts. These cells play an important role in tissue repair, with their contractile nature aiding wound closure. However, whilst these cells are vital for wound repair, they have negative effects in the asthmatic lung, resulting in secretion of collagens, pro-inflammatory cytokines and increased contractility, which perpetuates the inflammation and constriction observed in asthma. [23,30,89,90]

Myofibroblasts are found in greater number in the asthmatic airway, [89,91] where the enhanced levels of TGFβ present results in the increased differentiation of fibroblasts to myofibroblasts. [29,92,93] Several other sources have also been proposed for the increased numbers of myofibroblasts in the asthmatic lung. These include dedifferentiation of smooth muscle cells and a migration out of the muscle bundles, an influx of fibrocytes from the circulation or epithelial to mesenchymal transition. The latter is known to occur during development as well as in cancer and has been hypothesised in the asthmatic airway, however in asthma the basement membrane is normal and there is no evidence of invasion of epithelial cells into the subepithelial layer. [30,94–96]

The increase in the number of fibroblasts and myofibroblasts results in thickening of the airways walls in asthma, with enhanced deposition of many extracellular matrix proteins such as collagens I, III and IV and proteoglycans within the lamina reticularis. [91] Previous studies have reported an inverse relationship between both the airway wall thickness with FEV_1 and the level of extracellular matrix protein present. [97] This increase in extracellular matrix is also associated with increased subepithelial fibrosis.

Airway remodelling in the form of smooth muscle hyperplasia and hypertrophy leads to AHR in response to innocuous stimuli causing difficulty in

breathing.[14] This increased airway wall thickening leads to further airflow obstruction, which limits lung function.

Epithelial-Mesenchymal Trophic Unit (EMTU)

With the epithelium and underlying mesenchymal layer both undergoing substantial changes due to the inflammation and remodelling aspects of asthma, this results in the production of a large number of autocrine and paracrine mediators which perpetuate these effects. The involvement of both epithelial and mesenchymal cells in the regulation of the airway microenvironment, normally observed during lung development and wound repair, is described as the EMTU and this appears to be reactivated in asthma.[98,99] In this hypothesis, fibroblasts, especially those in close proximity to the epithelium, are proposed as the main regulator of localised inflammation by acting as sentinel cells (Figure 1.4).[98]

It is thought that continued activation of this EMTU occurs in response to damage to the airway, with epithelial cells producing TGFβ, which induces a phenotypic change of fibroblasts to myofibroblasts, which in turn produce a wide range of inflammatory and fibrotic mediators. Therefore, this continuous wound healing scenario is thought to perpetuate remodelling and in some patients lead to fixed airflow obstruction.[1,98–101]

Aetiology of Airway Remodelling

The aetiology of the airway remodelling observed in asthma is unclear. It is unknown whether this is caused by the persistent inflammation seen in the asthmatic airways resulting in continuous cycles of damage and repair or whether this actually occurs prior to any asthmatic symptoms. There have been recent paediatric studies in children as young as 3 where significant airway remodelling, such as fibroblast proliferation and increased collagen deposition leading to thickening of the laminar reticularis, is observed before the development of asthma symptoms.[102,103] Studies investigating the airways of children with moderate[102] and severe[104] asthma indicate thickened

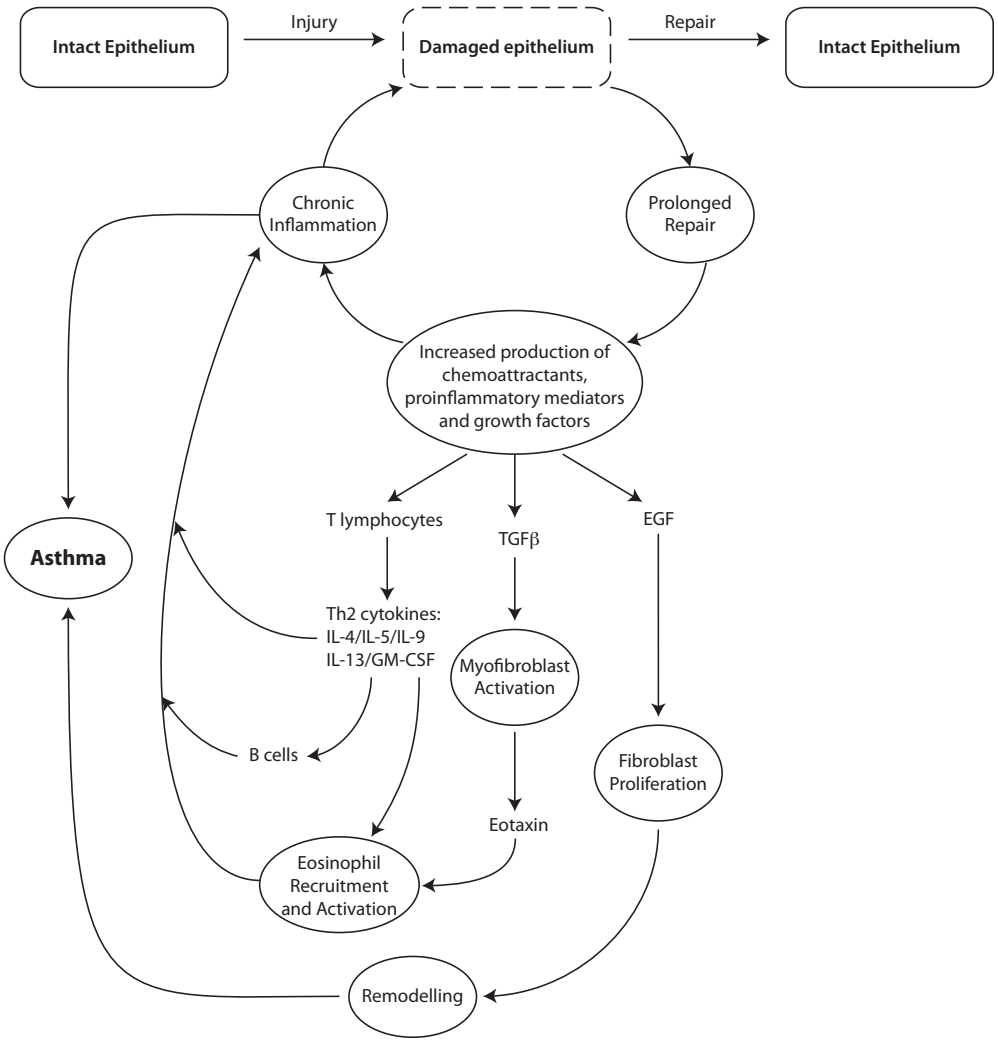

Figure 1.4: The role of the Epithelial-Mesenchymal Trophic Unit (EMTU) in asthma. In response to epithelial damage, the EMTU appears to be reactivated in asthma, resulting in increased communication between the epithelium and mesenchymal cells. The enhanced production of Th2 cytokines and growth factors such as transforming growth factor β (TGFβ) and epidermal growth factor (EGF) induces fibroblast proliferation and activation of myofibroblasts. In turn, these fibroblasts induce further inflammation and remodelling effects, which are key in the pathogenesis of asthma. [1,100,101]

basement membranes present in these children is comparable to that in adult asthma, which again is indicative of a rapid early remodelling response. [102,104]

Recent investigations in mild asthmatics have also highlighted that bronchoconstriction in the absence of eosinophilic inflammation induces airway remodelling. Here patients challenged with methacholine demonstrated similar increases in both epithelial TGFβ and goblet cell staining as well as increased collagen band thickness to those patients challenged with allergen, where eosinophil recruitment was also observed. [105]

1.5 Classification of Asthma

As the severity of symptoms and the frequency of asthma exacerbations can vary dramatically between patients, organisations such as the Global Initiative for Asthma (GINA), the British Thoracic Society (BTS) and the American Thoracic Society (ATS) have each produced guidelines which can be used by physicians when treating asthmatic patients. Whilst there are some differences between the classifications, these generally result in patients grouped into 4 or 5 steps, each with differing levels of treatment. The treatment protocols described by these organisations require constant monitoring of patients in order to maintain control of the asthma symptoms, whilst taking the least medication possible. [14,15]

The GINA guidelines describe four levels of asthma, as determined by the frequency of exacerbations and the symptom severity. The drugs used to manage asthma vary depending on whether the disease is mild intermittent, mild persistent, moderate persistent or severe. [14]

In recent years because of the heterogeneity of symptoms observed between asthma patients, there has been a significant interest in development of a non-biased method of categorising patients using cluster analysis. These clusters have been established in populations in Europe via U-BIOPRED (Unbiased BIOmarkers in PREDiction of respiratory disease outcomes) and the USA via the Severe Asthma Research Program (SARP). [106,107] These

studies have looked at a large number of healthy and asthmatic subjects (>1500 in SARP alone) to try to distinguish differences between the groups, with the aim of better identifying treatments based on these clusters. [106]

1.6 Treatments for Asthma

Current asthma therapies (Table 1.1) reduce the symptoms associated with the disease but are unable to provide an absolute cure. With the correct use of these medications many patients will only suffer from occasional symptoms and fewer exacerbations, [108] however this is not the case for all patients and many asthmatics are not adequately treated with conventional therapies.

Treatments	**Mild Intermittent**	**Mild Persistent**	**Moderate Persistent**	**Severe Persistent**
	Short Acting β_2 Agonist as required			
ICS Dose		Low	Med/High	High
LABA	−	−	+	+
OCS	−	−	Consider short-course	+
LT Modifier	−	+/−	+/−	+/−
Other	−	−	−	Anti-IgE etc.

Table 1.1: Standard asthma therapy based on disease severity. ICS = Inhaled Corticosteroids, LABA = Long Acting β_2 Agonist, LT = Leukotriene, OCS = Oral Corticosteroids. Adapted from [14]

Patients with asthma require "reliever" medication for rapid symptomatic relief in the event of an exacerbation. Short acting β_2 adrenergic agonists (SABA), such as salbutamol, act as smooth muscle relaxants to reverse the contraction observed during asthma attacks. [109]

As well as "reliever" medication, "controller" therapies are also required by patients with more persistent asthma, which are designed to reduce

symptoms and exacerbations. These include long acting forms of β_2 agonists (LABA), such as salmeterol, [110] as well as inhaled and oral corticosteroids. [14]

Beclomethasone, an inhaled glucocorticosteroid (ICS) is prescribed in more persistent asthma to suppress airway inflammation and reduce AHR, thus improving lung function. [14] Steroids suppress acute and chronic inflammation by targeting cytokine protein synthesis. In severe asthma cases when inhaled steroids are not sufficient, oral corticosteroids, such as prednisolone, are also used. [14] These drugs only help in some cases of asthma, with many severe asthmatics described as "steroid refractory" as these provide little relief from their symptoms. [111] Both inhaled and oral corticosteroids have many negative side effects including oral candidiasis and adrenal suppression. [70,112] Therefore, the minimum possible dose possible of these drugs is given, and this is generally as a combination therapy with LABA to provide better symptomatic relief. [14]

As many severe asthmatics are not adequately treated by β_2-agonists or steroids, other drugs such as leukotriene modifiers (such as Montelukast or Zafirleukast) or anti-IgE therapy (Omalizumab) are often used as an adjuvant. These, again, aim to reduce the inflammation aspects of the disease by blocking some of the key inflammatory mediators. [14,15]

1.7 Aetiology of Asthma

Whilst the aetiology of asthma has been linked to childhood atopy in many patients, this is not the case for all asthmatics. Evidence suggests that asthma originates early in life and that the structural and molecular changes that occur at this time lead to the characteristic symptoms of asthma. Whilst airway remodelling has been characterised in young children who later develop asthma, [103,113] it is unclear if these changes occur during *in utero* development, or after birth.

Respiratory viruses have also been implicated in the aetiology of asthma, however there is conflicting data at present, with some reports suggesting that

children who have documented respiratory syncytial virus (RSV) are more prone to asthma. [114] However there have also been studies showing protection from asthma from RSV infection. [115] More recently, rhinovirus (RV) infection has been identified as an important early risk factor for asthma. [116] One key study investigating the role of both RSV and RV in the aetiology of asthma found that around 90 % of children aged 3 who had experienced wheezing with RV developed asthma by the age of 6. Whilst RSV-related wheeze was also observed to increase this risk (relative risk of 2.6), it was the RV-induced wheezing illness which had the greatest effect (relative risk of 9.8). [117] Respiratory viruses have also been associated with a worsening of asthma symptoms, as these viruses appear to target the lower airways of asthmatics, compared with the higher airways in healthy individuals. It has been estimated that approximately 50-75 % of all asthma exacerbations are due to RV infections. [118]

As well as environmental risks such as viruses, there is also a strong trend of heritability which has been observed in twin studies. [119–121] It has therefore been postulated that asthma is a multifactorial disorder involving the interaction of both environmental and genetic factors. [122,123]

1.8 Asthma Genetics

There has been a considerable amount of work in recent years attempting to determine which genes may be responsible for the symptoms of asthma. However, asthma is a complex and heterogeneous disease, which cannot be easily defined and attributed to a single gene. Instead it is likely that combined polymorphisms in a variety of genes lead to a predisposition towards asthma.

Through the use of candidate gene analysis positional cloning and genome wide association studies, many genes have been linked to asthma susceptibility, including *ADAM33*, *TNF*, *TGFB1*, *IL4R* and *IL13* [124] but there has been much debate over the identified genes and polymorphisms due to variations in cohorts and environmental factors.

1.8.1 *ADAM33*

An association between A Disintigrin And Metalloprotease (*ADAM*) 33 and both asthma and AHR was first established in 2002 via linkage analysis. [123] This has since been confirmed in several other populations, [125] however the exact role of *ADAM33* has yet to be determined.

A knockout mouse for *ADAM33* was developed in an attempt to elucidate the function and role in asthma, although no abnormalities were observed and no differences were found between wildtype and $ADAM33^{-/-}$ mice when studying the ovalbumin model of allergic asthma. [126]

In humans, on the other hand, *ADAM33* polymorphisms have been found to correlate with reduced lung function. [127,128] This has also been observed in children at age 3 and 5, highlighting that differences in the lungs of asthmatic patients may happen before the cycles of persistent inflammation and remodelling occur. [129]

ADAM33 is mainly mesenchymally located [130] but no differences have been observed in the pattern or level of expression between healthy and asthmatic individuals. [131,132] However, a soluble form of ADAM33 (sADAM33) has been detected in the bronchioalveolar lavage (BAL) fluid of asthmatic but not healthy individuals, and the levels correlated with both the severity of asthma and decreased lung function, measured as % predicted FEV_1. [133]

Recently ADAM33 has been linked to remodelling and angiogenesis, and production of sADAM33 was enhanced by TGFβ_2, the expression of which is also augmented in the asthmatic lung. [134–136]

1.8.2 *IL4* & *IL13* Gene Cluster

Another key area of interest has been the IL-4 gene cluster. Located on chromosome 5, the IL-4 gene cluster encodes the β_2-adrenergic receptor (*ADRB2*). [50–52] as well as the Th2 cytokines IL-3 (*IL3*), IL-4 (*IL4*), IL-5 (*IL5*), IL-9 (*IL9*), IL-13 (*IL13*), and GM-CSF (*CSF2*), [55,56] which are key in the pathogenesis of asthma and allergic inflammation.

IL4 and *IL13* are two of the most studied candidate genes for both allergic inflammation and asthma, with multiple single-nucleotide polymorphisms (SNPs) detected in each.

IL13

SNPs have been found in clusters at both the 5′ and 3′ ends of the *IL13* gene. In particular, two polymorphisms have been identified in the 5′ promotor region. One such mutation, *IL13*-1512AC, which is also referred to by the RefSNP accession ID (rs number) rs1881457, has been linked with higher levels of IgE in skin prick test positive children.[137] *IL13*-1112CT (rs1800925), on the other hand, has been associated with an increased risk of allergic sensitisation, asthma, airway hyperresponsiveness, high levels of IgE and atopic dermatitis,[137–141] although this has not been replicated in all studies.[142,143] The presence of this mutation in the promotor region results in increased transcription of the *IL13* gene[144] leading to increased IL-13 secretion by Th2 polarised T cells.[145]

IL13+2044GA (rs20541) is a non-conservative point mutation in exon 4 of the gene, which results in the exchange of an arginine residue for glutamine at position 130 (R130Q), resulting in an overactive IL-13 variant.[146] This R130Q form of IL-13 is found in 20 % of Caucasians and is more prominent in asthmatics than healthy controls.[142,147] Associations have also been observed with atopy,[142] total serum IgE levels[137,139,143,148,149] and atopic dermatitis.[139,142,150,151] This gain of function mutation is also often found to be co-expressed with the promotor polymorphism *IL13*-1112 resulting in increased expression of an overactive *IL13* variant.[152,153]

Several polymorphisms have also been identified within the 3′ untranslated region (UTR) of the gene. Of particular interest is *IL13*+2525GA (rs1295685), which has been associated with raised cord serum IgE levels,[154] which in turn is related to a higher risk of developing atopy or asthma by age 4.[155,156]

IL4

SNPs within the *IL4* gene have also been studied in detail. One such polymorphism, *IL4*-589CT (rs2243250) located in the promoter region of the gene and found in approximately 27 % of Caucasians, [157] has been shown as a risk factor for atopy, allergic rhinitis, and wheeze. [158,159] Whilst some studies have found links between this polymorphism and both total IgE [157] and specific IgE for housedust mites, [158] this has not been replicated in all studies. [160]

This mutation is however, generally considered to be associated with asthma. [157,159–162] In particular Sandford, Chagani *et al.* determined this as a risk factor for severe near-fatal or fatal asthma [163] whilst Burchard, Silverman *et al.* found associations to reduced FEV_1 in asthmatic subjects. [164]

When studying the effect of this polymorphism *in vitro*, Rosenwasser, Klem *et al.* also found the presence of the T allele resulted in increased *IL4* gene expression. [157]

Related Genes

Polymorphisms identified in other genes associated with Th2 inflammation have also been linked to asthma and atopy.

One such gene is *IL4RA*, which is located on chromosome 16. The *IL4RA* gene encodes IL-4Rα which is part of the IL-4 and IL-13 receptor system. [53–56,165] SNPs identified in exon 12 of *IL4RA* include rs1805011, rs1805015 and rs1801275, all of which result in mis-sense mutations within the intracellular signalling portion of IL-4Rα, as well as rs1805010, which is located extracellularly. [163,164,166–173]

The mutation *IL4RA*+1902GA (rs1801275) is found in approximately 36 % of Caucasians [166] and results in the the exchange of the polar, uncharged amino acid glutamine at position 576 for positively charged arginine. This SNP has been associated with atopy [166] and was reported to induce enhanced

signal transduction through IL-4Rα,[166,174] although this could not be confirmed in other studies.[168,175] This polymorphism has also been associated with hyper IgE syndrome, severe atopic asthma[166,169,173] and is considered a risk factor for reduced lung function in asthmatics in some, but not all populations.[163,173] Whilst some individual studies have reported no linkage with atopic asthma in their cohorts,[170,176] meta-analysis of a number of studies demonstrated a significant association between the risk of asthma, in particular atopic asthma and this polymorphism.[177]

The *IL4RA* SNP rs1805010 located in exon 5 results in the exchange of Ile for Val at position 50 in the extracellular portion of the receptor. This mutation has been associated with atopic asthma as well as increased responsiveness of IL-4Rα to IL-4 leading to increased STAT6 phosphorylation, resulting in enhanced cell proliferation and upregulation of IgE synthesis.[167,175] This polymorphism was also found to associate with more severe asthma.[178] However meta-analysis of nine individual studies found no overall association with asthma.[177] This may be due to differences between cohorts,[173] or could be more strongly associated in certain populations. It may be that the strong linkage disequilibrium observed between this and other polymorphisms associated with asthma, such as *IL4RA*-Q551R, have led to misleading results.[162,173]

Gene-gene interactions between polymorphisms in *IL4RA* and *IL13* have also been observed in asthma.[171] One particular pair of SNPs includes rs1805015, which results in exchange of polar uncharged serine for non-polar hydrophobic proline at position 478 of *IL4RA* and rs1800925 in *IL13*, was found to increase the risk of developing asthma five-fold.[171]

1.9 IL-4 & IL-13

Consistent with their genetic association with atopy and asthma, IL-4 and IL-13 appear play a critical role in the pathogenesis of asthma and allergic inflammation.[53,54,165]

The Th2 humoral-immune phenotype, which is driven by IL-4, is generally associated with the clearance of parasitic worms.[179] Production of IL-4 and IL-13 in response to helminth infections, such as *Heligmosomoides polygyrus* or *Schistosoma mansoni* results in increased gut contractility and mucus production, allowing rapid clearance of the infection, as well as the production of a protective Th2 inflammatory response, which downregulates the otherwise pathological Th1 response.[179] Whilst the effects of IL-4 and IL-13 are beneficial in response to helminth infection, similar responses are observed in the asthmatic lung, where these cytokines appear to have been misdirected towards innocuous antigens, and are subsequently the driving force of many of the symptoms observed.

Despite only sharing around 25 % homology, consisting of a conserved core of 25 amino acids,[53] IL-4 and IL-13 have similar but non-redundant roles (Figure 1.5). These shared roles arise from the use of a common receptor, IL-4Rα:IL-13Rα1.[180–182]

IL-4 and IL-13 both cause eosinophilia (by inhibiting apoptosis and increasing growth), eosinophil chemotaxis and eosinophilic inflammation through the production of eotaxin (also known as Chemokine (C-C motif) ligand (CCL) 11) by fibroblasts.[161,184–186] IL-4 and IL-13 also contribute to airway remodelling, a hallmark of asthma, by inducing goblet cell hyperplasia and increasing mucin gene expression, resulting in the hypersecretion of mucus.[80,161,187] Both cytokines have been shown to increase IgE levels by acting on B lymphocytes, promoting class switch of immunoglobulins to IgE and increasing expression,[188–190] as well as decreasing transcription of Interferon-γ,[45] thereby encouraging a switch to the Th2, whilst also suppressing the Th1 phenotype.[45,191]

IL-4 and IL-13 also exhibit some distinct roles, for instance IL-4 promotes differentiation towards the Th2 phenotype,[192] therefore increasing the number of Th2 cells, whilst inhibiting differentiation to Th1 cells,[16] which IL-13 is unable to do. IL-4 plays an important role in B cell activation by increasing expression of Class II Major histocompatibility complex (MHC) molecules, as well as promoting immunoglobulin (Ig) synthesis and class switch to IgG_4

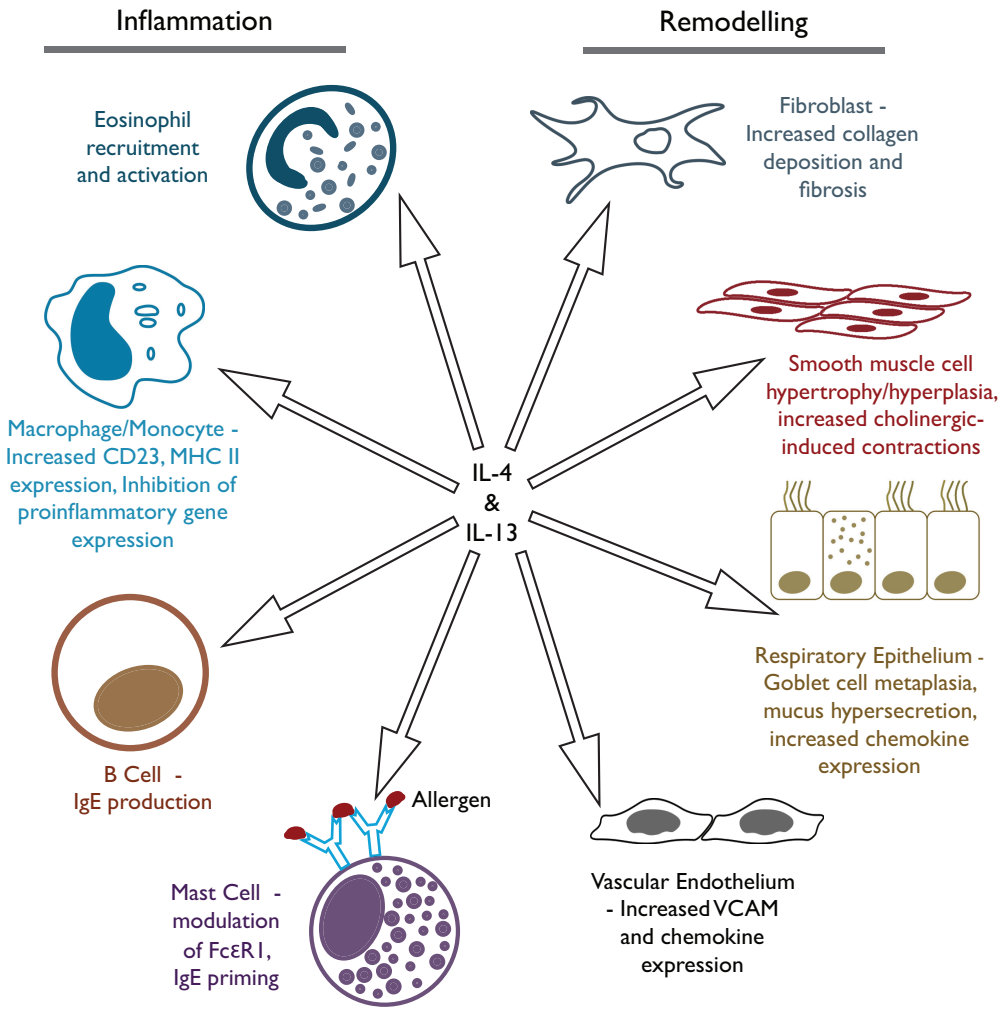

Figure 1.5: The effects of IL-4 and IL-13 on the airway. IL-4 and IL-13 exert a wide range of effects on the airway, both directly, and through the aid of other mediators. Their shared functions are brought about by the fact that both IL-4 and IL-13 share a receptor IL-4Rα:IL-13Rα1. Adapted from [183]

and IgE.[188,189] IL-4 exerts its effects on monocytes and macrophages, and promotes growth of mast cells, basophils and eosinophils.[193] IL-4 is also involved in eosinophil recruitment, with the aid of Vascular Cell Adhesion Molecule-1 (VCAM-1),[25] and induces fibroblast chemotaxis and activation.[55] IL-4 is derived from T lymphocytes, eosinophils and cells of basophil and mast cell lineage.[55]

IL-13, on the other hand, is synthesised by $CD4^+$ and $CD8^+$ T cells, and unlike IL-4, IL-13 does not activate T cells and is unable to promote differentiation to the Th2 phenotype.[55] IL-13 directs T lymphocytes, monocytes, basophils and eosinophils to sites of inflammation, resulting in airflow obstruction.[183] IL-13 also elicits effects on monocytes, resulting in morphological changes and upregulation of both the low affinity receptor for IgE (FCεRII) and MHC II antigens.[194] IL-13 increases goblet cell density and instigates goblet cell metaplasia,[195,196] and also induces VCAM-1,[25] thus working synergistically with IL-4. IL-13 has also been shown to promote fibrosis, by way of increased collagen deposition, resulting in airway remodelling in asthma.[161,197] In mice, overexpression of IL-13 specifically induces airway fibrosis and many of the inflammatory features of asthma.[161]

Various studies have found that asthma exacerbations, caused by allergen challenge, result in an increase in both the number of cells expressing IL-4 and IL-13 mRNA within the BAL fluid,[198,199] as well as an increase in levels of these cytokines.[184,199]

More recently there have been several studies investigating the levels of both IL-4 and IL-13 present in the airways of healthy and asthmatic volunteers. Brightling, Symon *et al.* found that both IL-4 and IL-13 were present within the smooth muscle to a much greater extent in asthmatic subjects than healthy controls.[200] In this group of asthmatic patients however, approximately 20 % did not show any measurable staining.[200] Berry, Parker *et al.* found similar results within the submucosa when looking for IL-13 positive cells.[201] This investigation also measured sputum concentrations of IL-13 and found higher concentrations in the sputum of mild asthmatic

patients, although, as before, this seemed to only occur within a subset of patients. [201]

Saha, Berry *et al.* studied the levels of IL-13 in bronchial biopsies and sputum from asthmatics with varying degrees of disease severity. [202] Here the authors reported much greater staining of IL-13 in sputum obtained from mild and severe asthmatics compared with healthy controls. Interestingly though, there appeared to be less IL-13 present in sputum obtained from moderate asthmatics and although this group was slightly smaller than the others there was a significant difference between the mild and the moderate asthmatic groups. When investigating the levels of IL-13 in the submucosa and airway smooth muscle they again found increased IL-13 present in mainly the mild and severe asthmatics. The authors also noted a correlation between the subjects who demonstrated sputum eosinophilia and those who expressed IL-13 positive cells. [202] In particular, these studies highlight some of the variation observed between asthma phenotypes, suggesting that IL-13 may be of particular importance in a subset of asthmatic subjects.

Recently Woodruff, Modrek *et al.* have proposed Th2-driven inflammation to be one of the defining phenotypes of one subgroup of asthmatics. [203] In this study the "Th2 high" group were found to express higher levels of the Th2 cytokines IL-5 and IL-13, as well as *MUC2* and *MUC5AC* than the "Th2 low group". This "Th2 high" subset also exhibited greater airway hyperresponsiveness, eosinophilia, subepithelial fibrosis and increased serum IgE levels. In many of these cases the "Th2 low" group were indistinguishable from the healthy control subjects. [203]

Another interesting difference between the two groups was the response to steroids, as it was only in the "Th2 high" group that these were able to improve lung function. [203] Whilst it is unclear what proportion of asthmatics would fall into these two categories, and what the driving force behind the "Th2 low" groups' symptoms may be, the Th2 phenotype driven by both IL-4 and IL-13 is clearly important in the pathogenesis of asthma.

1.9.1 Novel Asthma Therapeutic Approaches Targeting IL-4, IL-13 & Their Receptors

In recent years there has been extensive work investigating potential therapies for asthma based on IL-4 and IL-13. These include monoclonal antibodies (mAb) against either cytokine or one of their receptor subunits, a double mutein form of IL-4, and both antisense oligonucleotides and small interfering RNAs (siRNA) against IL-4Rα.

The initial results from the preclinical stages of these trials seemed hopeful, however the results obtained during the clinical trials have been broadly disappointing. More recent data has suggested that this is, at least partially, due to the broad recruitment of patients and it is now thought that a more stratified approach to asthma therapies may be more beneficial due to the heterogeneity of the disease phenotypes. As there is a subset of asthmatics who appear to be driven by a Th2 phenotype, with IL-13 in particular playing a key role in their asthma symptoms,[203,204] recent trials have focussed on this specific group of patients and have been more successful.

The current status of these clinical trials is reviewed below. Where available clinicaltrials.gov references are provided in brackets, last accessed in October 2011.

Anti-IL-4Rα Monoclonal Antibodies

AMG-317 is a human MAb which binds to IL-4Rα preventing both IL-4 and IL-13 signalling. In a recent phase 2 trial (NCT00436670), AMG-317 was found to decrease levels of circulating IgE and reduce eosinophilia, however this drug did not demonstrate clinical efficacy for reducing asthma symptoms of the patient group studied. Although neither the primary or secondary endpoints of this study were matched, some patients, particularly the most severe asthmatics of the cohort, did appear to benefit from this drug, with a trend towards a decrease in asthma exacerbations.[205,206]

IL-4Rα Double Mutein

An IL-4 receptor α (IL-4Rα) antagonist, in the form of an IL-4 double mutein, attenuates both IL-4 and IL-13 *in vitro* and is currently undergoing clinical trials. [207] This drug has the benefit of suppressing two key Th2 cytokines, which is important due to redundancy of their actions.

This IL-4Rα antagonist, also known as Pitrakinra, consists of an IL-4 mutant (R121D/Y124D) which selectively binds the IL-4Rα subunit and prevents both binding of the wildtype forms of IL-4 and IL-13, as well as subsequent dimerisation with either γc or IL-13Rα1, rending this receptor incapable of signalling. [207,208]

Initial randomised control trials (NCT00535028 and NCT00535031) demonstrated a trend towards a decrease in asthma related adverse events, as well as a significant decrease in the number of exacerbations which required β_2 agonist relief. The severity of the late asthmatic response was also decreased with treatment, where a smaller reduction in FEV_1 was observed in response to allergen challenge in those who were given the drug compared with a placebo. [207]

Efficacy of this drug was observed against the allergen-provoked early- and late-phase responses, but not against the acquired increase in bronchial hyperresponsiveness, after both subcutaneous and inhaled administration, although the latter appears to be the preferred method due to direct targeting of the relevant tissues. [207,209]

In a phase IIb trial, which finished in mid-2010, where pitrakinra was given to more than 500 uncontrolled asthmatics, clinical efficacy was only found in a subgroup of the population studied, which consisted of 125 patients with eosinophilic asthma (NCT00801853). [210]

Recently pharmacogenetic analysis has identified that IL-4Rα mutations (including rs1801275 and rs1805011) may prevent the actions of pitrakinra in certain patients (NCT00535431). As novel asthma therapies tend to have greater cost implications than standard therapies this may need to be considered before administration. [211]

Soluble Human IL-4Rα

Altrakincept, a soluble recombinant human form of IL-4Rα (sIL-4Rα) has been tested for its ability to block the interaction between IL-4 and its receptor by sequestration.

In an initial phase I study, patients with moderate asthma were withdrawn from ICS therapy and treated with nebulized sIL-4Rα. Whilst the patients given the placebo experienced a reduction in lung function after steroid withdrawal, this was not the case with the treatment group. [212] A subsequent larger, 12 week phase II study in subjects with moderate and persistent asthma who were withdrawn from steroids for the study (NCT00017693), again demonstrated efficacy in preventing asthma symptoms and reduced lung function associated with steroid withdrawal. One of the issues of this study was patient retention, as between 47 % and 69 % of patients had to withdraw from the study by day 84 (withdrawal was mandated after an asthma exacerbation). [213]

However, when this drug was taken to phase III trial it was not found to provide clinical efficacy for the treatment of asthma, which may have been due to the limited bioavailability or degradation in the airways. [214]

IL-4Rα Antisense Oligonucleotides

A second-generation antisense oligonucleotide of IL-4Rα (AIR-645 and ISIS-369645), which targets *IL4RA* mRNA, has been tested for its ability to act as a dual inhibitor of IL-4 and IL-13. In mouse models this was found to reduce not only IL-4Rα expression, but also both airway inflammation and hyperresponsiveness. In 2009 reports published from initial studies (NCT00658749 and NCT00941577) with this 2′-O-methoxyethyl oligonucleotide found that the drug was well tolerated, however as yet results have not been published regarding the clinical efficacy of this drug in asthma. [215]

Anti-IL-4 Monoclonal Antibodies

Initial data from trials in non-human primates found that a humanized anti-IL-4 mAb, pascolizumab, demonstrated clinical potential for the treatment of asthma.[216] However, subsequent clinical trials in patients with symptomatic corticosteroid-naive asthma (NCT00024544) failed to provide any clinical benefit.

Anti-IL-13 Monoclonal Antibodies

Anrukinzumab, also known as IMA-638, is a humanised monoclonal antibody against IL-13.[217] In non-human primate studies, treatment with IMA-638 was found to reduce both the number of inflammatory cells and the levels of inflammatory mediators within BAL after exposure to *Ascaris suum*, which induces acute airway inflammation.[218] However, when this antibody was tested in a double-blind randomised, placebo-controlled trial of mild atopic asthmatics, although a significant attenuation in the early and late phase asthmatic response was observed after 14 days, this was small and overall little clinical benefit of treatment was found, with no effect on allergen-induced AHR or sputum eosinophil counts. This trial was run concomitantly with one studying IMA-026, which also acts as an IL-13 mAb, but unlike IMA-638 there was no benefit even at 14 days.[219] This work was extended with a larger, phase II study in patients with persistent asthma (NCT00425061) monitoring changes in peak expiratory flow rate over 16 weeks, and again this drug did not demonstrate clinical efficacy and is therefore not being developed further.[214] Instead IMA-638 is currently being investigated as a therapy for ulcerative colitis (NCT01284062).

Another IL-13 MAb, QAX576, is currently being evaluated in a phase II randomised, double-blind trial as "add-on" intravenous therapy for persistant asthmatics whose symptoms are not controlled by ICS and LABA. This study is in the process of recruiting patients, and initial data is expected at the end of 2011 (NCT01130064).

CAT-354 (Tralokinumab), a humanized antiIL-13 mAb [220] has also undergone phase II trials for the treatment of moderate-to-severe asthma. Final data collection for the first phase II trial in asthma occurred in August 2010, although no data has been published as of October 2011 (NCT00873860). A larger 52 week, phase IIb, double-blind study to evaluate the efficacy of this drug in severe uncontrolled asthma is due to start in 2011, with data due at the beginning of 2014 (NCT01402986). This study aims to evaluate asthma exacerbation rates as well as various measures of pulmonary function and quality of life scores.

Recently lebrikizumab (MILR1444A), another monoclonal antibody against IL-13, has also undergone clinical trials for asthma ranging from mild to severe (NCT00781443, NCT00930163, NCT00971035 and NCT01423318). The results from one of these studies (NCT00930163), where the cohort consisted of severe, steroid refractory asthmatics, were published this year and has been considered largely successful. [221]

In this cohort total serum IgE and peripheral-blood eosinophil count were used to distinguish asthmatics with predominantly Th2/IL-13 related symptoms. Blood periostin levels were also used as a biomarker for this as it is induced by IL-13. Here, patients with higher levels of periostin at baseline, due to a predominance of IL-13-mediated inflammation, responded better to the drug than both the lower periostin and placebo groups. For example a 14 % increase in the prebronchodilator FEV_1 was observed after 12 weeks in the high periostin group, compared with 5.1 % in the low periostin and 4.3 % in the placebo groups.

The fact that better efficacy of this drug was found to be related to the baseline periostin levels of the patients, where many other similar therapies have failed, demonstrates that a stratified approach to therapy by directly-targeting the patients with more Th2-mediated inflammation may provide greater benefit.

1.10 IL-4 & IL-13 Receptors

It has been suggested that the relatively low success rate of novel therapies based on IL-4/IL-13 is due to their shared receptor complex. IL-4 and IL-13 receptors are present on many human airway cells and addition of either IL-4 or IL-13 leads to initiation of a phosphorylation cascade resulting in STAT6 activation. [27,183]

IL-4 can signal through the type I receptor by binding to Interleukin-4 receptor α (IL-4Rα) and γc, a chain that is common among several cytokine receptors (Figure 1.6). [222,223] The type II receptor (IL-13Rα1:IL-4Rα) enables IL-13 signalling and acts as an alternate receptor for IL-4 (Figure 1.6). [180,223] IL-13 binds to IL-13Rα1 and this complex recruits IL-4Rα for stabilisation of the interaction. [224] IL-4, on the other hand, binds with high affinity to the IL-4Rα subunit and does not require the addition of either γc or IL-13Rα1 to stabilise this interaction. [224,225] Addition of one of these two subunits to the receptor complex is, however, required for signalling. [180,222] Interestingly, IL-13 has a second receptor subunit, IL-13Rα2. This binds IL-13 with high affinity but is unable to activate the STAT6 pathway.

1.10.1 Intracellular Signalling

IL-4 and IL-13 signalling occurs through the Janus Kinase (JAK) Signal Transducer and Activator of Transcription (STAT) pathway (Figure 1.6). Upon ligand binding and receptor dimerisation the IL-4 and IL-13 receptors autophosphorylate, enabling a subsequent signalling cascade. [226] JAK1 forms a physical complex with the IL-4Rα subunit allowing signalling to occur. [227] JAK3, on the other hand, binds to the γc chain, [228,229] whilst JAK2 and Tyrosine kinase 2 (Tyk2) bind to the IL-13Rα1 chain. [230,231] In all of these cases, the JAKs are activated by phosphorylation, which then leads to phosphorylation of STAT6. [232,233] This activation is necessary for IL-4 and IL-13 to mediate their effects by transcription of a plethora of inflammatory mediators and matrix proteins. [234]

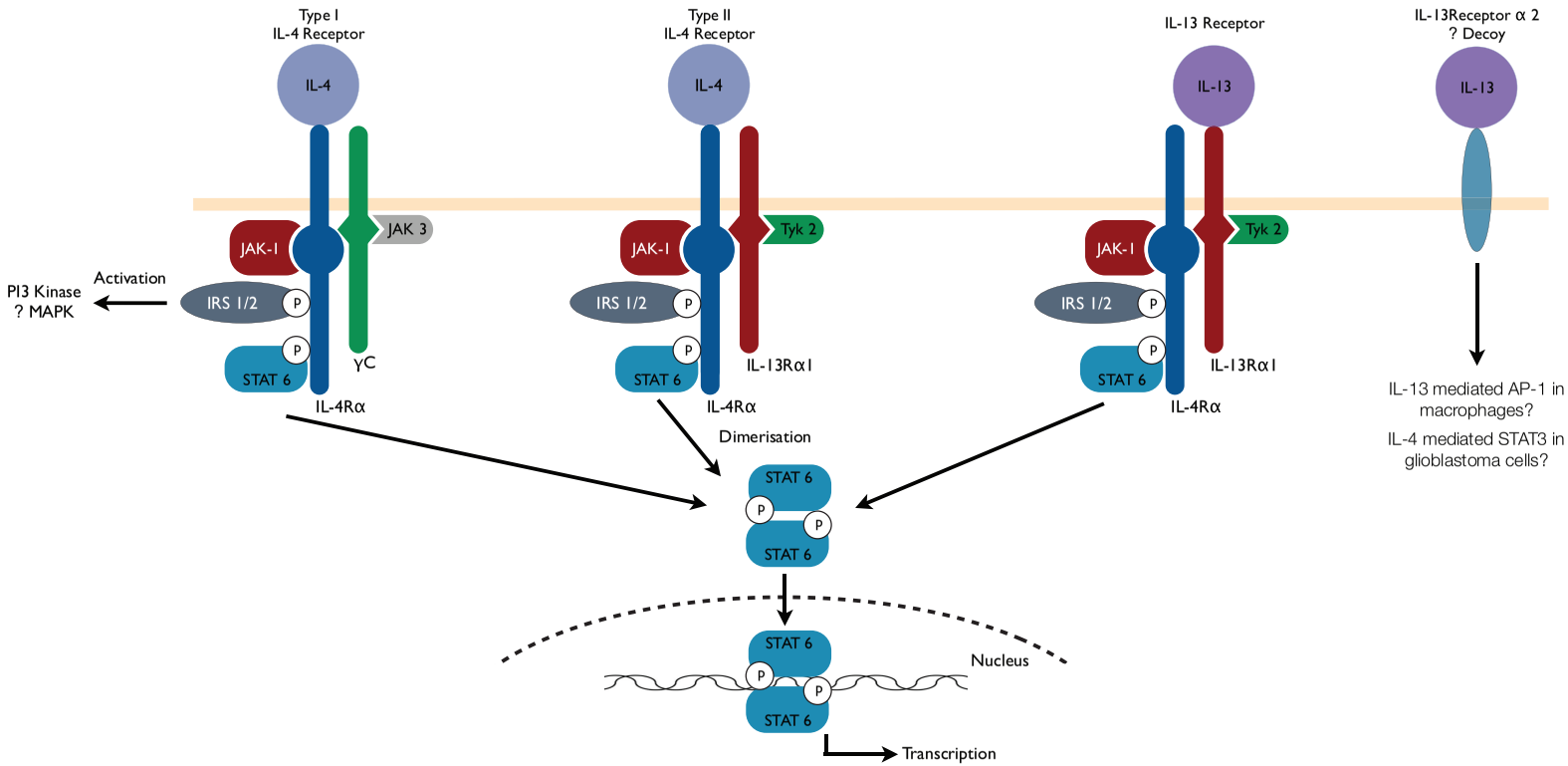

Figure 1.6: The different receptor subtypes for IL-4 and IL-13. There are two receptors available for IL-4: IL-4Rα:γc and IL-4Rα:IL-13Rα1. The latter also enables IL-13 signalling. Signalling through these receptos occurs through the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway. A second receptor exists for IL-13, termed IL-13Rα2. Very little is known about how this receptor mediates its effects, as it does not appear to have any signalling domains on its small intracellular tail. It has been suggested that this intracellular tail may interact with IL-4Rα, blocking the effects of IL-4 and possibly IL-13, as well as acting as a "decoy receptor" for the latter. Adapted from [183]

As there are some known differences between IL-4 and IL-13 responses it has been postulated that these may be due to the ability of the type I receptor, which can only bind IL-4, to induce a differential signalling pathway. Knockout studies have found several IL-4 mediated effects which are either not STAT6 or only partially STAT6-dependent, such as antigen-induced eosinophilia and VCAM-1 expression in mice. [235] More recently another study in mice found antigen-induced expression of both STAT6-dependent and -independent genes. [236] An alternative intracellular signalling pathway has therefore been proposed, although this has been less thoroughly investigated than the JAK/STAT system and remains relatively poorly understood.

This alternative pathway involves phosphorylation of one the homologous insulin receptor substrate (IRS) proteins, IRS 1 or 2, which are able to bind the IL-4Rα subunit. [237] This activation of IRS is thought to occur specifically due to IL-4 and not IL-13. [238] Experiments on cells containing the mutation Y497F of IL-4Rα, which prevents phosphorylation of IRS 1, demonstrate diminished ability to proliferate in response to IL-4. [239]

The phosphorylation of IRS 1/2 is thought to activate the p85 subunit of phosphoinositide 3-kinase (PI3K) leading to activation of the serine/threonine kinase Akt and potentially mitogen activated protein kinase (MAPK) pathways. [239] The IRS activation of the PI3K pathway has been linked to the proliferation of Th2 cells and induction of gene expression in alternatively activated macrophages. [238] Blockade of the PI3K pathway in haematopoetic cells has been shown to suppress IL-4-mediated protection from apoptosis, although the mechanism for this remains unclear. [240] Much of this research, however, has been performed in animal models or shown by indirect mechanisms and the role of this pathway in primary human cells remains unclear.

1.11 IL-13Rα2

As well as IL-4Rα/IL-13Rα1, IL-13 also binds to IL-13Rα2, originally thought to be a "decoy receptor" (Figure 1.6). [241–243] IL-13Rα2 contains a

short intracellular tail, is unable to initiate a signal through STAT6, [242,244] and its regulatory mechanism remains poorly understood.

IL-13 binding to IL-13Rα2 occurs very rapidly, with high affinity and does not dissociate easily. [243] This is commonly seen with negative regulators supporting the idea of IL-13Rα2 being a "decoy receptor". A knockout mouse for IL-13Rα2 has been shown to exhibit similar traits to mice overexpressing IL-13, again substantiating the notion that IL-13Rα2 limits the effects of IL-13 *in vivo*. [245,246] However, recently it has been shown that IL-13Rα2 also acts as a negative regulator for IL-4 and was found to physically associate with IL-4Rα (but not IL-4) in the presence of IL-4, attenuating the effects of both IL-4 and IL-13 in an auto-regulatory manner, and therefore is not solely acting as a "decoy". [165,224,247–250]

1.11.1 Intracellular Pools of IL-13Rα2

Intracellular stores of IL-13Rα2 have been detected in epithelial cells, with evidence that this receptor is mainly localised to the cytoplasm until Interferon (IFN) γ induces rapid mobilisation to the cell membrane. [251,252] As IL-13Rα2 is able to modulate Th2 cytokine responses it has been postulated that the presence of these pools enables rapid regulation of these cytokines, which would not be possible if *de novo* synthesis was required. Previous work has also demonstrated the upregulation of surface IL-13Rα2 by IL-13 pretreatment. [165,253] The fact that both IL-13 and IFNγ can upregulate IL-13Rα2 supports the idea that IL-13Rα2 acts to negatively regulate the Th2 response.

1.11.2 Soluble IL-13Rα2

In mice, soluble IL-13Rα2 (sIL-13Rα2) is released from the cell surface to regulate the effects of IL-13. This is thought to be formed by alternative splicing of the wild-type IL-13Rα2, [254,255] although enzyme (MMP8) and allergen (house dust mite) dependent cleavage has also been observed. [256,257] This form, however, has not been detected in either bronchoalveolar lavage fluid or serum from humans, [255,258] suggesting that Th2 cytokine regulation differs

in mice and humans. sIL-13Rα2 does not contain the short intracellular tail and whilst it can regulate IL-13, by sequestration, it is unable to bind IL-4 and the truncation of the tail prevents binding to IL-4Rα,[165,259] hindering suppression of this cytokine. This especially highlights the importance of the intracellular tail of IL-13Rα2 in the regulation of IL-4.

1.11.3 The Role of IL-13Rα2 in Other Diseases

IL-13Rα2 has been found to play an important role in various fibrotic diseases, such as colitis, as well as cancers, including glioma.[249,260–266]

Fibrosis

IL-4 and IL-13 are key cytokines in the immune response to helminth infections. However in severe infections, such as schistosomiasis, where eggs laid in the hepatic portal vein become trapped in the liver and intestine, production of large quantities of these cytokines in an attempt to fight the infection can lead to tissue destruction and fibrosis.[179] As both IL-4 and IL-13 are important in the pathogenesis of schistosomiasis, much research has been focussed on the role of IL-13Rα2, which is able to suppress the actions of both of these cytokines. In mice treated with sIL-13Rα2 a significant reduction in tissue fibrosis and collagen deposition was observed, however without regulation of IL-4, this is still not completely suppressed.[47,267]

Cancer

Whilst IL-4 is normally associated with differentiation and growth of B and T cells, in the brain under normal conditions this cytokine inhibits the proliferation of astrocytes.[268–270] However, whilst IL-4 decreases proliferation in normal cortex, this is not the case in high grade glioma, where aberrant growth is observed.[271] IL-4 has been found to exhibit pro-tumour activities in rodent experimental glioma, with knockdown of this cytokine acting as an effective therapy in animal models.[272] Interestingly though, this appears to

be specific to higher-grade forms of this cancer as treatment with *IL4* gene transfer appears to actually have therapeutic benefits in low grade glioma. [273]

Astrocytic tumours (gliomas) are the most common brain tumours found in adults and are almost always genetic in aetiology. [274] Glioblastoma multiforme (GBM) is a highly malignant, aggressive form of brain tumour, which arises from astrocytes or their precursors. [274] Astrocytes exhibit many functions in the central nervous system including coordination of inflammation and immune responses in the brain, [275,276] however in GBM these functions are suppressed. IL-13Rα2 is highly expressed in glioblastoma cells but is generally absent in normal astrocytes and lower grade gliomas. [248,277,278] Due to this high expression, IL-13Rα2 has recently been proposed as a biomarker for GBM. [277] This glioblastoma specific expression of IL-13Rα2 has been postulated to prevent the growth arrest normally associated with IL-4 in astrocytes.

1.11.4 Potential Signalling Pathways for IL-13Rα2

Despite previously being considered to only act as a "decoy receptor" due to the short intracellular tail lacking any obvious signalling motifs, in recent years there has been research suggesting that IL-13Rα2 may actually be able to instigate a signal.

Two groups in particular have dominated this research, one proposing an IL-13 induced Activator Protein 1 (AP-1) signal leading to fibrosis, and another suggesting a STAT3 signal in response to IL-4 in GBM.

Signalling Via AP-1

Recent studies in macrophages, as well as animal models of colitis and airway fibrosis by Fichtner-Feigl *et al.* have suggested that IL-13Rα2 is able to transduce a signal, through an AP-1 variant containing c-Jun and Fra-2, which in turn activates the *TGFB1* promotor, leading to fibrosis (Figure 1.7). [261–265]

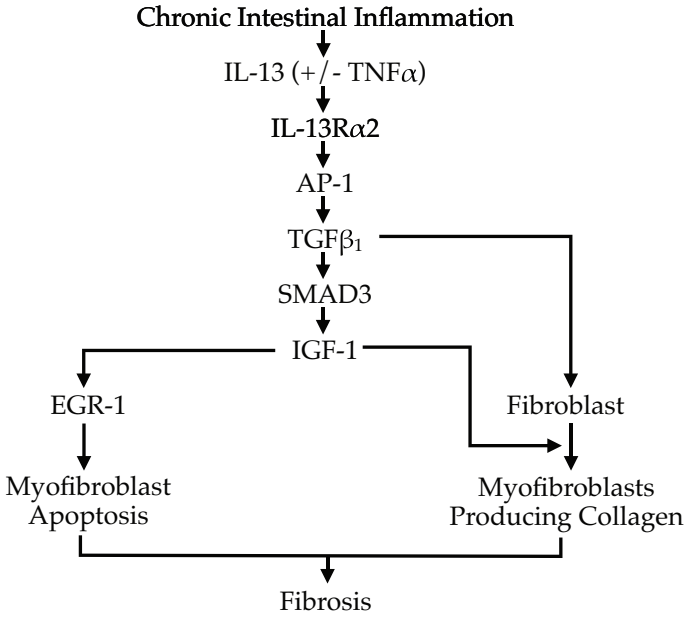

Figure 1.7: Proposed mechanism for IL-13-induced activation of AP-1 via IL-13Rα2. In experimental models of colitis it has been suggested that IL-13-mediated signalling via IL-13Rα2 induced fibrosis. This occurs via activation of AP-1, which drives increased *TGFB1* transcription and induction of a fibrogenic pathway involving insulin-like growth factor 1 (IGF-1) and early growth-response gene product 1 (EGR-1). TGFβ_1 induces the transition of these cells to become myofibroblasts, as well as collagen deposition by both fibroblasts and myofibroblasts. Myofibroblast apoptosis is also observed. As proposed in Fichtner Feigl, Young *et al.* [264]

In oxazolone-induced colitis and bleomycin-induced lung fibrosis mouse models, combined treatment with IL-13 and TNFα was found to induce activation of the *TGFB1* promotor. In both THP-1 cells, which naturally express IL-13Rα2, and an IL-13Rα2 overexpression model in monomac6 cells, AP-1 was required for activation of the *TGFB1* promotor. The authors have proposed that this IL-13 induced activation of AP-1 initiates a fibrogenic pathway, with increased expression of TGFβ_1, insulin-like growth factor 1 (IGF-1) and early growth-response gene product 1 (EGR-1). These are key factors involved in fibrogenic processes, which in turn leads to increased production of collagen by myofibroblasts. [264] Somewhat unexpectedly, apoptosis of myofibroblasts is also observed in response to activation of EGR-1.

However, it has been suggested that myofibroblast function varies throughout the cell life-cycle as a self-regulatory mechanism and that this may aid removal of pre-existing myofibroblasts, which can act to inhibit collagen production by younger myofibroblasts. [279]

Blockade of IL-13Rα2 expression reduced TGFβ_1 and collagen production and prevented bleomycin-induced lung fibrosis. Therefore Fichtner-Feigl *et al.* hypothesised that IL-13 induced fibrosis occurs via IL-13Rα2. [261–265] As airway remodelling, in the form of subepithelial fibrosis, is a hallmark of asthma, activation of the *TGFB1* promotor via IL-13Rα2 could be an important mechanism in this disease.

Signalling Via STAT3

Whilst the work by Fichtner-Feigl *et al.* [261–265] has examined the possibility of an IL-13-mediated signal via IL-13Rα2, other studies by Rahaman *et al.* have investigated a potential IL-4-mediated signal in glioblastoma. [249,280]

Unlike normal astrocytes and low grade gioma where IL-4 exhibits anti-tumour effects, glioblastoma cells are considered refractory to IL-4 and IL-13 as no STAT6 activation occurs after treatment with these cytokines. [249] Recent studies by Rahaman, Sharma *et al.* confirmed that this is due to the high levels of IL-13Rα2 present in these cells. [249] They have since demonstrated that in these cells STAT3 is aberrantly activated by IL-4, which is partially mediated by IL-13Rα2, as demonstrated by knock-down experiments. [280] The exact mechanism by which this occurs is unknown as no physical contact is required between STAT3 and IL-13Rα2. [280]

Ramahan, Vogelbaum *et al.* propose that IL-4 upregulates the BCL2 family of anti-apoptotic proteins, including Bcl-2. Bcl-X_L, Mcl-1, in GBM mainly via STAT3 (Figure 1.8), although the PI3K/AKT pathway may also be involved. [280] In this proposed mechanism the IL-13Rα2 subunit appears to bind to the IL-4Rα subunit, which has previously been shown to occur in the presence of IL-4. [165,249] Rahaman, Vogelbaum *et al.* suggest that this may induce a conformational change allowing the recruitment and activation of STAT3, which is not normally observed. [280]

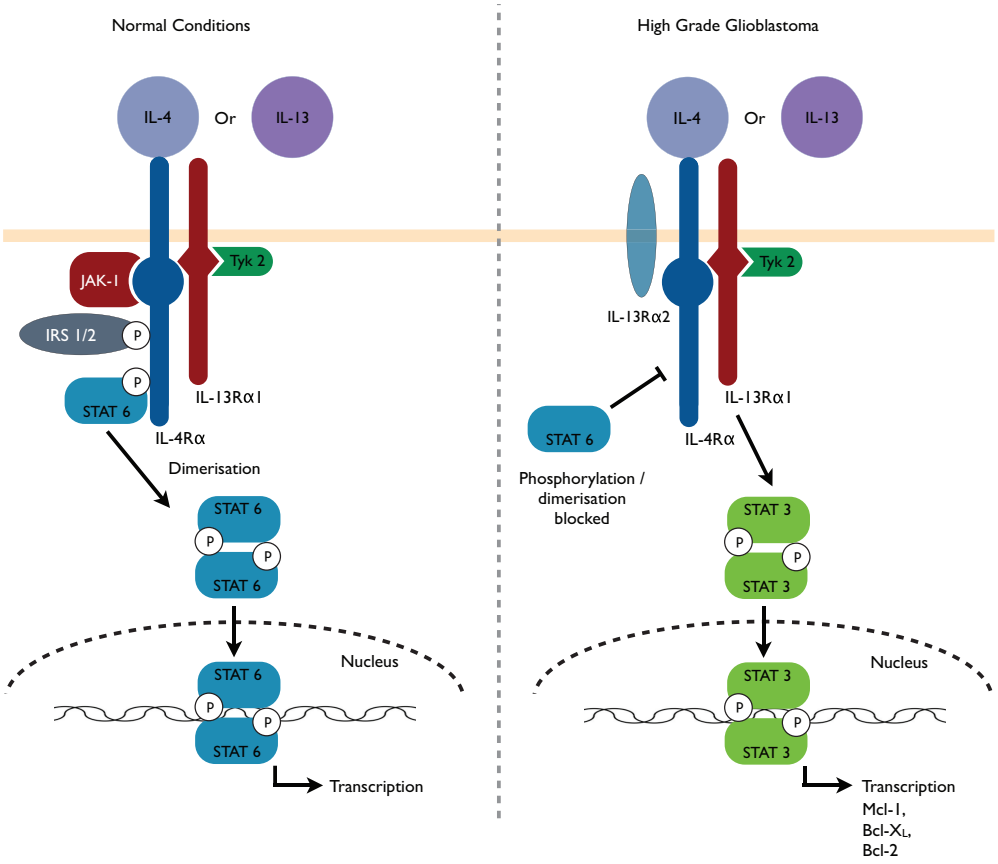

Figure 1.8: Proposed mechanism for IL-4-induced activation of STAT3 via IL-13Rα2. Under normal conditions, stimulation of cells with either IL-4 or IL-13 results in signalling via the type II receptor, IL-4Rα:IL-13Rα1. This leads to activation of the JAK/STAT pathway, with dimerisation of phosphorylated STAT6 inducing transcription of IL-4 and IL-13 responsive genes. However, it has recently been suggested that in high-grade glioma the IL-13Rα2 subunit, which is able to bind to IL-4Rα, interferes with either STAT6 phosphorylation or dimerisation and instead alternative activation of STAT3 occurs. Upon entering the nucleus, this dimerised STAT3 acts as a transcription factor leading to upregulation of Bcl-2. Bcl-X_L and Mcl-1. As proposed by Rahaman, Vogelbaum *et al.* [280]

Whilst previous studies have shown that IL-4 signalling is regulated by IL-13Rα2,[165] the mechanism for this remains unclear. The proposal by Rahaman, Vogelbaum *et al.* that IL-13Rα2 may actually induce a signal in response to IL-4 could provide an explanation for this regulatory mechanism.[280]

1.11.5 The Effect of SNPs on IL-13Rα2 Regulation

Genetics have been found to affect the regulation of IL-13 by IL-13Rα2. At least one polymorphism of IL-13, associated with asthma, demonstrates differing regulation by IL-13Rα2 than the wildtype form. The rs20541 variant of IL-13 is found in 20 % of Caucasians and is more prominent in asthmatics than healthy controls.[147] IL-13Rα2 is unable to regulate this variant form of IL-13 as effectively as wildtype IL-13. This isoform demonstrates slower association with IL-13Rα2 and mediates increased eotaxin production and STAT6 activation in a low IL-13Rα2 environment in comparison to the wildtype IL-13.[281]

1.11.6 IL-13Rα2 in Asthma

Previous work has shown substantial variation in the responsiveness of both bronchial fibroblasts and epithelial cells to IL-4 and IL-13 and that this inversely correlates with IL-13Rα2 levels in the cells, which varies between donors.[165,250,281] It is unclear at present whether this differential expression of IL-13Rα2 accounts for any differences in the asthma phenotype, such as the "Th2 high" and "Th2 low" groups which have been noted in recent years. With IL-13Rα2 able to regulate the effects of both IL-13 and IL-4, this receptor is important in the modulation of Th2 responses.

Therefore, as previous studies have shown that IL-4 signalling is regulated by IL-13Rα2,[165] which could be related to the recent suggestion that this "decoy receptor" may actually induce a signal in response to IL-4, this project will attempt to address the mechanism by which this regulation occurs, to provide greater understanding of the pathogenesis of asthma.

1.12 Objectives & Hypothesis

The hypothesis I wish to test is that IL-13Rα2 is responsible for the regulation of IL-4 mediated signalling in human bronchial fibroblasts and that regulation by IL-13Rα2 is altered in asthma.

The aim of this study is to investigate the mechanism by which IL-13Rα2 regulates IL-4 mediated signalling. This could lead to the opportunity to develop a therapy for asthma. IL-13Rα2 is a tempting therapeutic target not only because it a naturally occurring protein, but it is also able to attenuate both IL-4 and IL-13, which are key cytokines in the instigation of inflammation in the lung. Previous attempts to attenuate either IL-4 or IL-13 individually have been largely unsuccessful due to their overlapping responses and their importance in the maintenance of the immune response in other areas of the body.

Determining the mechanism by which IL-13Rα2 inhibits IL-4 signalling would have therapeutic value as this knowledge could be used to develop small molecule inhibitors.

1.12.1 Objectives:

1. Is the surface expression of IL-4 and IL-13 receptors modulated?

i Characterise the levels of IL-13Rα2 and the IL-4 and IL-13 signalling receptors in human bronchial fibroblasts (HBFs) from healthy and asthmatic subjects.

ii Determine whether receptor expression is modulated by treatment with Th2 or Th1 cytokines.

iii Confirm whether differing levels of IL-13Rα2 or the IL-4/13 signalling receptors observed between subjects affect the responsiveness of the HBFs to IL-4 and IL-13.

2. Can the levels of IL-13Rα2 be modulated?

i Develop a model to modulate the levels of IL-13Rα2 in HBFs using siRNA against IL-13Rα2 in high expressing cells and overexpression of IL-13Rα2 in low expressing cells.

ii Confirm that modification of the surface expression of IL-13Rα2 affects the responsiveness of cells to both IL-13 and IL-4.

3. Does IL-13Rα2 signal?

i Establish whether IL-4 or IL-13 are able to instigate a STAT3 signal in HBFs.

ii Ascertain whether this STAT3 signal is mediated by IL-13Rα2.

Chapter 2

Materials & Methods

2.1 Materials

The Interleukin (IL)-13 used in these studies was a gift from Novartis, Horsham, UK. All other reagents were obtained from suppliers as detailed below.

2.1.1 Cell Culture

Advanced BioMatrix, Tuscon, USA Bovine Collagen Type I (5005-B).

Becton Dickinson, Oxford, UK Matrigel (356231).

Greiner, Gloucestershire, UK Cryovials (126278).

Invitrogen, Paisley, UK Dulbecco's Modified Eagles Medium (DMEM) (11960044), Heat-Inactivated Foetal Bovine Serum (FBS) (10108165), Penicillin Streptomycin (25030-024), L-Glutamine (25030-024), Sodium Pyruvate (11360-039), Minimum Essential Medium Non Essential Amino Acids (11140-035), Trypsin-EDTA (15400054), Hank's balanced salt solution (HBSS) without $Ca^{2+}Mg^{2+}$(14170-0880).

Lonza, Wokingham, UK BEBM Basal Medium (CC-3171), BEGM Single-Quot Kit Supplements & Growth Factors (CC-4175) UltraCulture (12-725F).

Nutacon, Leimuiden, The Netherlands PureColl rat tail Collagen I (5005-B).

Sigma-Aldrich, Poole, UK Bovine insulin, human transferrin, and sodium selenite (ITS) liquid media supplement 100× stock solution (I3146), Dimethylsulphoxide (DMSO) (D2650), Trypan Blue (T8154).

ThermoFisher Scientific, Loughborough, UK Nunc Cell Culture Flasks and Plates.

2.1.2 Cell Treatments

Invivogen, Nottingham, UK Polyinosinic-Polycytidylic acid (Poly I:C) (tlrl-pic).

National Institute for Biological Standards and Control (Health Protection Agency), Potters Bar, UK IFNβ (00/572).

PeproTech, London UK IL-4 (200-04), IL-6 (200-06), IFNγ (300-02).

R&D Systems, Abingdon, UK Monoclonal Anti-Human IL-6 Receptor (MAB227), Anti-Human IL-13Rα2 antibody (AF146).

Sigma-Aldrich, Poole, UK Cycloheximide (C4859), Dexamethasone (D2915).

Stratech, Newmarket, UK Monoclonal Antibody to Human Interferon α/β R2 (IFNAR2) (21385-1).

2.1.3 siRNA Transfection

Dharmacon, ThermoFisher Scientific, Loughborough, UK Pool of small interfering RNA (siRNA) against IL-13Rα2 (L-004598-00) and Control siRNA (D-001810-10-05), 1× siRNA buffer (B002000-UB-100).

Invitrogen, Paisley, UK OptiMEM (11058-021).

Roche Applied Science, Burgess Hill, UK X-treme Gene siRNA Transfection Reagent (04 476 093 001).

2.1.4 Vector Transfection

American Type Culture Collection (ATCC), Manassas, USA BEAS-2B cell line (CRL-9609).

Cell Signaling Technology, New England BioLabs, Hertfordshire, UK *Hind*III Restriction Endonuclease (R0104), *Sal*I Restriction Endonuclease (R0138).

Invivogen, Nottingham, UK F-12 + GlutaMAX-1 Nutrient Mixture (Hams) (31765), Geneticin (10131-019), pcDNA3.1 (–) vector.

Origene, Cambridge, UK *IL13RA2* cDNA NM_00640 (SC125642).

Qiagen, Crawley, UK Effectene Transfection Reagent (301425).

2.1.5 RNA Extraction, Reverse Transcription & Quantitative Polymerase Chain Reaction (PCR)

Ambion Applied Biosciences, Warrington, UK DNase Kit including 10× DNase Buffer (AM1906).

BioRad, Hertfordshire, UK Plate seals (MSB-1001), Thin Walled 96-Well PCR plates (MLL-9651).

Invitrogen, Paisley, UK Trizol (15596018).

PrimerDesign, Southampton, UK Precision MasterMix (Precision-ic), Primers (PP-hu-900) for IL-6, IL-4Rα, IL-13Rα1, IL-13Rα2, γc and Ubiquitin C/Phospholipase A2 housekeeping genes (HK-PP-hu-900), Reverse Transcription Kit (RT-Std).

Sigma-Aldrich, Poole, UK Chloroform (C2432), Ethanol (32221), Isopropanol (I9516).

2.1.6 Flow Cytometry

BD Biosciences, Oxford, UK Allophycocyanin (APC)-conjugated mouse IgG_{2B} negative control (555745), FACSAria, FACSDiva software v5.0.3.

Dako, Cambridgeshire, UK Polyclonal rabbit anti-mouse immunoglobulins/FITC Rabbit F(ab')2 (F031302).

GenProbe, Cedex, France Unconjugated mouse anti-human IL-13Rα2 (852.120.000), PE-conjugated mouse anti-human IL-13Rα2 (852.122.010), mouse IgG_1 (857.070.000), mouse IgG_{2A} (857.080.000).

Invitrogen, Paisley, UK Heat-Inactivated FBS (10108165).

Promokine, Heidelberg, Germany Alexafluor-405 (PK-PF-LK-405-05) and Alexafluor-488 (PK-PF-LK-488-05) antibody dye conjugation kits.

R&D Systems, Abingdon, UK APC-conjugated mouse anti-human IL-13Rα1 (FAB1462A), APC-conjugated mouse anti-human IL-4Rα (FAB230A), Carboxyfluorescein (CFS)-conjugated mouse anti-human IL-13Rα1 (FAB1462F), unconjugated mouse anti-human γc (MAB2841), CFS-conjugated mouse IgG_{2B} (IC0041F) and APC-conjugated mouse IgG_{2A} (IC003A).

Sigma-Aldrich, Poole, UK Bovine Serum Albumin (BSA) Fraction V (A3059), Dulbecco's Phosphate Buffered Saline (PBS) without $Ca^{2+}Mg^{2+}$ (D8537), Ethylenediaminetetraacetic acid (EDTA) (03690).

2.1.7 SDS-PAGE & Western Blotting

BioRad, Hertfordshire, UK 30 % Acrylamide/Bis-Acrylamide (37:1) (161-0157), filter paper (1704085), Tris-Glycine-SDS (161-0772), Pre-cast SDS-PAGE gels (Criterion) (345-0112), Precision Plus kaleidoscope colour protein standard (161-0395), Mini-Protean Tetra Cell, Mini Trans-Blot Cell, MOPS buffer (161-0788), GS800 densitometer and QuantityOne software.

Cell Signaling Technology, New England BioLabs, Hertfordshire, UK Rabbit anti-human Phospho-STAT3 (Tyr705) (#9131), Rabbit anti-human STAT3 (#9132), Rabbit anti-human Phospho-STAT6 (Tyr641) (#9361), Rabbit anti-human STAT6 (#9362).

Dako, Cambridgeshire, UK Swine Anti-Rabbit Secondary (P0217), Rabbit Anti-Mouse Secondary (E0464).

Fotospeed, Corsham, UK Print Developer (FX20), Rapid Fixer (PD5).

GE Life Sciences, Buckinghamshire, UK Enhanced Chemiluminescence (ECL) Plus (RPN2132), Hybond-P Polyvinylidine fluoride (PVDF) Transfer Membrane (RPN303F), Hyperfilm^{TM} ECL X-Ray Film (28906837).

Marvel, Dublin, ROI Skimmed Dried Milk.

Roche Applied Science, Burgess Hill, UK Complete Protease Inhibitor Cocktail Tablets (11697498001), PhosStop Phosphatase Inhibitor Cocktail Tablets (04906837001).

Sigma-Aldrich, Poole, UK Ammonium persulphate (APS) (A9164), β-mercaptoethanol (B-8026), Bromophenol Blue (M7522), Bovine Serum Albumin (BSA) Fraction V (A3059), Coomassie Brilliant Blue R (B0149), Glacial Acetic Acid (27221), Glycine (G7126), Hydrochloric Acid (HCl) (07102), Isopropanol (24137), Methanol (24229), N,N,N',N'-tetramethylethylenediamine (TEMED) (T7024), Ponceau Red Stain (P7170), Sodium Chloride (NaCl) (S9625), Sodium Dodecyl Sulphate (SDS) (L6026), Tris base (T1503), Tween-20 (P1379).

2.1.8 Enzyme-Linked ImmunoSorbant Assay (ELISA)

R&D Systems, Abingdon, UK Eotaxin DuoSet ELISA Kit (DY320), Eotaxin Quantikine ELISA Kit (DTX00) IL-6 Quantikine ELISA Kit (D6050).

2.1.9 Methylene Blue Assay

Sigma-Aldrich, Poole, UK Borate disodium tetraborate (Borax) (B9876), Ethanol (32221), Formaldehyde (F8775), Hydrochloric Acid (HCl) (07102), Methylene Blue (M-9140), NaCl (S9625).

2.2 Patient History

A panel of 23 patients was used for this study, which includes 15 asthmatics and 8 non-asthmatics. Cells were obtained from the Synairgen Research Ltd. Biobank of Primary Cells and were provided under MTA to the University of Southampton. The cells were used under ethics approval granted by the Southampton and Southwest Hampshire Research Ethics Committee (ethics number: 05/Q1702/165 Investigation of pathophysiological mechanisms in airways diseases such as asthma and COPD). Samples were assigned a patient number to protect patient identity and collected from Southampton General Hospital by Synairgen Research Ltd.

Age and sex matched patients were categorised based on GINA guidelines (Tables 2.1 & 2.2).[14] No differences were found in FEV_1 or % predicted FEV_1 between the groups. PC_{20} data were not collected for healthy controls, who had no previous history of asthma. Some healthy control patients were atopic, but this was more frequently observed in the asthmatics ($p < 0.01$).

2.2.1 Healthy Control Subjects

Code	**Age**	**Sex**	**Atopy**	**FEV_1**	**% FEV_1**	**αSMA**
A55	27	M	No	4.64	94.6	10
A56	60	M	No	3.63	114.5	5-10
A57	31	M	No	4.41	98.7	20
A60	23	M	Yes	4.75	97.4	20
A63	24	F	No	3.73	115.1	15-20
A67	35	M	Yes	5.14	120.0	20
A72	24	M	Yes	4.77	115.9	10-15
A75	32	F	No	3.16	132.9	20
Mean	32.0	2/6 [a]	5/3 [b]	4.28	111.1	16
SE	4.3			0.24	4.67	1.8

Table 2.1: Healthy control subject patient history. Healthy controls with no previous history of asthma were given code A + number. [a] = Female/Male, [b] = No/Yes, PC_{20} data were not collected for healthy control volunteers. αSMA = % α smooth muscle actin, % FEV_1 = % predicted FEV_1 , SE = Standard Error.

2.2.2 Asthmatic Subjects

Code	**Age**	**Sex**	**Atopy**	**FEV_1**	**% FEV_1**	**PC_{20}**	**Medication**	**αSMA**
B11	22	M	Yes	5.02	107.0	7.0	SABA	20
B12	54	M	Yes	3.21	104.0	1.2	SABA	25
B22	21	F	Yes	3.87	104.3	11.8	SABA, ICS	10-15
B24	34	M	Yes	4.30	101.8	2.5	None	5-10
B25	20	M	Yes	5.16	114.7	8.0	SABA	20
B28	26	F	No	2.93	88.5	0.0	SABA, ICS	20
B29	22	F	Yes	3.95	105.0	1.1	SABA, ICS	10
B32	51	F	Yes	2.68	132.4	15.5	SABA	30
B33	23	M	Yes	4.79	102.5	6.1	SABA	30
B35	21	M	Yes	4.87	110.7	>16	SABA, ICS	5
C11	34	M	Yes	3.58	97.0	1.0	SABA, ICS 200 μg BD	10-15
C22	26	M	Yes	4.57	99.0	0.0	ICS+LABA 200 μg BD	<5
C23	29	M	Yes	3.87	93.0	4.0	ICS+LABA 100 μg BD, SABA, NICS	10-15
C30	36	F	Yes	2.85	87.0	2.6	SABA, ICS 100 μg BD, NICS BD	10
C32	24	M	Yes	4.81	95.0	16	ICS+LABA 100 μg BD, SABA	20
Mean	29.5	5/10 [a]	1/14 [b]	4.03	102.8	6.2		16
SE	2.8			0.22	2.90	1.5		2.2

Table 2.2: Asthmatic patient history. Mild-intermittent (B) or mild-moderate asthmatics (C) grouped based on GINA guidelines. [14] A PC_{20} of 0 mg/ml indicates a decrease with normal saline in the absence of methacholine. ICS presented as Beclometasone dipropionate equivalent dose. All medication taken as required unless dose stated. [a] = Female/Male, [b] = No/Yes, BD = 2× daily, % FEV_1 = % predicted FEV_1 , ICS = Inhaled Corticosteroid, ICS+LABA = Combined therapy of ICS and LABA, LABA = Long Acting β_2 Agonist, NICS = Nasal ICS, SABA = Short Acting β_2 Agonist, SE = Standard Error.

2.3 Methods

2.3.1 Cell Culture

Primary human fibroblasts (HBFs) were obtained by outgrowth from bronchial biopsies of either healthy or known asthmatic patients, as previously described. [250] Cells were used between passage number 3 and 7.

Primary bronchial epithelial cells (PBECs) were obtained from bronchial brushings from three healthy control subjects with no previous history of asthma or atopy, as previously described. [250] These cells were kindly provided at passage 2 on collagen I coated 12 well plates by Drs Emily Swindle and Cornelia Blume.

All culture reagents were pre-warmed before addition to the cells to prevent heat-shock. Primary fibroblasts were routinely cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM nonessential amino acids (DMEM Complete). Epithelial cells were cultured in Bronchial Epithelial Growth Medium (BEGM). Both fibroblasts and epithelial cells were incubated in a humidified Heraeus incubator at 37°C, 5 % CO_2. All cell culture work was undertaken in a Class II biological safety laminar flow hood.

2.3.2 Trypsinisation of Confluent Cell Monolayers

Fibroblasts were grown as monolayers on tissue culture plastic to 90 % confluency before passaging.

Trypsin-EDTA concentrate (10×) was diluted before use to a 1× working solution using Hank's balanced salt solution (HBSS) without $Ca^{2+}Mg^{2+}$. The cell monolayer was washed twice with HBSS to remove all traces of serum, Ca^{2+} and Mg^{2+}, which can inhibit the action of trypsin. The monolayer was washed with 1 ml of trypsin for a 75 cm^2 flask and the cells were incubated

for 1 minute (or until the majority of the cells had become loose, as visible under the microscope) at 37°C, 5 % CO_2. The flasks were tapped sharply to detach the cells and the trypsin was inactivated by addition of DMEM Complete.

Cells were either frozen as stocks, or seeded into 75 cm^2 flasks at a seeding density of 0.75×10^6 cells/flask. For experiments cells were seeded in 6 or 12 well plates at a density of 5×10^4 and 1×10^5 cells/well respectively.

Where indicated (in Chapter 5) some cells were seeded onto a Matrigel-coated well (200 μl/well of a 12 well plate) or within Matrigel (500 μl/well) for 3-dimensional culture. Matrigel is liquid at 4°C, but forms a gel at 37°C. The Matrigel-coated wells were prepared in advance by adding Matrigel to the well and incubating at 37°C prior to addition of the fibroblasts. When the cells were grown in 3D culture, however, the cells were premixed with chilled Matrigel prior to seeding. In this instance media was added to the culture after 10 minutes incubation at 37°C to allow gel polymerisation.

2.3.3 Viable Cell Counting

The Trypan Blue exclusion method was used to count the number of viable, live cells. Non-viable cells stain blue, whilst viable cells with intact cell membranes exclude the dye. Before counting, 20 μl of cell suspension was diluted into 20 μl HBSS and 10 μl of 0.4 % (w/v) Trypan Blue.

An Improved Neubauer haemocytometer (depth 0.1 mm; 1/400 mm^2) was used to count the number of viable cells present within the 1 mm^2 central square. This was repeated for both the top and bottom chambers for accuracy. The viable cell count per millilitre was then calculated using the following equation:

$$\text{Viable cells/ml} = (\text{viable cell number}) \times (\text{dilution factor used}) \times 10^4.$$

Cells were diluted in DMEM Complete, to the appropriate concentration and plated into wells or culture flasks, as required.

2.3.4 Cryogenic Storage & Regeneration of Cell Stocks

Post-trypsinisation, the cell suspension was pelleted at 300 g for 5 minutes at 4°C. The cells (approximately 1×10^6 cells per vial) were resuspended in 1 ml of chilled DMEM Complete containing 10 % (v/v) dimethylsulphoxide (DMSO), to act as a cryoprotectant and frozen in 2 ml cryovials at a rate of -1°C per minute to -80°C overnight in a cryogenic freezing container, before being transferred to liquid nitrogen vapour at -150°C, for long-term cryogenic storage.

For regeneration, cell stocks were warmed rapidly by the addition of pre-warmed DMEM Complete. Once defrosted, the cells were pelleted and resuspended in 15 ml DMEM Complete to remove all traces of DMSO and transferred into a 75 cm^2 flask for routine culture.

2.3.5 Treatments

Before treatment, fibroblasts were grown to ∼95 % confluence and serum starved using UltraCulture supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (SFM) for 24 hours to make the cells quiescent. In some instances fibroblasts were pre-treated with 10 ng/ml IL-4 or IL-13 during this initial 24 hour period. The medium was replaced with fresh UltraCulture in the presence or absence of recombinant human Interleukin (IL)-13, IL-4, IL-6, Interferon (IFN) β, IFNγ or Poly I:C at varying concentrations (see Chapters 3 – 5 for details). Where indicated these challenges were supplemented with dexamethasone treatment.

For PBECs the media were exchanged for BEBM containing 10 μg/ml bovine insulin, 5.5 μg/ml human transferrin, 5 μg/ml sodium selenite and 0.1 % bovine serum albumin (BSA) (starvation media) for 24 hours prior to treatment. Subsequently the media were exchanged for fresh starvation media in the absence or presence of IL-13 or IFNγ for up to 24 hours.

For experiments analysing *de novo* protein synthesis a 30 minute pre-treatment step of 50 μl cycloheximide (CHX) was included after the 24 hour starvation period.

2.3.6 siRNA Against IL-13Rα2

Fibroblasts were seeded at 2×10^4 cells per well of a 12 well plate in DMEM supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM nonessential amino acids but without antibiotics (siRNA Media) and used once they reached ~30-40 % confluence. A pool of siRNA against IL-13Rα2 or control siRNA (varying concentrations from 0 to 50 nM) was diluted in OptiMEM (up to 100 μl total) in one eppendorf, and XtremeGene transfection reagent (2 μl) was combined with OptiMEM (98 μl) in a separate eppendorf. The XtremeGene and siRNA were mixed and incubated for 15 minutes at room temperature. During this incubation the existing media on the cells was exchanged with 800 μl fresh siRNA media before addition of the siRNA:Xtreme Gene drop-wise onto the cells in duplicate.

Twenty-four to 96 hours after addition of the siRNA the cells were homogenised in Trizol and analysed by RT-qPCR or trypsinised and analysed by flow cytometry.

2.3.7 Overexpression of IL-13Rα2

BEAS2B cells overexpressing IL-13Rα2 were provided by Dr Allison-Lynn Andrews and Dr Ali Tavassoli (School of Chemistry). Briefly, IL-13Rα2 was amplified by PCR from cloned cDNA using the following primers:

Forward	*Sal*I	GTTGTTCTCGAGATGGCTTTCGTTTGCTTGGC
Reverse	*Hind*III	GTTGTTAAGCTTTCATGTATCACAGAAAAATTCTGG

The PCR product was digested with *Sal*I and *Hind*III restriction endonucleases, and cloned into pcDNA3.1 (-) vector between the *Sal*I and *Hind*III

restriction sites (Figure 2.1). The fidelity of the cloned sequence was confirmed by sequencing. The cytomegalovirus (CMV) promotor in this vector drives high levels of expression without the need for doxycycline regulation. This system allows the establishment of stable cell lines by Geneticin selection.

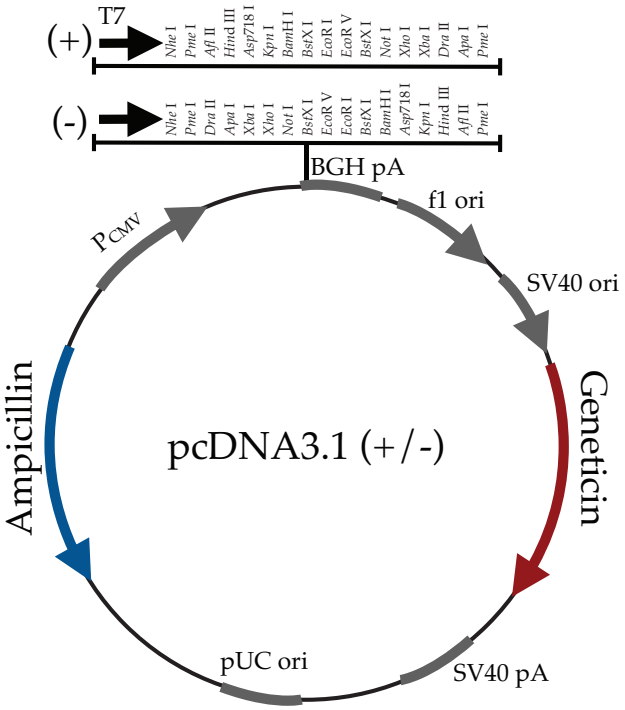

Figure 2.1: Vector map. The IL-13Rα2 cDNA was inserted at the *Eco*R I site. Transcription was driven by a CMV promotor. Vector contains an ampicillin resistance gene and a neomycin (Geneticin) resistance gene, which are used for selection. P_{CMV} = Cytomegalovirus promotor, Ori = origin of replication. SV40 ori = SV40 early promotor & origin, SV40 pA = SV40 early polyadenylation signal

BEAS2B cells were transfected with either a pcDNA3.1 vector containing IL-13Rα2 (B2B-FL) or an empty vector (B2B) by lipofection. Stably transfected cells (i.e. cells which had accepted the DNA into their genome) were selected using Geneticin (600 μg/ml). This was reduced to a maintenance dose of 200 μg/ml for routine cell culture. Levels of IL-13Rα2 surface expression were determined by flow cytometry.

2.3.8 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

For gene expression studies, fibroblasts were serum starved for 24 hours before treatment with various stimuli for up to 48 hours. The cells were washed and the RNA was extracted from the cells using the Trizol method. The RNA was subsequently reverse transcribed to cDNA before analysis by RT-qPCR.

RNA Extraction

Trizol is a mono-phasic solution of phenol and guanidine isothiocyanate, which enables single-step RNA isolation from cells by maintaining the integrity of the RNA during cell homogenisation. [282] The expected yield of RNA from 1×10^6 fibroblasts is between 5 and 7 μg.

The cell monolayer was washed with HBSS and then incubated with Trizol for 5 minutes at room temperature to allow cellular lysis, as well as dissociation of soluble protein factors from the RNA. This was followed by disruption by pipetting, prior to transfer to an RNase and DNase free sterile 1.5 ml eppendorf. The samples were stored at -20°C until required.

The samples were thawed on ice and 200 μl of chloroform was added per ml of Trizol used. The samples were mixed vigorously by hand and incubated for 10 minutes before centrifugation at 12,000 g for 15 minutes at 4°C. The top aqueous layer containing the RNA was then transferred to a fresh eppendorf and the remaining interphase and organic phase layers were left intact and stored at -20°C, to allow future extraction of DNA via ethanol precipitation. Protein could be recovered from the organic phenol phase using isopropanol precipitation. Isopropanol (500 μl, equivalent to the volume of the aqueous layer) was then added and the samples were mixed thoroughly by shaking and stored overnight at -80°C to improve the RNA yield. The samples were incubated on ice for 15 minutes, thoroughly mixed by vortexing for 5 to 10 seconds and centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant was discarded, and the pellet containing the RNA

was washed with 1 ml 75 % ethanol. The pellet was re-centrifuged at 7,500 g 4°C for 5 minutes, the ethanol was removed and the pellet was allowed to air dry for 10 minutes before DNase treatment.

DNase Treatment

To remove trace amounts of genomic DNA present within Trizol extracted RNA, the samples were treated with DNase. The DNase (1 μl of 2 U/μl stock) was diluted in distilled water (17 μl) containing 10× DNase buffer (2 μl) before use. Each RNA pellet was resuspended in 20 μl and incubated at 37°C for 1 hour, before addition of 5 μl neutralisation buffer to prevent degradation of the RNA. After a 2 minute incubation at room temperature the samples were spun at 12,000 g for 2 minutes to separate the slurry from the RNA.

RNA Quantification

A NanoDrop ND-1000 Spectrophotometer was used to determine both the quantity and quality of the RNA in each sample, by measuring absorbance at the 230, 260 and 280 nm wavelengths.

The scanning area was wiped clean with a distilled water soaked tissue before injection of 1 μl of distilled H_2O to act as the blank. Following this, 1μl of sample was added to the scanning area and detected. The quality of the RNA was analysed by the ratio between the wavelengths 260 and 280 nm (a value of between 1.8 and 2 is desired). As 1 μg RNA was used for each reverse transcription reaction the volume of RNA required per sample was determined by dividing 1,000 by the ng/μl value provided.

Reverse Transcription

Reverse transcription was performed on all Trizol-extracted RNA samples to obtain cDNA, for analysis using quantitative polymerase chain reaction (qPCR).

The RNA (1 μg) was added in the initial, annealing step of reverse transcription along with 2 μl Oligo dT primer dTNP mix and 1 μl Random hexamer primer. This was then made up to 10 μl using RNase/DNase free H_2O. This reaction was carried out within a thin-walled 200 μl tube to allow rapid heating and cooling. The samples were heated to 65°C for 5 minutes, followed by immediate cooling in an ice water bath. For the extension step, a master mix containing 4 μl 5× MMLV buffer, 0.2 μl RNase/DNase free H_2O and 0.8 μl MMLV enzyme was added to each of the tubes. The samples were then mixed by flicking the tubes, and incubated at 42°C in a water bath for 1 hour. After incubation, the cDNA was diluted 1:10 in DNase/RNase free water and stored at -20°C.

Quantitative PCR

Real time quantitative polymerase chain reaction (RT-qPCR) [283] was performed using the BioRad CFX system. 2.5 μl of cDNA template was transferred to a well of a thin walled 96 well plate, together with 1 μl primer and probe (see Appendix A for sequences) and 12.5 μl (22.5 μl for housekeeping genes, where two fluorophores were multiplexed) Precision Master Mix. Each sample was measured in duplicate, and all changes in gene expression were compared to two previously determined housekeeping genes (Ubiquitin C and Phospholipase A2 (UBC/A2)). All primers and probes were pre-labelled with FAM (6-carboxyfluoroscein), except Phospholipase A2 which is labelled with Cy5.5 to enable multiplexing of the housekeeping genes. Controls for the reverse transcription reaction (water used during the reaction, no-RNA and no-enzyme reactions) and the PCR were included in each run.

Data obtained from the RT-qPCR were analysed using the $2^{\Delta\Delta}$CT method. [284] During amplification of cDNA the fluorescent dye is released, resulting in increased fluorescent intensity in samples with more of the gene of interest. The number of cycles of amplification required for the level of fluorescent intensity to reach a threshold (the CT value) is related to the amount of target gene cDNA present, with a lower CT value indicating

greater gene expression (a decrease of 1 $^{\Delta}$CT is equal to a doubling of the target gene). The CT values for the gene of interest were normalised to the geometric mean of two housekeeping genes (UBC/A2). These data were subsequently normalised to the baseline control value of one subject. [285]

2.3.9 Flow Cytometric Analysis of IL-4 & IL-13 Receptor Subunits

Cells were characterised for the surface expression levels of IL-4Rα, IL-13Rα1, IL-13Rα2 and γc by flow cytometry. Fibroblasts were seeded into 12 well plates at a density of approximately 2×10^4 cells per well, and grown until ∼95 % confluent, as previously described. The cells were serum starved for 24 hours and treated with either IFNβ, IFNγ, IL-4, IL-13, or Poly I:C for up to 24 hours prior to analysis, as described in the results section. Where appropriate a washout period was also included, consisting of the media being exchanged for fresh ultraculture for 3 hours when indicated.

The cells were trypsinised, and the trypsin was inactivated by addition of 1 ml DMEM complete. The cell suspension was then washed in 2 ml FACS buffer (1× PBS, 0.5 % BSA, 2 mM EDTA) and centrifuged at 300 g for 5 minutes at 4°C. Samples were incubated on ice in the dark for 45 minutes with an appropriate antibody or isotype control (Table 2.3) in 100 μl FACS buffer containing 10 % human serum.

Antibody	**Fluorophore**	**Dilution**	**Isotype**	**Manufacturer**
IL-4Rα	APC	1:10	Mouse IgG_1	R&D Systems
IL-13Rα1	APC	1:10	Mouse IgG_{2B}	BD Pharmingen
IL-13Rα1	CFS	1:10	Mouse IgG_{2B}	R&D Systems
IL-13Rα2	FITC 2°	1:200	Mouse IgG_1	GenProbe
IL-13Rα2	PE	1:10	Mouse IgG_1	GenProbe
γc	AF-405	1:20	Mouse IgG_{2A}	GenProbe

Table 2.3: Flow cytometry antibody dilutions. 2° = Secondary antibody, AF = Alexafluor, APC = Allophycocyanin, CFS = Carboxyfluoroscein, FITC = Fluoroscein Isothiocyanate, PE = Phycoetherin

Prior to use the γc and mouse IgG_{2A} isotype controls were labelled with a Promokine Alexafluor-405 dye, according to the manufacturers instructions. In initial experiments the IL-13Rα2 antibody and mouse IgG_1 isotype control were obtained in an unconjugated format and used with a rabbit anti-mouse Fluorescein isothiocyanate (FITC) labelled secondary antibody. In later experiments the fluorophore used for the IL-13Rα1 and isotype control was changed from allophycocyanin (APC) to carboxyfluoroscein (CFS) and the IL-13Rα2 antibody and isotype controls were purchased with a directly conjugated fluorophore (phycoetherin (PE)) in order to use all the fluorescent antibodies together in one reaction. In both cases the original antibody clone from the same company was used.

Cells were washed with 2 ml FACS buffer to remove any excess antibody, centrifuged at 300 g for 5 minutes and resuspended in 250 μl FACS buffer. Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3. Mean fluorescent intensities (MFI) were normalised to isotype controls giving specific MFI (SMFI).

2.3.10 SDS-PAGE & Western Blotting

Sample Preparation

Fibroblast monolayers were washed after treatment with ice cold phosphate buffered saline (PBS) and lysed in boiling sample buffer (62.5 mM Tris-HCl pH 6.8, 10 % glycerol, 5 % β-mercaptoethanol, 2 % SDS, 0.01 % Bromophenol blue), which contains a blue dye, enabling visualisation of the running front as it moves through the gel. The samples were scraped from the base of the well using a cell scraper and stored in eppendorf tubes at -20°C until required. Before western blotting, the DNA within the samples was sheared by sonication using a probe sonicator for 15 seconds at an amplitude of 2.5 μM and the samples were boiled at 95°C for 5 minutes to denature the proteins. The samples were pulse spun and cooled to room temperature before loading into an sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

SDS-PAGE

SDS-PAGE was performed using a 10 % polyacrylamide resolving-gel with a 4 % stacking gel, cast to a 1 mm thickness (see Appendix A) using the Tetra Cell system. The 10 % concentration was chosen to provide the best resolution of proteins between 40 and 100 kiloDaltons (kDa). The resolving layer was overlaid with water-saturated isopropanol to exclude oxygen, an inhibitor of polymerisation. After allowing at least 45 minutes for the gel to polymerise, the isopropanol was removed and the gel surface was washed with water before addition of a 4 % polyacrylamide stacking layer, and a 10-well comb was inserted. Twenty-five microlitres of sample was loaded per lane and 5 μl of PrecisionPlus kaleidoscope protein standard was added to enable a molecular weight comparison of the sample protein bands. The gels were run in at a constant 100 V (up to 300 mA) in Tris-Glycine-SDS (TGS) buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1 % SDS) until the dye front reached the bottom of the gel.

Where indicated, the SDS-PAGE gel was immersed in Coomassie Brilliant Blue stain for 1 hour and destained in 25% methanol, 10% glacial acetic acid overnight.

Western Blotting

The gels were preshrunk in Transfer buffer (TGS buffer containing 20 % methanol) for 15 minutes, before western blotting. A polyvinylidene difluoride (PVDF) membrane was prepared by pre-wetting with methanol for 3 minutes, followed by transfer buffer for 10 minutes.

The gel and PVDF were sandwiched between blotting paper and Scotchbrite^{TM} pads, and inserted into a transfer chamber filled with transfer buffer with the PVDF closest to the anode. The protein bands within the gel were transferred to PVDF at a constant 90 V (up to 350 mA) on ice, with constant stirring to ensure heat dissipation, for 2 hours to allow for complete transfer of high molecular weight proteins.

Ponceau Staining

Ponceau stain was used to confirm transfer of the proteins from the gel to the membrane. This stain binds reversibly to the protein bands on a PVDF membrane, and can be easily removed by washing with water without affecting the protein bound to the membrane.

After electrophoretic transfer, the membrane was washed for 15 minutes in Tris-buffered saline containing 0.05 % Tween 20 (TBS-Tween) to remove any excess salts. The membrane was then fully immersed in ponceau stain (0.1 % (w/v) Ponceau S in 5% acetic acid (w/v)) for 5 minutes. Excess stain was removed by rinsing in distilled water, until the background was clear. The presence of protein on the membrane was indicated by red bands.

Antibody Incubation

After ponceau staining the membrane was washed in TBS-Tween for 10 minutes to remove any remaining red stain before incubating in 5 % low-fat milk powder in TBS-Tween (blocking buffer) for 30 minutes at room temperature with rocking to block non-specific binding sites.

The primary antibody was diluted in TBS-Tween containing 5 % (w/v) bovine serum albumin fraction V (BSA), as directed by the manufacturer, (see Table 2.4 for details) and incubated overnight, with rocking at 4°C.

The membrane was then washed in TBS-Tween for 30 minutes with three changes in buffer to remove any excess antibody. An appropriate horseradish peroxidase (HRP) conjugated secondary antibody was selected and diluted in blocking buffer (see Table 2.4 for details) and incubated for one hour at room temperature, followed by four, 10 minute washes in TBS-Tween.

Protein Detection

Enhanced Chemiluminescence (ECL) plus was used for detection of the protein bands. The ECL plus reagents were equilibrated to room temperature

Antibody	**Species**	**Concentration**	**Dilution**
Phospho-STAT3 (Tyr705)	Rabbit	43 μg/ml	1:1000
Pan-STAT3	Rabbit	89 μg/ml	1:1000
Phospho-STAT6 (Tyr641)	Rabbit	42.6 μg/ml	1:1000
Pan-STAT6	Rabbit	100 μg/ml	1:1000
HRP Conjugated Anti-Rabbit 2°	Swine	1300 μg/ml	1:2000

Table 2.4: Western blotting antibody dilutions. 2° = Secondary antibody, HRP = Horseradish Peroxidase

before use and used according to the manufacturers instructions. The membrane was incubated in ECL plus for five minutes, before draining the excess reagent by dabbing the corner of the membrane against a tissue. The membrane was carefully placed protein side up between two sheets of transparent OHP film, ensuring no air bubbles were present and the sheets were sealed shut in an X-ray film cassette and taped in place to prevent movement during exposure. In a dark room autoradiography film was exposed to the membrane for 1 minute. The X-ray film was developed in Print Developer for 1 minute with rocking, and fixed in Rapid Fixer for one minute with rocking before immersion in water. If necessary, this initial exposure was used to determine the subsequent exposure times, which varied from generally between 5 seconds and 5 minutes.

Protein bands were quantified using a densitometer and normalised to the pan-protein using QuantityOne software.

2.3.11 Enzyme-Linked ImmunoSorbant Assay (ELISA)

Conditioned media from cells were analysed for levels of eotaxin or IL-6 by ELISA. The principals of this assay are summarised in Figure 2.2.

Sample Preparation

Fibroblasts were seeded at 5×10^4 cells per well in 12 well plates and grown to $\sim$95 % confluence. For analysis of eotaxin production, cells were serum

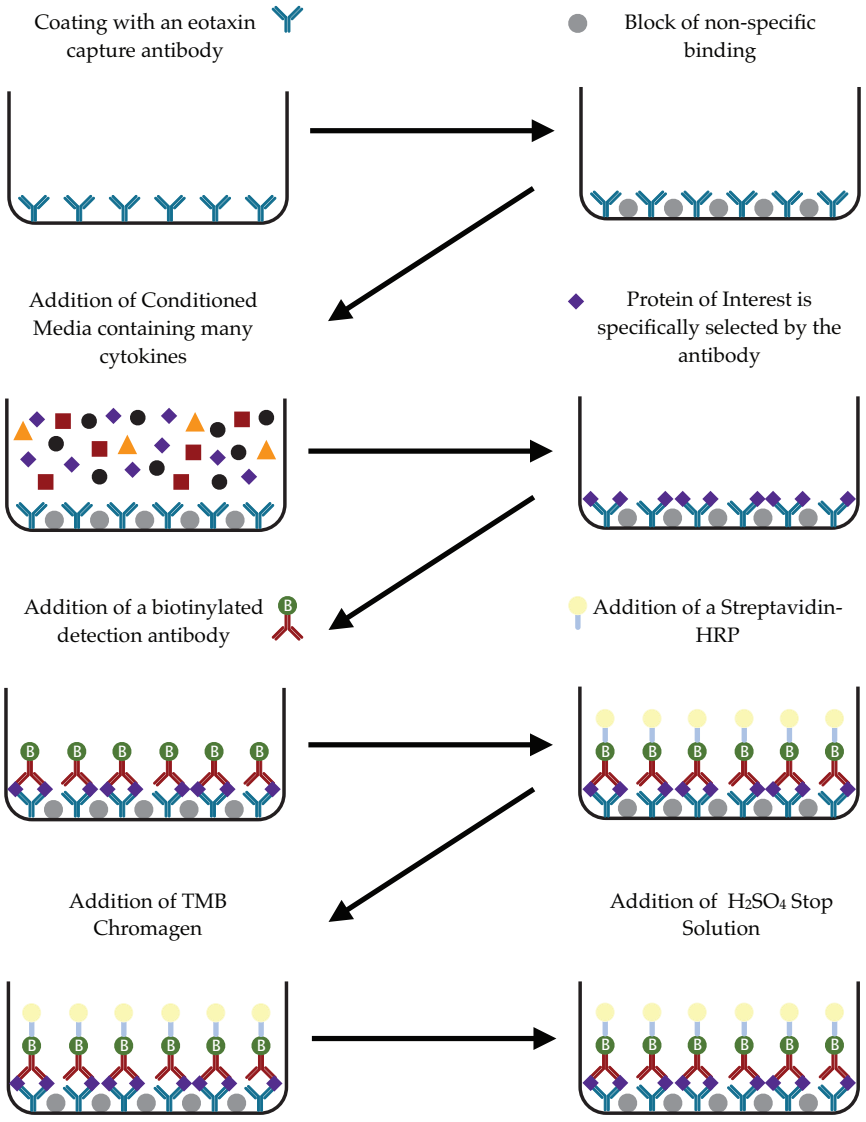

Figure 2.2: The principals of an enzyme-linked immunosorbant assay (ELISA). A sandwich ELISA detects a specific protein of interest from conditioned media. The wells are initially coated with a capture antibody and blocked for non-specific binding before addition of the conditioned media containing the analyte, which is specifically retained by the capture antibody. After washing, a biotinylated detection antibody, which also detects the desired protein, is then added. Streptavidin-HRP binds to this biotin and turns the chromagen blue. Acid stops the reaction, resulting in a yellow colour proportional to the amount of the protein of interest present. Protein quantitation can be determined by measuring the absorbance and normalising to a standard curve. HRP = Horseradish Peroxidase, TMB = 3,3′,5,5′-tetramethylbenzidine, H_2SO_4 = Sulphuric Acid.

starved in the presence or absence of 10 ng/ml IL-4 or IL-13 or 10 μg/ml Poly I:C for 24 hours. The cells were incubated for 3 hours with fresh medium before further treatment (as described in Chapters 3 & 4). In some cases cells were also pretreated during this 3 hour washout step with a blocking antibody against IL-13Rα2. For analysis of IL-6, conditioned medium was collected at baseline after 24 hours serum starvation.

In both instances media were clarified by centrifugation (300 g for 5 minutes) before analysis.

Eotaxin ELISA

Conditioned media was analysed for levels of Eotaxin using a DuoSet ELISA Kit. The eotaxin capture antibody was reconstituted in 1 ml Dulbecco's phosphate buffered saline (DPBS) to a concentration of 360 mg/ml and the eotaxin detection antibody was diluted in DPBS containing 1 % BSA Fraction V (reagent diluent) to a concentration of 18 mg/ml. The antibodies were frozen at -20°C in aliquots for up to 6 months and reconstituted by a 1:180 dilution in their respective reconstitution buffers before use. During each incubation the plate was covered with a fresh plate seal.

Maxisorp 96 well ELISA plates were incubated with 100 μl/well 2 mg/ml eotaxin capture antibody overnight at room temperature before aspirating each well and addition of 300 μl/well wash buffer (0.05 % Tween-20 in DPBS) by multichannel pipette. To remove the buffer the plate was inverted and impacted against clean paper towels ensuring complete removal of the liquid at each step. This process was repeated three times for every wash cycle. To prevent non-specific binding the wells were incubated with 300 μl/well reagent diluent for one hour at room temperature with rocking, before repeating the washing step.

The conditioned media samples were diluted 1:10 before use and 100 μl of sample was added per well. A standard curve using recombinant human eotaxin was created by reconstituting a 120 ng stock in 0.5 ml reagent

diluent and further diluting 1:60 to form a 7 point standard curve with 2-fold dilutions ranging from 1000 to 15.6 pg/ml eotaxin (Figure 2.3). Each sample or standard was added in duplicate and incubated for 2 hours at room temperature, before washing 3 times as before.

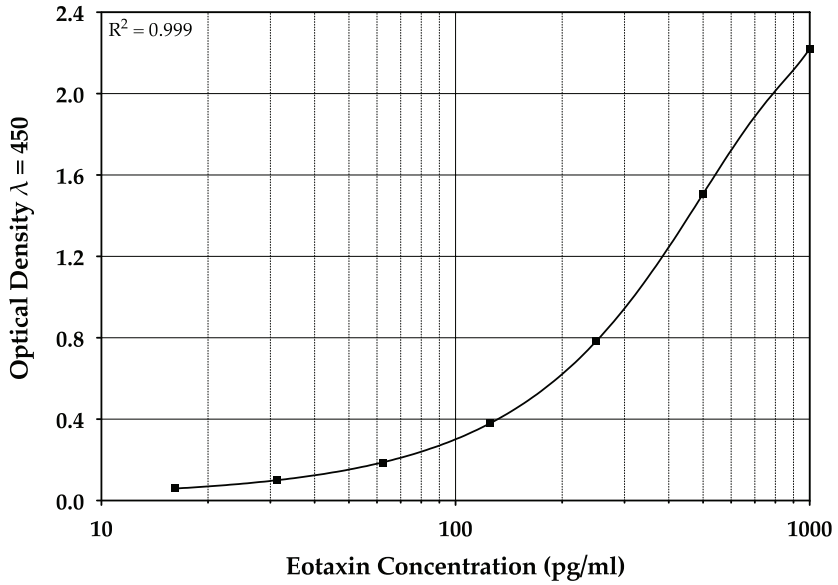

Figure 2.3: A typical eotaxin standard curve.

An eotaxin detection antibody was diluted 1:180 with reagent diluent to 100 ng/ml and 100 μl was added to each well and incubated for 2 hours at room temperature. The wells were subsequently washed as before. Streptavidin conjugated horseradish peroxidase (HRP) was diluted 1:200 with reagent diluent and 100 μl was incubated per well for 20 minutes before washing as previously described.

To enable colorimetric quantification of the eotaxin present in each well, 100 μl of single solution TMB was added per well and incubated in the dark, resulting in the development of a blue colour correlating to the concentration. After 20 minutes the reaction was stopped by the addition of 50 μl stop solution (1 M H_2SO_4), converting the chromagen to yellow. The optical density at 450 nm was immediately determined using a MultiScan plate reader, with wavelength (λ) correction at 570 nm.

The detection limit of this assay is 15.6 pg/ml. The manufacturer tested this assay for specificity using a panel of 82 cytokines and growth factors, including IL-4, IL-6, IL-13 and TGFβ, and no significant cross-reactivity or interference was observed. Inter-assay variation was determined to be less than 11.6 %. [286]

IL-6 ELISA

Media obtained from fibroblasts at baseline (post 24 hours of serum starvation) were analysed for levels of IL-6 using a Quantikine ELISA kit according to the manufacturers instructions. The plates were pre-blocked to prevent non-specific binding and pre-coated with a mouse monoclonal primary antibody against IL-6. Conditioned media samples (diluted 1:6) and IL-6 standards (reconstituted to 300 pg/ml and serial diluted to 3.12 pg/ml providing a 7 point standard curve as directed by the manufacturer (Figure 2.4) were diluted in Calibrator Diluent RD5T (specific for cell culture supernatant samples) and incubated in duplicate for 2 hours at room temperature with rocking.

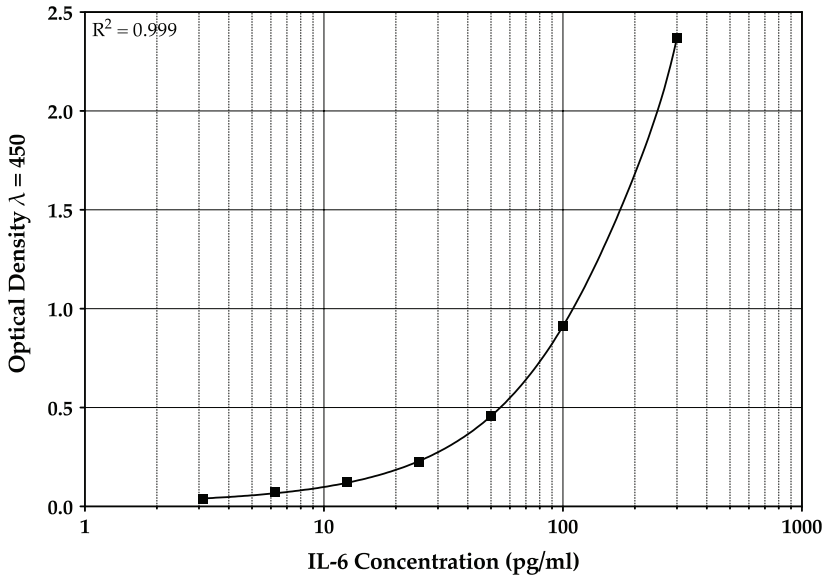

Figure 2.4: A typical IL-6 standard curve.

The wells were washed 4 times with 300 μl wash buffer (provided as a 25× buffered surfactant with preservatives, diluted 1:25 immediately before use with distilled water) per well using a multichannel pipette before addition of 200 μl/well of the IL-6 conjugate (polyclonal antibody against IL-6 conjugated to horseradish peroxidase). After 2 hours of incubation at room temperature, the wells were again rinsed 4 times with washed buffer and incubated with 200 μl substrate solution (equal parts Colour Reagent A (stabilised hydrogen peroxide) and Colour Reagent B (stabilised tetramethylbenzidine (TMB) chromagen)) for 20 minutes at room temperature in the dark before addition of 50 μl stop solution (1 M H_2SO_4), which converts the blue chromagen to yellow. The plates were tapped gently to ensure even colour development and read at 450 nm with λ correction at 570 nm, using a MultiSkan plate reader.

The minimum detectable concentration for this assay is 3.12 pg/ml. No cross-reactivity or interference was observed by the manufacturer with a panel of 25 cytokines, growth factors and receptors including TGFβ, IL-4 and IL-6 soluble receptor. Inter-assay variation was determined to have a coefficient of variance of less than 3.7 %. [287]

2.3.12 Methylene Blue Growth Assay

Methylene blue is a cationic dye, which binds to negatively charged cell constituents and can be used to determine cell number after treatment based on measurement of cell biomass. [288] When conditioned media was collected for assaying by ELISA the cells were fixed in Formal Saline (4 % (v/v) formaldehyde in 0.9 % (v/v) saline solution) for at least 30 minutes. Cells were subsequently stained with methylene blue dye (see Appendix A for details) for 30 minutes, and excess dye was carefully washed off with tap water. The dye was eluted using 1:1 ethanol:HCl (see Appendix A for details) and allowed to sit for 30 minutes to ensure full elution of the dye. The eluted dye was read at 630 nm using a MultiSkan plate reader.

To determine the number of cells present a standard curve was prepared (Figure 2.5). Cells were serial diluted when seeded in triplicate in a 96 well plate from 5×10^5 cells/well in doubling dilutions to form a 12 point standard curve. The cells were allowed at least 6 hours to attach to the plate before being fixed in formal saline and stained with methylene blue as above.

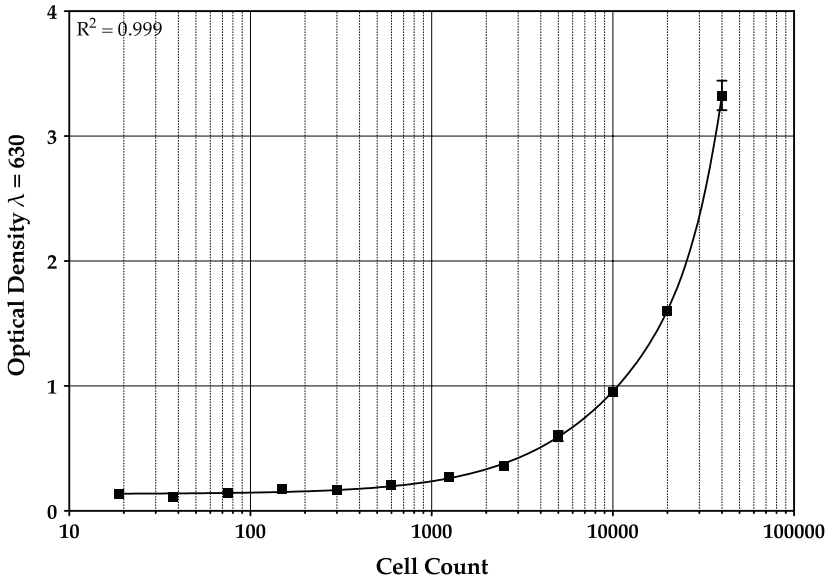

Figure 2.5: Methylene blue standard curve.

2.3.13 Statistics

Data collected were initially analysed by ANOVA, or a Kruskal-Wallis test if data were non-parametric, to determine if there was any statistical significance. When appropriate Bonferroni or Dunn's post tests were included. Statistical analyses on single comparisons were performed using a paired Student's t-test or Wilcoxon Matched Pairs test if data were not normally distributed. (GraphPad Prism v5.0c, GraphPad Software Inc., San Diego, USA).

Results were considered significant if $p < 0.05$ ($\star = p < 0.05$, $\star\star = p < 0.01$, $\star\star\star = p < 0.001$).

Chapter 3

Characterisation of the IL-4 & IL-13 Receptors

3.1 Introduction & Objectives

Previous studies have demonstrated natural variation in the responsiveness of HBFs from different subjects to IL-4 and IL-13. [281,289] It has been suggested that this may be due to differential expression of IL-13Rα2, which has previously been shown to be upregulated in response to IL-13 treatment. [165,245,253] However, the expression levels of the signalling receptors, IL-4Rα, γc and IL-13Rα1, may also play an important role in the regulation of these cytokines. [290]

Whilst these receptors have been studied individually there has been little work investigating the expression and regulation of IL-4Rα, γc, IL-13Rα1 and IL-13Rα2 together. Where co-expression of more than one of these receptors has been investigated, only limited numbers of subjects have been studied, and thus it has not been possible to undertake comprehensive analysis of the involvement of each of these receptors in the regulation of IL-4 and IL-13. Therefore this chapter will undertake a systematic review of the dynamics of each of the different IL-4 and IL-13 receptor subunits to determine if it is upregulation of IL-13Rα2 expression alone that is responsible for the differences in responsiveness to IL-4 and IL-13 in bronchial fibroblasts, or in combination with downregulation of the signalling receptors.

The objectives of this study were to:

i) characterise the levels of each IL-4 and IL-13 receptor subunit expressed by fibroblasts from healthy and asthmatic subjects,

ii) determine if the expression is affected by treatment with various cytokines and

iii) establish whether differences between subjects correlate with functional responsiveness of HBFs to IL-4 or IL-13.

3.2 Results

3.2.1 Characterisation of the Responsiveness of Fibroblasts to IL-4 & IL-13

Fibroblasts are known to produce eotaxin, a chemoattractant for eosinophils in response to both IL-4 and IL-13.[161,184,185] This can therefore be used as a functional readout for the responsiveness of HBFs to these cytokines. To confirm this, bronchial fibroblasts obtained by outgrowth of bronchial biopsies from healthy and asthmatic volunteers (for clinical details see Tables 2.1 and 2.2) were initially treated with varying concentrations of IL-4 or IL-13 for 24 hours. The conditioned media was collected and analysed for levels of eotaxin production by ELISA. The cells were fixed and the level of eotaxin production was normalised to cell number, which was determined by methylene blue assay.

In the case of both IL-4 (Figure 3.1*a*) and IL-13 (Figure 3.1*b*), a dose dependant increase was observed in eotaxin production, with 10 ng/ml of either treatment resulting in the largest induction of eotaxin ($p < 0.001$).

Although statistical significance was also observed at 1 ng/ml ($p < 0.01$), after challenge with 10 ng/ml of either IL-4 or IL-13 a greater difference was observed between subjects, where some produced much larger quantities of eotaxin than others. Whilst there was considerable variation in eotaxin

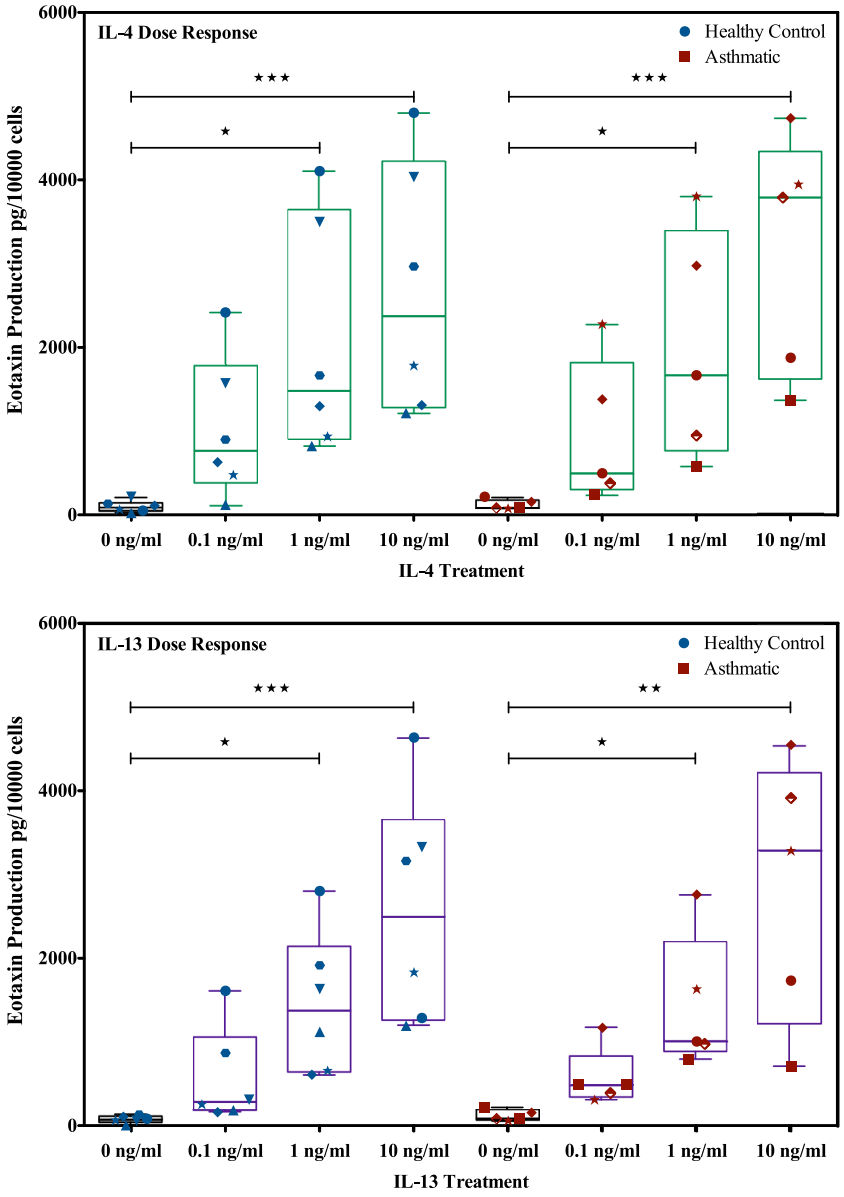

Figure 3.1: Induction of eotaxin by fibroblasts in response to IL-4 or IL-13 treatment. HBFs were grown until ∼95 % confluent, before 24 hours serum starvation. Cells were subsequently incubated in the presence of between 0 and 10 ng/ml IL-4 or IL-13 for 24 hours and eotaxin production was analysed by ELISA of the conditioned media. Eotaxin levels were normalised to cell number, determined by methylene blue assay. $n = 6$ healthy and 5 asthmatic subjects. $\star\star = p < 0.01$ $\star\star\star = p < 0.001$.

production by the different subjects, there was no difference observed between fibroblasts obtained from asthmatics versus healthy controls.

Previous work by Andrews, Nasir *et al.* has also demonstrated that addition of a pretreatment step with IL-13 during the initial serum starvation stage, causes desensitisation of the HBFs to both IL-13 and IL-4 and results in a decrease in the production of eotaxin and STAT6 phosphorylation normally observed in response to both cytokines. [165] To confirm this, and to see if this also occurs if the cells are pretreated with IL-4, HBFs were serum starved in the presence or absence of 10 ng/ml IL-4 or IL-13 for 24 hours. The media were then exchanged for 3 hours to ensure all traces of the pretreatment were removed (washout period), before the subsequent treatment with either IL-4 or IL-13 for 24 hours. The supernatant was collected and analysed for changes in eotaxin production by ELISA and the cell number was determined by methylene blue as previously described.

As observed in Figure 3.1, treatment with either IL-4 or IL-13 resulted in a significant increase in production of eotaxin ($p < 0.01$). However, if the cells were exposed to a pretreatment step using either IL-13 or IL-4 during the initial serum starvation period eotaxin production was significantly attenuated ($p < 0.05$) (Figure 3.2). This suggests that longer term exposure with either cytokine results in the implementation of a regulatory pathway, which desensitises the cells to the ligand preventing an uncontrolled response.

It has previously been suggested that both the variation observed between subjects at baseline and this phenomenon of reduced responsiveness after pretreatment is linked to the IL-13Rα2 expression levels on the cells. [165]

3.2.2 The Role of IL-13Rα2 in Reducing Responsiveness to IL-4 & IL-13

To determine if the levels of this receptor could be responsible for these effects the cells were initially characterised for baseline levels of IL-13Rα2 by flow cytometry (representative FACS plots for high and low expressers, compared to a relevant isotype control shown in Figure 3.3*a*). HBFs from these known

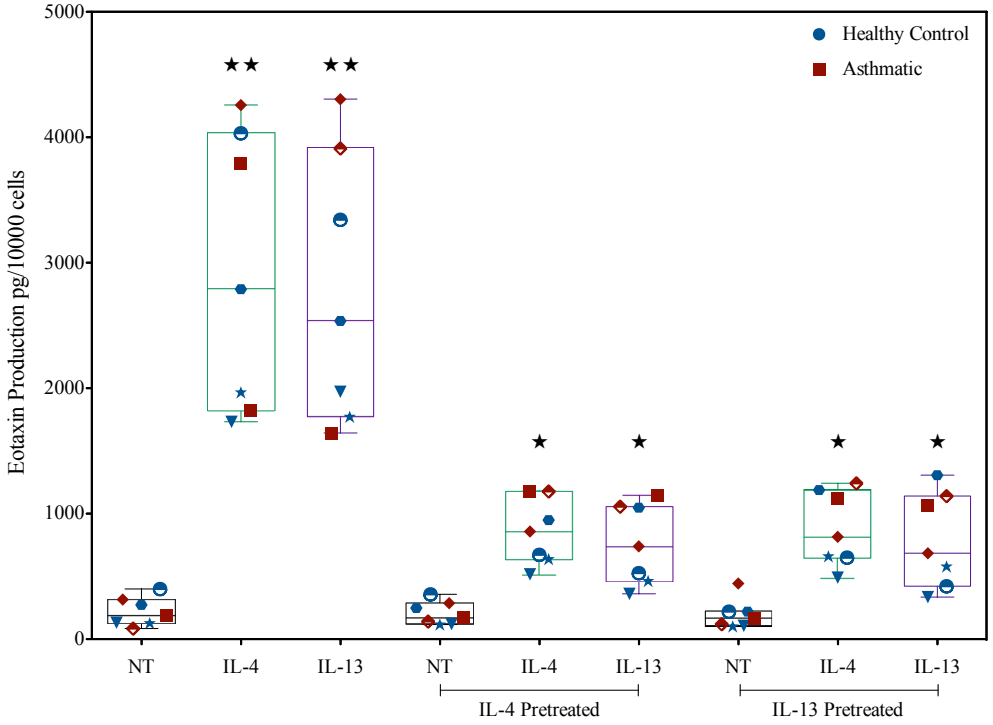

Figure 3.2: The effect of IL-4 or IL-13 pretreatment on fibroblast responsiveness. HBFs were seeded in 12 well plates at a density of 5×10^4 cells/well and grown until ∼95 % confluent, before 24 hours serum starvation in the presence or absence of 10 ng/ml IL-4 or IL-13. Cells were subsequently exposed to a 3 hour washout period with fresh media before a further 24 hour incubation in the presence or absence of 10 ng/ml IL-4 or IL-13 for 24 hours. Eotaxin production was analysed by ELISA of the conditioned media and levels were normalised to cell number, determined by methylene blue assay. $n = 4$ healthy and 3 asthmatic subjects. Statistical significance was determined using a Friedman test, with Dunns post-test. $\star = p < 0.05$ $\star\star = p < 0.01$.

high or low basal expressers of IL-13Rα2 were serum starved for 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13. The cells were, as before, exposed to a 3 hour washout period prior to treatment for a further 24 hours with fresh media containing either IL-4, IL-13 or no cytokine. Eotaxin levels and cell number were determined as previously described.

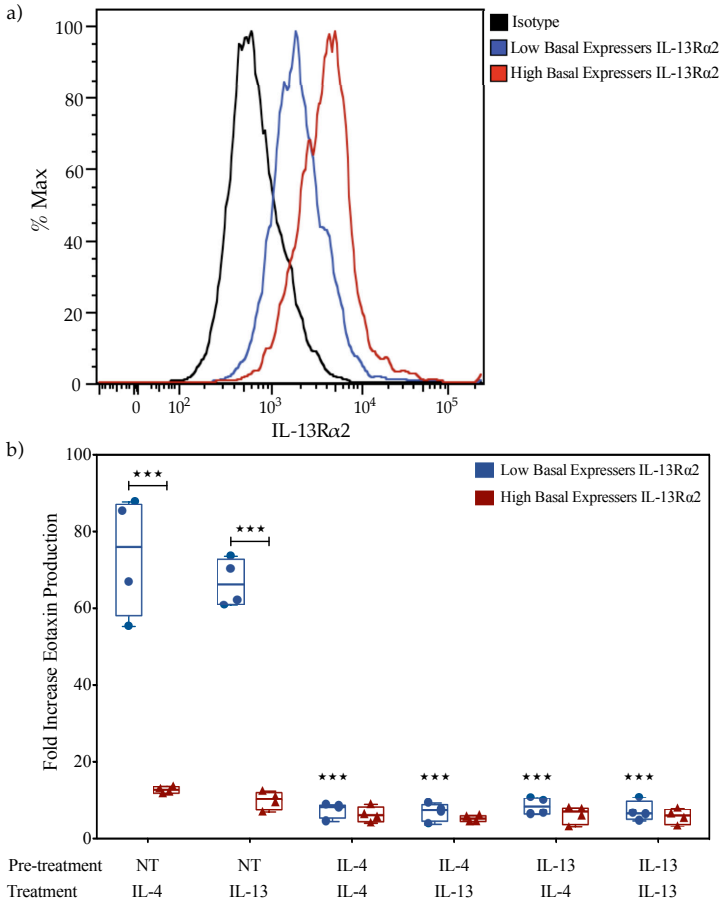

Figure 3.3: The effect of basal IL-13Rα2 expression on eotaxin production. Baseline levels of IL-13Rα2 surface expression were determined by flow cytometry. Low and high basal expressers of IL-13Rα2 were selected and grown until $\sim$95 % confluent, before serum starvation for 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13. The media was exchanged for 3 hours and cells were subsequently incubated with 10 ng/ml IL-4, IL-13 or media for 24 hours. Eotaxin production was analysed by ELISA and was normalised to cell number using a methylene blue assay. a) A typical FACS plot (of at least 4 subjects) showing IL-13Rα2 staining of untreated cells from a high or low expresser of IL-13Rα2 in comparison to a FITC-labelled isotype control. b) Eotaxin release by low expressers of IL-13Rα2 in response to IL-4 or IL-13 was greater than release by high expressers and was suppressed by pretreatment with either cytokine. $n = 4$ high and 4 low expressers of IL-13Rα2. Data expressed as median +/− IQR. $\star\star\star = p < 0.001$.

In this instance the variation in responsiveness to IL-4 or IL-13 was negatively related to the surface expression levels of IL-13Rα2 (Figure 3.3*b*), where low expressers of IL-13Rα2 produced high levels of eotaxin in response to treatment, whilst high basal expressers of IL-13Rα2 only released marginal amounts of eotaxin into the media (Figure 3.3*b*). The low basal expressers of IL-13Rα2 demonstrated approximately a 65 to 75 fold increase in eotaxin production in response to IL-13 and IL-4 respectively in the absence of any pretreatment, compared with 9 to 13 fold increase observed from cells naturally expressing high baseline levels of IL-13Rα2. This appears to suggest a functional response to IL-13Rα2 surface expression levels.

When the fibroblasts were pretreated with either IL-4 or IL-13, the production of eotaxin significantly decreased in the low basal expressers ($p < 0.001$). The high basal expressers of IL-13Rα2, on the other hand, demonstrated a trend towards a decrease in the levels of eotaxin produced after pretreatment in comparison to those without this pretreatment step, however this did not achieve statistical significance. In this instance it may be that, if IL-13Rα2 levels are responsible for these differences, the existing high expression of surface IL-13Rα2 is already suppressing eotaxin production and therefore the addition of a pretreatment step to desensitise the HBFs to these cytokines has little impact. Both IL-4 and IL-13 had similar effects, both in the level of induction of eotaxin, and the degree of attenuation of this due to pretreatment.

As the desensitisation of the cells to IL-4 or IL-13 appeared to be related to IL-13Rα2 it was necessary to confirm that this effect was due to the regulatory activity of this receptor.

3.2.3 Characterisation of the Levels of IL-13Rα2 Expression in Different Subjects

Kinetic experiments were consequently undertaken to determine whether the receptor levels were affected by IL-4 or IL-13 treatment. As previous data have demonstrated that 24 hours of treatment with IL-13 resulted in

increased surface IL-13Rα2 levels, which, in turn, was able to inhibit both IL-4 and IL-13 induced eotaxin production by HBFs,[165] initial experiments focussed on IL-13Rα2. HBFs were serum starved for 24 hours prior to treatment with 10 ng/ml IL-4 or IL-13 for 0, 4, 8, 24 and 48 hours before assessment of IL-13Rα2 mRNA levels by RT-qPCR.

All the subjects studied demonstrated more than a 2-fold increase in IL-13Rα2 mRNA in response to both IL-4 and IL-13 treatment after 4 hours (Figure 3.4). This increase became significant in response to either treatment after 8 hours ($p < 0.001$) and continued to rise over a 48 hour period. Whilst the levels of IL-13Rα2 mRNA produced increased in all subjects, at the later timepoints the increase observed was markedly variable between subjects, which could be due to differences in regulation of IL-13Rα2 gene expression between subjects affecting the levels of mRNA produced, and may affect the amount of protein subsequently produced.

These initial analyses, however, only determined changes in the level of mRNA expression due to treatment and not differences in surface expression of IL-13Rα2 between subjects. It is this surface expression level that is important as the increase in mRNA observed may not translate to a change at the protein level. Therefore, to establish whether the surface expression levels of IL-13Rα2 are affected by treatment, HBFs obtained from healthy and asthmatic volunteers were serum starved for 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13 before characterisation of surface receptor levels by flow cytometry.

A significant shift in fluorescence was observed due to the specific detection of surface IL-13Rα2 at baseline on the HBFs in comparison to a matched isotype control (Figure 3.5*a* and *b*). As with the mRNA, IL-13Rα2 surface expression significantly increased when HBFs were treated with either IL-4 (Figure 3.5*a* and *c*) or IL-13 (Figure 3.5*b* and *d*) ($p < 0.001$). There was no overall difference observed between HBFs obtained from asthmatic or healthy volunteers (Figure 3.5*c* and *d*), either at baseline or with treatment.

Whilst all subjects exhibited specific staining at baseline, the degree of staining varied greatly between subjects. There was also substantial variation

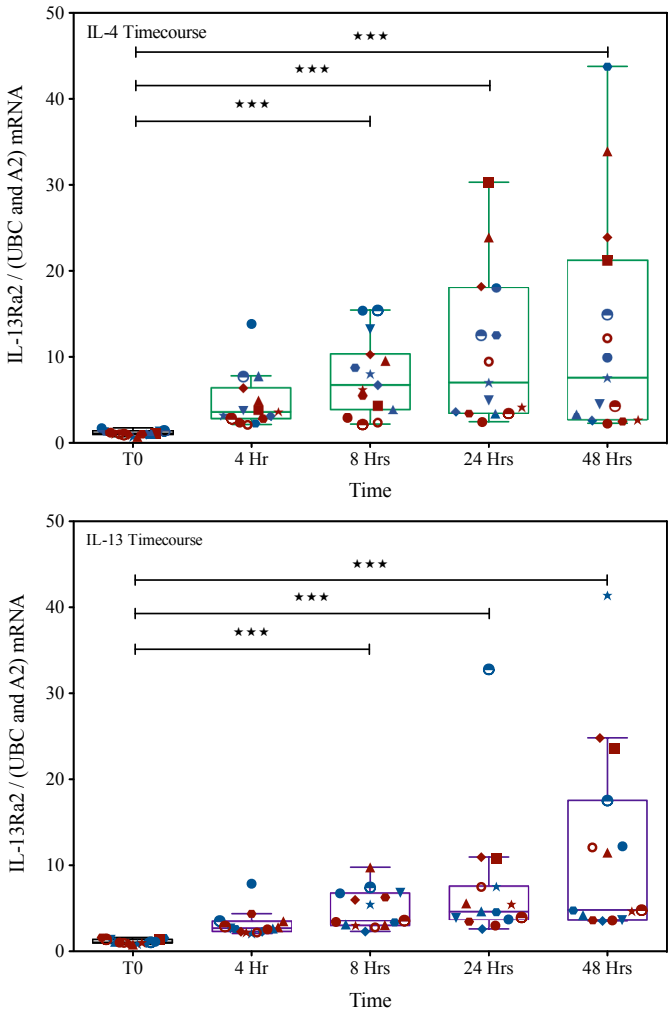

Figure 3.4: Induction of IL-13Rα2 mRNA by IL-4 and IL-13. HBFs were grown to ~95 % confluence and were serum starved for 24 hours prior to treatment with 10 ng/ml a) IL-4 or b) IL-13 for between 0 (T0) and 48 hours. RNA was extracted using the Trizol method. RT-qPCR analysis compared IL-13Rα2 mRNA levels to the geometric mean of two housekeeping genes (Ubiquitin C and Phospholipase A2). Data have been normalised to the average $^{\Delta}$CT of IL-13Rα2 in the unstimulated controls. Symbols represent individual volunteers (7 healthy and 7 asthmatic subjects) and are expressed as median, interquartile range and standard Tukey whiskers. Statistical significance was determined using a Friedman test, with Dunns post-test. $\star\star\star = p < 0.001$.

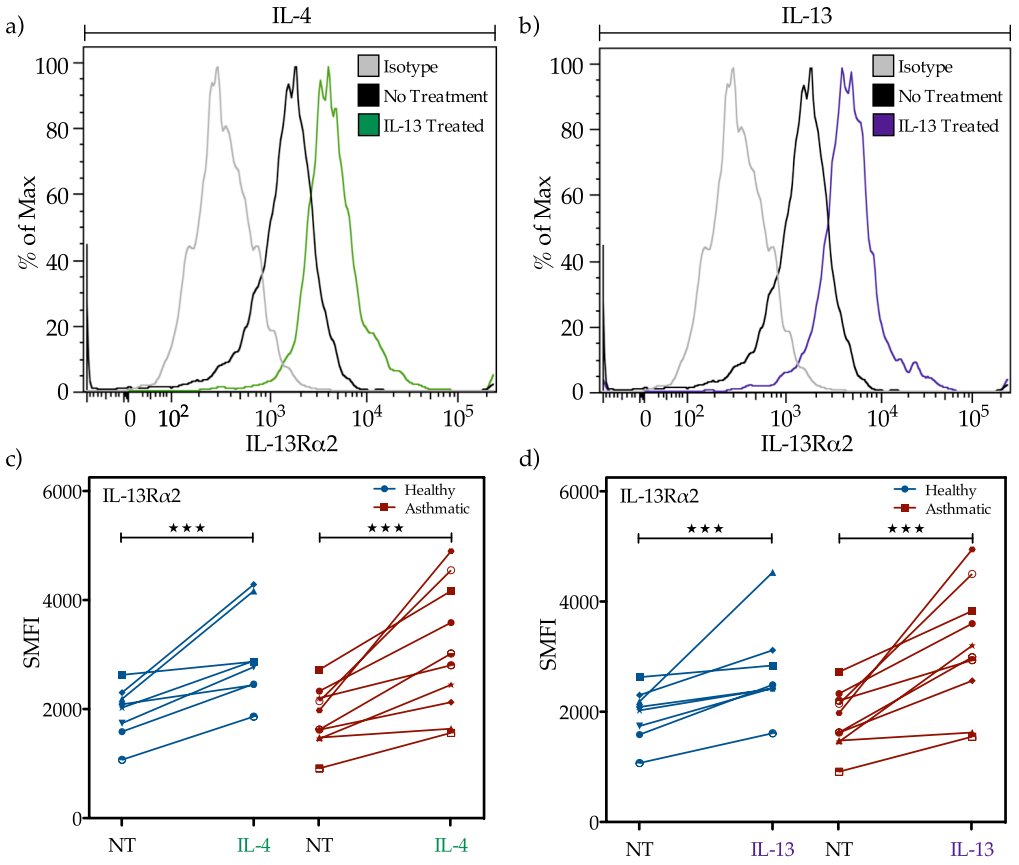

Figure 3.5: The effect of IL-4 or IL-13 treatment on IL-13Rα2 surface expression on fibroblasts from healthy and asthmatic subjects. HBFs were serum starved for 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13. 1×10^5 cells/subject were incubated with either an antibody against IL-13Rα2 or an appropriate isotype control before incubation with a FITC conjugated secondary antibody and analysis on a FACSAria. A typical FACS plot (of at least 18 subjects) showing the staining of untreated cells (black), in comparison to an isotype (grey) and the shift caused by (a) IL-4 or (b) IL-13 pretreatment. Challenge with (c) IL-4 or (d) IL-13 caused a significant increase in IL-13Rα2 surface expression. The magnitude of increase varied between subjects but no difference was found between healthy controls compared with asthmatic subjects. Data were normalised to an isotype control and expressed as specific mean fluorescent intensity (SMFI). $n = 8$ healthy and 10 asthmatic subjects. Statistical analysis was determined using Friedman test, with Dunns post-test. $\star\star\star = p < 0.001$.

between subjects in the level of increase of IL-13Rα2 surface expression in response to IL-4 or IL-13 treatment (Figure 3.5*c* and *d*), with HBFs from some subjects expressing more than twice as much IL-13Rα2 as at baseline, whilst others only marginally increased their surface expression levels. The level of increase in surface IL-13Rα2 expression was generally consistent for each subject in response to both IL-4 and IL-13. These data are also in line with the mRNA expression level of IL-13Rα2 as an increase in mRNA at 8 hours could lead to an increase in surface expression by 24 hours.

The differential response by subjects with similar baseline levels of IL-13Rα2 to both IL-4/13 cytokine stimulation, suggests that high IL-13Rα2 at baseline may not be sufficient to prevent a response. This could be due to a number of factors, such as the degree of signalling receptor expression or their intracellular signalling components, or the storage of differing quantities of IL-13Rα2 in intracellular pools.

Intracellular Pools of IL-13Rα2

It has previously been observed that in epithelial cells IL-13Rα2 is mainly intracellular and that this can be mobilised to the surface by IFNγ.[251] It was therefore of interest to confirm whether fibroblasts also increase their surface expression of IL-13Rα2 in response to IFNγ and subsequently if this is due to the presence of intracellular pools or if *de novo* protein synthesis is required.

Fibroblasts were serum starved for 24 hours before treatment with varying concentrations of IFNγ for up to 24 hours. Primary bronchial epithelial cells provided by Drs Swindle and Blume were also included as a positive control. The surface expression of IL-13Rα2 was determined by flow cytometry as previously described.

Treatment of fibroblasts with 1 or 10 ng/ml IFNγ for either 2 or 8 hours was insufficient to increase the expression of IL-13Rα2 on the cell surface (Figure 3.6*a*). Fibroblast IL-13Rα2 surface expression did however increase in response to IFNγ after 24 hours of stimulation. At this timepoint both

1 ng/ml and 10 ng/ml IFNγ treatment resulted in a significant increase in surface expression of IL-13Rα2 ($p < 0.01$ and $p < 0.001$ respectively).

In comparison, the PBECs IL-13Rα2 surface expression increased after 2 hours (Figure 3.6*b*), reaching significance after 8 hours with both doses of IFNγ tested ($p < 0.001$ and $p < 0.01$ respectively). The fact that the epithelial cells demonstrated much faster kinetics than the HBFs with regard to upregulation of surface IL-13Rα2 suggests that the regulation of expression of IL-13Rα2 may differ between these cells.

Experiments were therefore undertaken to characterise the presence of intracellular pools of IL-13Rα2 in these different cell types. Initial attempts to study the presence of pools of IL-13Rα2 using cell permeabilisation were unsuccessful due to extremely high levels of non-specific staining observed with both the IL-13Rα2 antibody and the isotype control. Instead, an alternative approach for determining whether intracellular stores of IL-13Rα2 existed in fibroblasts was required. To this end cycloheximide (CHX) was used as this suppresses de novo protein synthesis,[291] and can thus indicate if pools of IL-13Rα2 exist within the cell.

Fibroblasts were treated with either IFNγ or IL-13 for up to 24 hours, with some of the cells also pretreated with 50 μg/ml CHX for 30 minutes immediately prior to addition of IFNγ or IL-13. Primary bronchial epithelial cells were again included as a positive control for the presence of intracellular pools of IL-13Rα2 in this assay. The surface expression of IL-13Rα2 was determined by flow cytometry.

When the cells were pretreated with CHX (Figure 3.6*c*) the increase in surface expression of IL-13Rα2 previously observed in response to both IFNγ and IL-13 was attenuated, suggesting that in fibroblasts *de novo* protein synthesis is required for this increased expression. In comparison the epithelial cells included as a positive control (Figure 3.6*d*) were able to upregulate surface expression levels of IL-13Rα2 in response to IFNγ and IL-13 both in the presence and absence of CHX pretreatment, indicating the existence of intracellular pools of IL-13Rα2 in these cells, which are not found in fibroblasts.

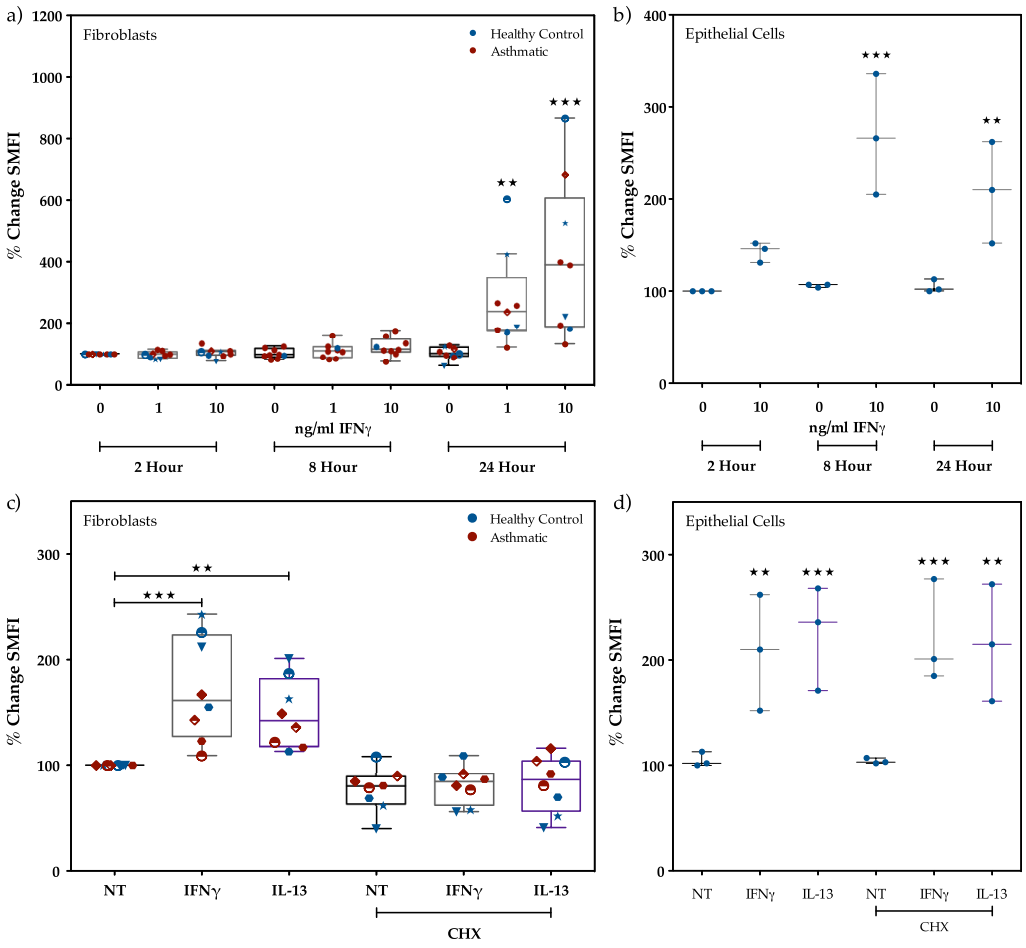

Figure 3.6: Characterisation of IL-13Rα2 intracellular pools in bronchial fibroblasts and epithelial cells. Fibroblasts were serum starved for 24 hours before treatment with a) 1 or 10 ng/ml IFNγ for up to 24 hours or c) 10 ng/ml IFNγ or IL-13. Some cells were also treated with 50 μg/ml cycloheximide (CHX) for 30 minutes prior to cytokine stimulation to block de novo protein synthesis. Epithelial cells were included as a positive control (b) and d)). Cells were incubated with an antibody against IL-13Rα2 or an isotype control and analysed on a FACSAria. Data were normalised to the isotype and the specific mean fluorescent intensity (SMFI). Fibroblasts $n = 4$ healthy and 5 asthmatic subjects. Epithelial cells n = 3 healthy subjects. Data are presented as median with standard Tukey whiskers. $\star\star = p < 0.01$ and $\star\star\star = p < 0.001$.

3.2.4 Expression of IL-4Rα, IL-13Rα1 & γc by HBFs

As intracellular pools of IL-13Rα2 were not found in primary human bronchial fibroblasts, it was thought that the variation in responsiveness by subjects expressing similar baseline surface levels of IL-13Rα2 observed in Figure 3.5 may instead be due to differential expression of the IL-4 and IL-13 signalling receptor subunits.

IL-4Rα is an essential component of both the type I and type II receptors. Whilst the γc subunit of the type I receptor can only bind IL-4, both IL-4Rα and IL-13Rα1, which constitute the type II signalling receptor, are able to bind both IL-4 and IL-13 respectively. A recent paper investigating human and mouse monocytes has suggested that levels of IL-4Rα and IL-13Rα1 may also be involved in regulating sensitivity to IL-4 and IL-13. [290] Therefore, the mRNA samples obtained earlier from HBFs treated with IL-4 or IL-13 for up to 48 hours were analysed by RT-qPCR for levels of IL-13Rα1, IL-4Rα and γc, to determine if these also varied between patients with IL-4 or IL-13 treatment.

Both IL-13Rα1 and IL-4Rα mRNA were detected in every subject, however the expression levels of neither IL-13Rα1 (Figure 3.7*a* and *b*) nor IL-4Rα (Figure 3.8*a* and *b*) were affected by either IL-4 or IL-13 treatment.

γc mRNA, on the other hand, was undetected in any of the samples, regardless of IL-4 or IL-13 treatment (data not shown). In this case the validity of the assay was confirmed using a positive control (BAL cell cDNA from healthy and asthmatic subjects provided by Dr Karl Staples), where mRNA expression was observed.

As with the IL-13Rα2 data, it was important to see how these observations made at the mRNA level compared to the surface expression of the receptor subunits. Therefore, the surface expression levels of the IL-4 and IL-13 signalling receptors on primary human bronchial fibroblasts were analysed by flow cytometry, following the same methods as before.

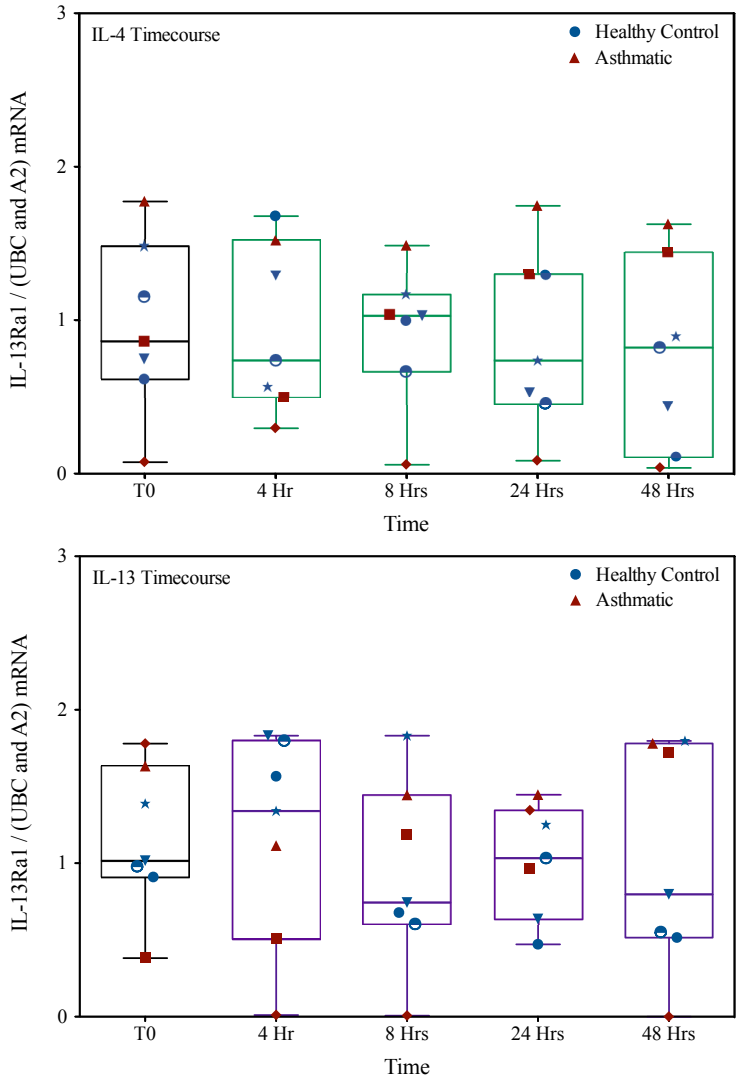

Figure 3.7: The impact of IL-4 and IL-13 on IL-13Rα1 mRNA expression by bronchial fibroblasts. HBFs were serum starved for 24 hours before treatment with 10 ng/ml a) IL-4 or b) IL-13 for 0 (T0), 4, 8, 24 or 48 hours. RNA was extracted using the Trizol method. PCR analysis compared IL-13Rα1 mRNA levels to the geometric mean of two housekeeping genes (UBC/A2). Data have been normalised to the average $^{\Delta}$CT of IL-13Rα1 in the unstimulated controls. n = 4 healthy and 3 asthmatic subjects. Data are presented as median with standard Tukey whiskers.

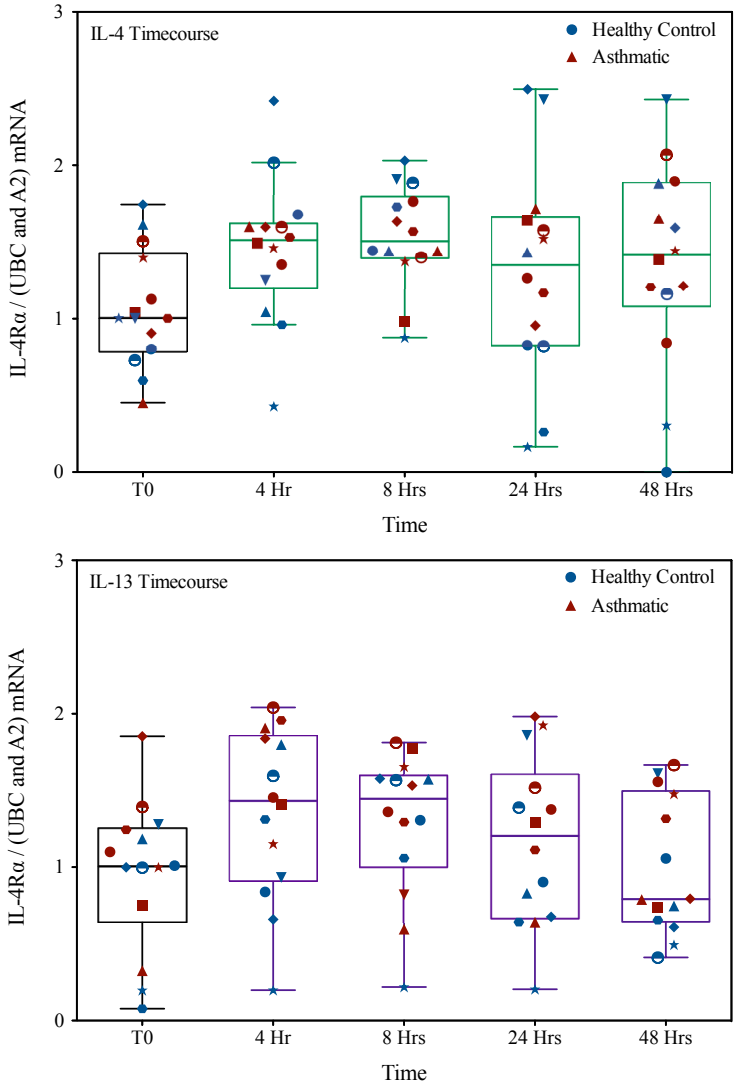

Figure 3.8: Characterisation of IL-4Rα mRNA levels in response to treatment with IL-4 or IL-13. HBFs were serum starved for 24 hours prior to challenge with 10 ng/ml a) IL-4 or b) IL-13 for 0 (T0) to 48 hours. RNA was extracted using the Trizol method. PCR analysis compared IL-4Rα mRNA levels to the geometric mean of two housekeeping genes (UBC/A2). Data have been normalised to the average $^{\Delta}$CT of IL-4Rα in the unstimulated controls. $n = 7$ healthy and 7 asthmatic subjects. Data are presented as median with standard Tukey whiskers.

Substantial surface expression of IL-13Rα1 was detected in HBFs from every subject and, as with the mRNA, was unaffected by treatment with IL-13 (Figure 3.9). The levels of the receptor were not significantly different between healthy or asthmatic subjects, however one subject demonstrated expression levels approximately $2-4$-fold higher than any other. This result was confirmed by repeating the experiment with the same end-result. This subject did not demonstrate significantly different levels of the other IL-4 or IL-13 receptors in comparison to other subjects and responded to IL-4 and IL-13 in a similar manner to cells from subjects with similar levels of the other IL-4 and IL-13 receptors suggesting that this high surface expression of IL-13Rα1 did not elicit a significant effect on the responsiveness of this subject to either IL-4 or IL-13.

Similarly to the mRNA data, no surface expression of γc was detected by flow cytometry (Figure 3.10). These data were confirmed to ensure there was not a problem with the antibody by analysing surface expression of PBECs, which are known to express this subunit.[80] In this instance 5 μg of the antibody demonstrated significant surface expression. For the HBFs up to 10 μg of antibody was tested on cells from 8 subjects with no expression detected at any concentration. These data imply that there is no γc expression by fibroblasts, correlating with previous data suggesting γc is only expressed by haematopoietic cells.[233]

IL-4Rα surface expression was observed in every subject at baseline, however, this was found to significantly decrease by approximately $50-60\,\%$ after 24 hours of treatment with either IL-4 (Figure 3.11*a*) ($p < 0.001$) or IL-13 (Figure 3.11*b*) ($p < 0.001$). To determine if this decrease was specific, HBFs were also treated with IFNγ before analysis of IL-4Rα levels. In this instance no significant difference in surface expression was observed between the treated and the untreated cells (Figure 3.11*c*). There was also no difference observed between disease phenotypes, either at baseline or in response to treatment, although expression levels did vary between subjects.

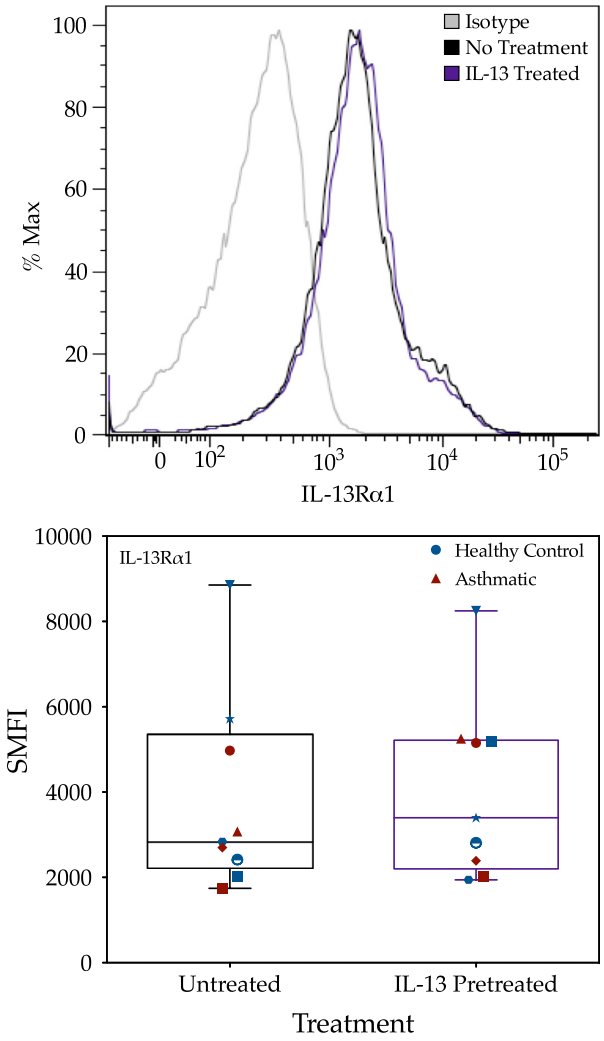

Figure 3.9: Determination of IL-13Rα1 surface expression on fibroblasts and the effects of IL-13 treatment. HBFs were seeded in 12 well plates and grown until ~95 % confluent, before 24 hours serum starvation in the presence or absence or 10 ng/ml IL-13. 1×10^5 cells/subject were incubated with either an APC labelled antibody against IL-13Rα1 or an APC-IgG_{2B} isotype control before analysis on a FACSAria. a) Histogram (typical of 8 subjects) demonstrating specific staining with no shift due to IL-13 treatment compared with unstained and isotype controls. b) No variation was observed due to treatment with IL-13. Data were normalised to an isotype control and expressed as specific mean fluorescent intensity (SMFI) $n = 5$ healthy and 4 asthmatic subjects. Data are median, interquartile range with standard Tukey whiskers.

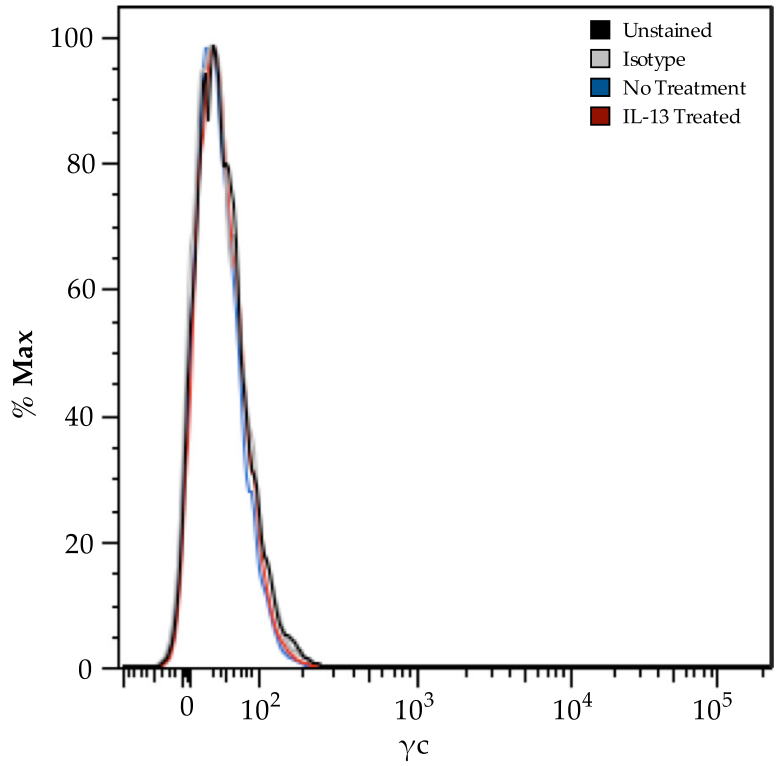

Figure 3.10: γc surface expression on fibroblasts from healthy and asthmatic subjects. HBFs were grown in 12 well plates until ∼95 % confluent, before 24 hours serum starvation in the presence or absence of 10 ng/ml IL-13. 1×10^5 cells/subject were incubated with an antibody against γc or IgG_{2A} isotype control conjugated to Alexafluor-405 and analysed on a FACSAria. No specific staining for γc was observed in any of the 8 subjects analysed.

The expression levels of IL-4Rα were found to vary between patients, however this was not found to correlate with either the baseline IL-13Rα2 expression, or the level of increased expression previously observed.

To further characterise this effect, HBFs were treated for 24 hours with varying doses of IL-4, before analysis of IL-4Rα levels by flow cytometry. The reduction in IL-4Rα surface expression due to challenge with IL-4 was found to behave in a dose dependent manner (Figure 3.12*a*), although this appeared to plateau after 1 ng/ml IL-4 treatment ($p < 0.01$), which had a similar effect to 10 ng/ml IL-4 ($p < 0.01$).

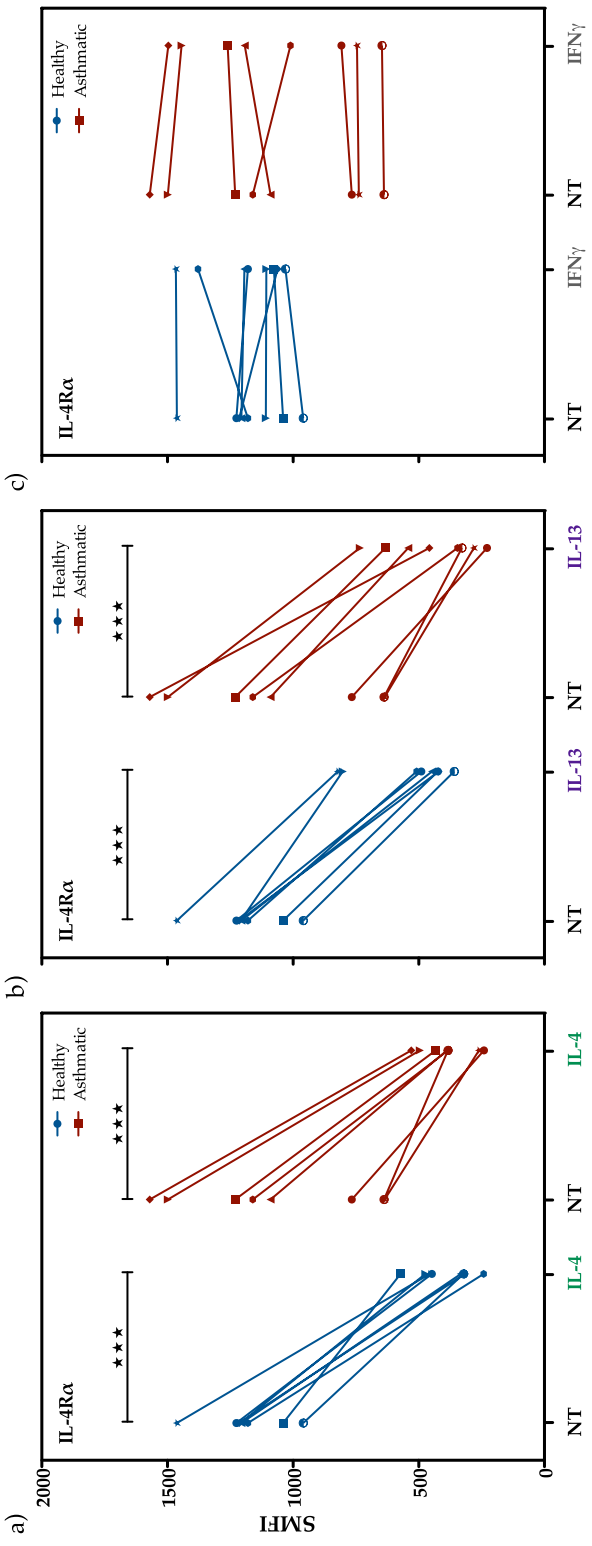

Figure 3.11: The effects of IL-4, IL-13 and IFNγ on IL-4Rα surface expression levels. HBFs were seeded in 12 well plates at 5×10^4 cells/well and grown until ~95 % confluent, before 24 hours serum starvation in the presence or absence of 10 ng/ml IL-4, IL-13 or Interferon (IFN) γ. 1×10^5 cells/subject were incubated with either an APC labelled antibody against IL-4Rα or an APC-IgG$_{2A}$ isotype control before analysis on a FACSAria. IL-4Rα levels decreased in response to either IL-4 or IL-13 but not IFNγ treatment. Data were normalised to an isotype control and expressed as specific mean fluorescent intensity (SMFI) $n = 8$ healthy and 8 asthmatic subjects. $\star\star\star = p < 0.001$.

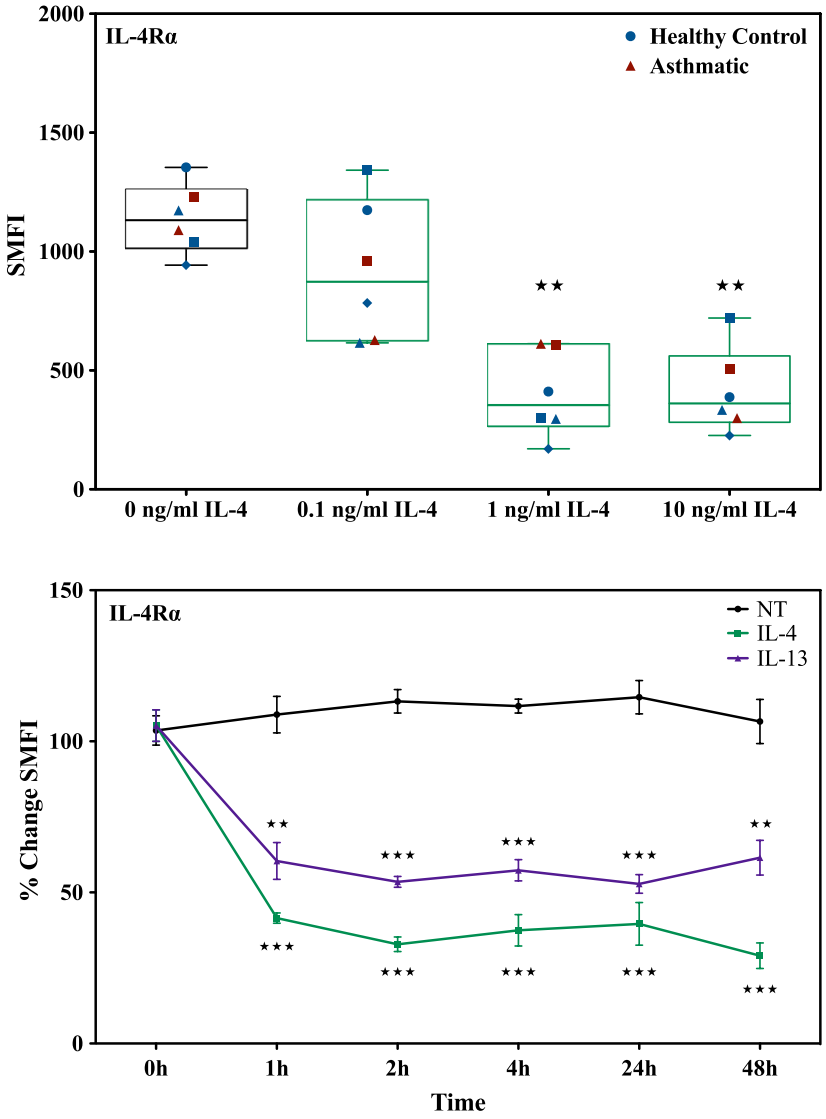

Figure 3.12: Dose and kinetic characterisation of IL-4Rα internalisation by HBFs. HBFs were grown until ~95 % confluent, before 24 hours serum starvation. Cells were treated with a) 0 to 10 ng/ml IL-4 for 24 hours or b) 10 ng/ml IL-4, IL-13 or serum-free media for up to 48 hours. 1×10^5 cells/subject were incubated with either an APC labelled antibody against IL-4Rα or an APC-IgG$_{2A}$ isotype control before analysis on a FACSAria. Data were normalised to an isotype control and expressed as a) specific mean fluorescent intensity (SMFI) b) % change in SMFI n = 4 healthy and 2 asthmatic subjects. Data are median with interquartile range. $\star\star = p < 0.01$ and $\star\star\star = p < 0.001$.

Downregulation of receptors following ligand stimulation is a well characterised phenomenon. To determine the kinetics of the reduction of IL-4Rα surface expression the cells were treated with 10 ng/ml IL-4 or IL-13 for between 1 and 48 hours and analysed by flow cytometry as before. In the case of both IL-4 and IL-13 treatment, a significant decrease occurred within 1 hour ($p < 0.01$ and $p < 0.001$ respectively), and the surface expression levels remained low over a 48 hour period (Figure 3.12*b*). At every timepoint, IL-4 appeared to cause a greater reduction (a $50 - 60\,\%$ decrease) in the surface expression of IL-4Rα in comparison to IL-13 (a $40 - 50\,\%$ reduction), however this difference was not found to be statistically significant.

In the experiments undertaken previously in this chapter HBFs were sometimes exposed to a pretreatment step with either IL-4 or IL-13 with a consequent desensitisation of the cells to these ligands. As challenge with either cytokine significantly decreased expression of IL-4Rα, it was necessary to determine if this diminished responsiveness observed after pretreatment could be attributed to the reduction in IL-4Rα surface expression as well as the increase in IL-13Rα2 levels. In the instances where a pretreatment step was included, the cells were always given a 3 hour washout period between treatments to ensure removal of any excess pretreatment that may have been left behind. Therefore, at the 24 hour timepoint, half of the cells were also exposed to a 3 hour washout period to determine if this decrease in surface expression of IL-4Rα could be reversed by exchanging the media.

As before, after either IL-4 or IL-13 treatment, IL-4Rα surface expression was reduced by approximately $50\,\%$ (Figure 3.13*a*), with the IL-4 treatment appearing to cause a greater reduction than the IL-13. When the washout step was included, however, the IL-13 treated HBFs were able to fully recover their surface expression levels of IL-4Rα to their untreated state. Whilst a similar response was observed for IL-4 ($p < 0.001$), the levels were still significantly lower than those observed on cells that had not been exposed to any cytokine treatment ($p < 0.001$). The differences observed here may be due to the differing binding affinities that these cytokines have for the IL-4Rα subunit, which may affect the internalisation of this receptor.

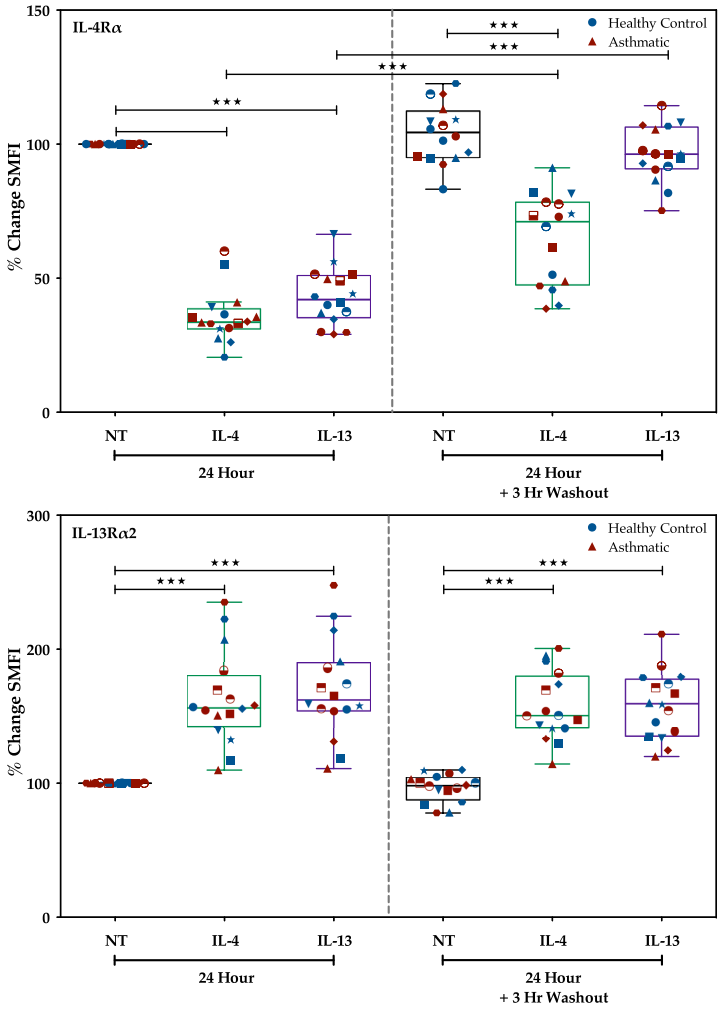

Figure 3.13: The effect of ligand removal on IL-4Rα and IL-13Rα2 surface expression. HBFs were grown until ~95 % confluent, before 24 hours serum starvation in the presence or absence or 10 ng/ml IL-4 or IL-13. Half of the cells were subsequently incubated with fresh media for 3 hours. 1×10^5 cells/subject were incubated with either antibodies against IL-4Rα and IL-13Rα2 or relevant isotype controls before analysis on a FACSAria. IL-4Rα levels significantly decreased in response to IL-4 or IL-13 treatment for 24 hours, whilst IL-13Rα2 levels significantly increased. Levels of IL-4Rα were restored during the 3 hour washout period after IL-4 or IL-13 treatment respectively but IL-13Rα2 levels were unaffected. Data were normalised to an isotype control and expressed as percentage change of specific mean fluorescent intensity (SMFI) $n = 8$ healthy and 8 asthmatic subjects. Data are median +/− IQR. $\star\star\star = p < 0.001$.

As the expression of IL-4Rα mostly recovers within this washout period these data suggest that the downregulation of IL-4Rα is unlikely to be the cause of the differences in responsiveness to IL-4 and IL-13 observed after pretreatment with either cytokine.

As the levels of IL-13Rα2 have previously been shown to increase in response to IL-4 or IL-13 challenge, the fibroblasts were also analysed for changes in levels of IL-13Rα2 surface expression with and without a washout period. As demonstrated in Figure 3.5, when treated with either IL-4 or IL-13 the surface expression levels of IL-13Rα2 significantly increased (Figure 3.13*b*). In contrast to the changes observed with the IL-4Rα expression, the washout period did not significantly affect either the baseline surface expression of IL-13Rα2 or the level of increase observed in response to either IL-4 or IL-13 challenge, with IL-13Rα2 expression remaining high in both instances.

3.2.5 Confirmation of IL-13Rα2 Involvement in the Attenuation of Responsiveness to Both IL-4 & IL-13

At this stage, the attenuation of responsiveness observed after HBFs were pretreated with either IL-4 or IL-13 could not be linked directly to IL-13Rα2. Although a link has been observed between IL-13Rα2 surface expression and eotaxin production, and pretreatment with either IL-4 or IL-13 was found to increase the levels of IL-13Rα2 it may be that pretreatment also induces other effects, such as an increase in Suppressor of Cytokine Signalling (SOCS) protein production. It was therefore necessary to undertake an experiment to determine whether this was a specific consequence of the IL-13Rα2 expression level, as previously suggested by Andrews, Nasir *et al.*.[165]

To investigate the involvement of IL-13Rα2 in the regulation of both IL-13 and IL-4, HBFs were serum starved for 24 hours in the presence or absence of IL-4 or IL-13 followed by a 3 hour washout period and treatment for 24 hours in the presence or absence of the two cytokines. In this experiment some of

the cells were exposed to either a neutralising antibody against the receptor (IL-13Ra2 NAb) or a relevant isotype control. The conditioned media were collected for analysis of eotaxin production by ELISA, which was normalised to the cell number determined by methylene blue assay.

As before, HBFs produced eotaxin in response to both IL-4 and IL-13 ($p < 0.001$) (Figure 3.14*a* and *d*) and, as previously reported this was significantly reduced with the addition or either IL-13 or IL-4 pretreatment ($p < 0.05$) (Figure 3.14*a* and *d*). Addition of an IL-13Rα2 NAb to cells restored both the IL-13 and IL-4 responses (Figure 3.14*b* and *e*), demonstrating that IL-13Rα2 negatively regulates IL-13, as previously described. [165] The fact that this was also observed with IL-4 challenge (Figure 3.14*e*), indicates that although IL-13Rα2 is unable to bind IL-4 directly, [165] it is able to negatively regulate this cytokine's actions. In both instances the isotype control had no effect on eotaxin production (Figure 3.14*c* and *f*) and no difference was observed between using either IL-4 or IL-13 in the pretreatment step.

Fibroblasts have also previously been shown to produce Interleukin 6 (IL-6) in response to IL-13, [292] and this could be used as another measure of responsiveness to confirm these results. It was therefore of interest to determine initially if both IL-4 and IL-13 could induce HBFs from asthmatic and healthy patients to release IL-6, and subsequently establish whether pretreatment with IL-13 and the use of an IL-13Rα2 NAb caused the same effects as observed with eotaxin production in Figure 3.14.

All the HBFs analysed produced IL-6 at baseline and this production was significantly increased by stimulation with either IL-4 or IL-13 for 24 hours ($p < 0.001$), with both cytokines inducing similar effects (Figure 3.15). Mirroring what was observed with eotaxin, pretreatment of the cells with IL-13 did not significantly affect the baseline production of IL-6 by the fibroblasts but did reduce the responsiveness of the cells to both IL-4 and IL-13. Again, as previously shown in Figure 3.14, addition of a neutralising antibody against IL-13Rα2 was able to restore the responsiveness of the cells to both IL-4 and IL-13.

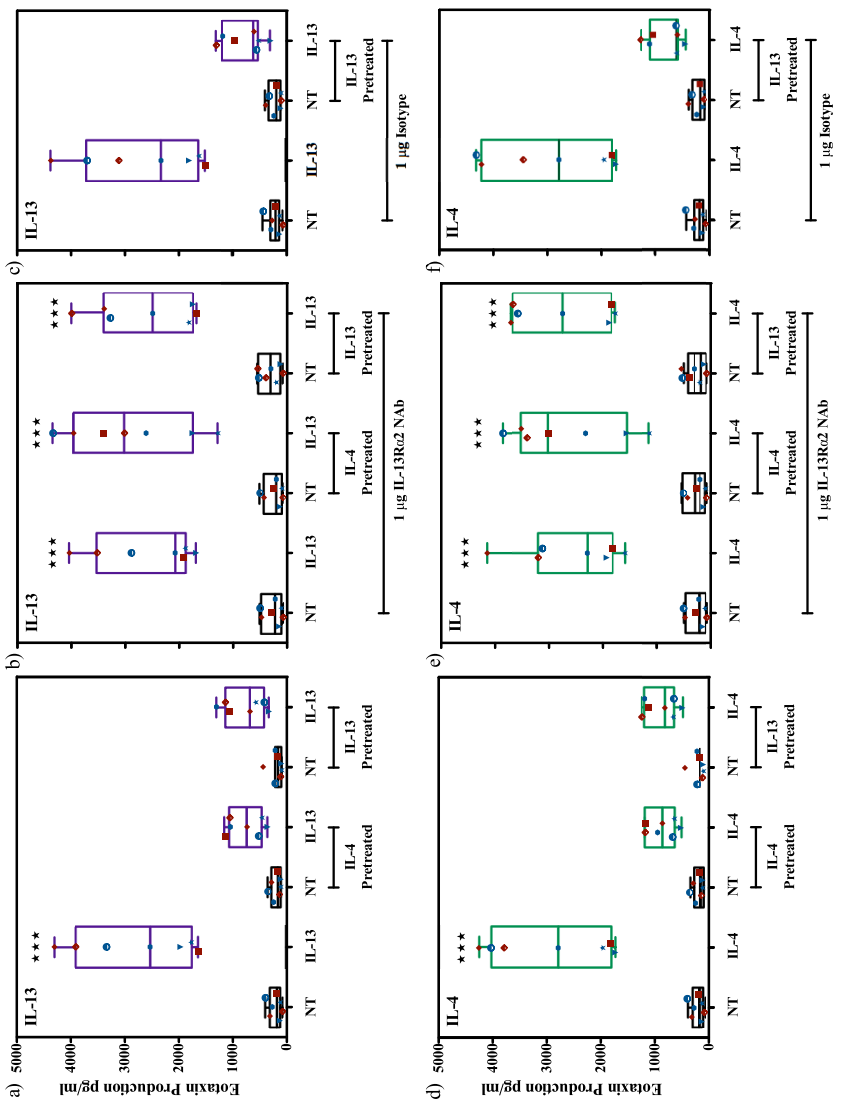

Figure 3.14: Eotaxin production after pretreatment with IL-4 or IL-13, and the effect of an IL-13Rα2 neutralising antibody (NAb). HBFs were serum starved 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13. Cells were washed out with fresh media for 3 hours. An IL-13Rα2 NAb or relevant isotype control was added to some of the cells as indicated at this point before subsequent incubation in the presence or absence of 10 ng/ml IL-4 or IL-13 and an IL-13Rα2 NAb where indicated for 24 hours. Eotaxin production was analysed by ELISA of the conditioned media. $n = 4$ healthy and 3 asthmatic subjects. Data expressed as median with IQR and Tukey whiskers. $\star\star\star = p < 0.001$.

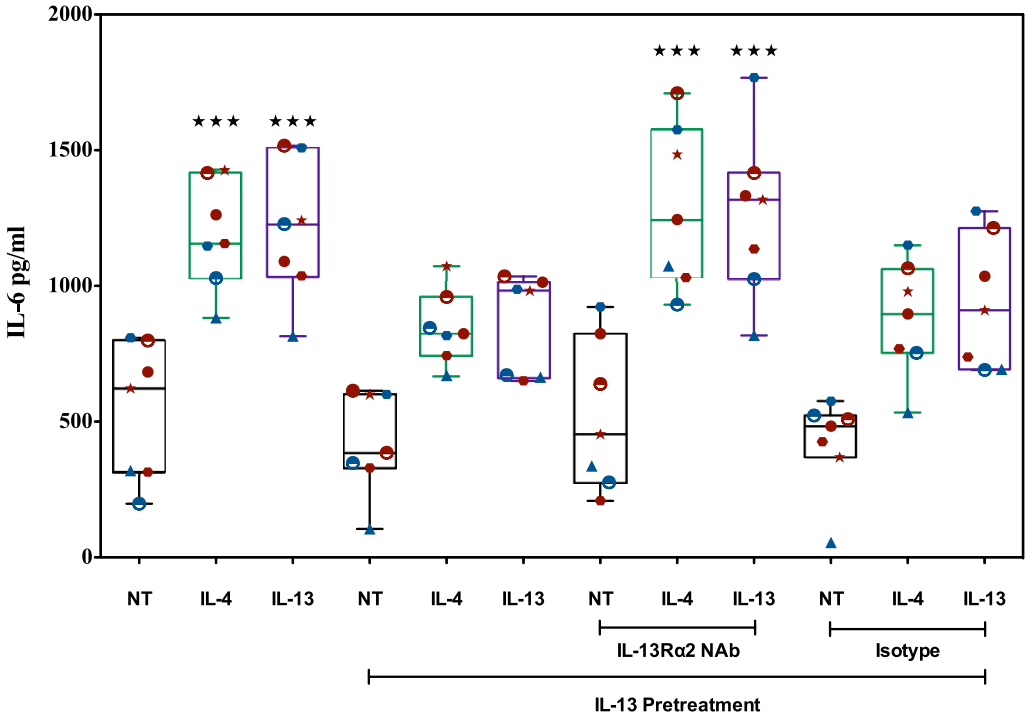

Figure 3.15: The effect of an IL-4 or IL-13 pretreatment step on IL-6 production, and the consequences of treatment with an IL-13Rα2 NAb. HBFs were grown in 12 well plates until ~95 % confluent and serum starved for 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13. Cells were washed out with fresh media for 3 hours during which half the cells were incubated with an IL-13Rα2 NAb or relevant isotype control. HBFs were incubated for a further 24 hours in the presence or absence of 10 ng/ml IL-4 or IL-13 ± IL-13Rα2 NAb where indicated. IL-6 production was analysed by ELISA of the conditioned media. $n = 3$ healthy and 4 asthmatic subjects. Data expressed as median with IQR and Tukey whiskers. $\star\star\star = p < 0.001$.

3.2.6 The Effect of Neutralising IL-13Rα2 at Baseline

Whist these experiments had focussed on the effects of pretreatment with IL-4 or IL-13 to upregulate the surface expression levels of IL-13Rα2, it was also important to determine the effect the IL-13Rα2 NAb at baseline. In particular it was of interest to establish whether a naturally high expresser of IL-13Rα2 could be made to behave like a naturally low expresser using this antibody to neutralising the receptor. To study this, HBFs from naturally low or high expressers of IL-13Rα2 were selected. The cells were serum starved for 24 hours in the absence of either cytokine and pretreated for 3 hours with either the IL-13Rα2 NAb or an isotype control before subsequent treatment with IL-4 or IL-13. The conditioned media were analysed for changes in eotaxin production, which was again normalised to cell number as determined by methylene blue assay.

As observed in Figure 3.3, in the absence of the IL-13Rα2 NAb the naturally low expressers of IL-13Rα2 produced significantly more eotaxin in response to both IL-4 and IL-13 than the naturally high expressers (Figure 3.16*a*) ($p < 0.001$). However, addition of the IL-13Rα2 NAb increased the cells responsiveness to both cytokines ($p < 0.001$). This effect was observed with both the low and high expressers of IL-13Rα2, although the NAb appeared to have a greater effect in the naturally higher baseline expressers.

As the dose initially studied did not completely restore the responsiveness to IL-4 and IL-13 to the levels observed in the naturally low expressers, a subsequent dose response of the antibody was performed. Here it was found that the higher concentrations of antibody were able to further reduce the efficacy of the IL-13Rα2 on the surface of the naturally high IL-13Rα2 expressers, resulting in greater release of eotaxin in response to treatment with IL-4 and IL-13, which was similar to that observed with naturally low expressers of IL-13Rα2.

These data therefore demonstrate a specific role for IL-13Rα2 in the regulation of both IL-4 and IL-13.

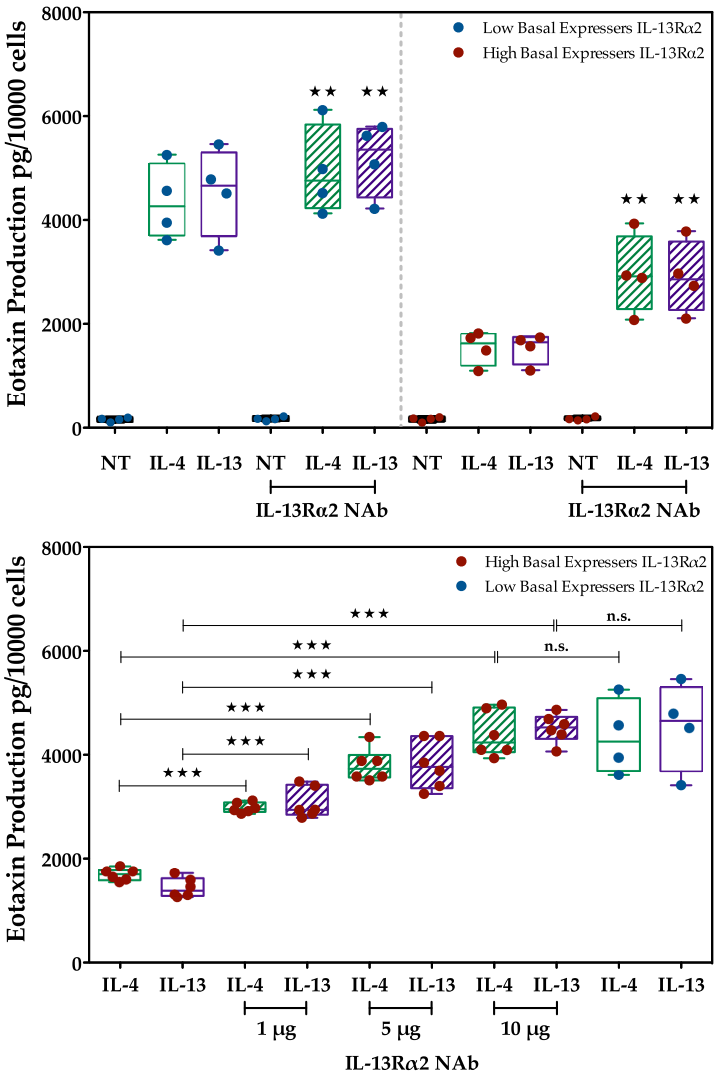

Figure 3.16: The effect of an IL-13Rα2 NAb on the responsiveness of HBFs to IL-4 and IL-13 at baseline. HBFs from known high or low expressers of IL-13Rα2 were seeded in 12 well plates and grown until ~95 % confluent, before 24 hours serum starvation. Cells were treated with 1 μg/ml IL-13Rα2 NAb for 3 hours before subsequent treatment with 10 ng/ml of either IL-4 or IL-13 for 24 hours. Conditioned media were collected and cells were fixed for quantitation of cell number by methylene blue assay. Eotaxin production was determined by ELISA and normalised to cell number. $n = 4$ low basal expressers and 4 high basal expressers of IL-13Rα2. Data are median with interquartile range. $\star\star = p < 0.01$ and $\star\star\star = p < 0.001$.

3.3 Discussion

Different subjects have previously been shown to vary in their responsiveness to IL-4 and IL-13.[289] This has been suggested to be related to the surface expression levels of IL-13Rα2, which acts as a negative regulator of these cytokines.[165,281,289] It was, therefore, hypothesised that the variation in surface expression levels of IL-13Rα2 would lead to differences in responsiveness to IL-4 and IL-13 in human bronchial fibroblasts. It was also hypothesised that the surface expression levels of IL-13Rα2 would increase in response to IL-4 and IL-13, and that this may be due to a regulatory loop.

Initial experiments investigated the production of eotaxin by HBFs from healthy and asthmatic subjects. All of the fibroblasts studied produced eotaxin in a dose dependent manner in response to challenge with either IL-4 or IL-13 (Figure 3.1). The amount of eotaxin produced in response to either IL-4 or IL-13 significantly varied between subjects, although was not found to be related to disease phenotype, and was most noticeable when treated with 10 ng/ml of either cytokine.

The responsiveness of the cells to both IL-4 and IL-13 was reduced when a pretreatment step with either cytokine was added, demonstrating the presence of a regulatory mechanism to prevent uncontrolled signalling (Figure 3.2). The mechanism previously proposed for this is the expression of IL-13Rα2,[165,281,289] and it was found that eotaxin production in response to IL-4 and IL-13 was related to the surface expression levels of this receptor, with high expressers of IL-13Rα2 producing less eotaxin than low expressers (Figure 3.3). The effect of pretreatment was also most noticeable with subjects which naturally expressed lower levels of IL-13Rα2 at baseline, where a significant attenuation was observed, which was not the case in the high baseline expressers.

Experiments were subsequently undertaken to determine the effect that IL-13 and IL-4 had on IL-13Rα2 expression levels to determine whether this was the only mechanism by which the pretreatment step reduced the sensitivity of these cells to both IL-4 and IL-13. IL-13Rα2 mRNA

significantly increased in response to both IL-4 and IL-13 treatment over time (Figure 3.4). A corresponding significant increase in IL-13Rα2 surface protein levels was also observed after 24 hours of treatment with either IL-4 or IL-13 (Figure 3.5). However, the basal expression and the extent of increase in IL-13Rα2 levels due to treatment varied dramatically between subjects. This is consistent with the variation observed in the eotaxin produced in response to IL-4 and IL-13, as well as previous reports of varied responsiveness to these cytokines by different subjects.[289] There was no variation due to disease phenotype, with no significant difference found between cells obtained from healthy controls or asthmatic patients. As IL-13 is also implicated in atopy[137–139,147] it is also important to note that the asthmatic patients used in these studies were atopic, whilst the healthy controls were non-atopic. Whilst it is not possible to rule out differences due to atopic status without studying atopic non-asthmatics, as the asthma phenotype may be having other underlying effects, it appears as if the levels of IL-13Rα2 at baseline do not correlate to an atopic phenotype.

The presence of intracellular pools of IL-13Rα2 have been observed in various cell types, in particular in epithelial cells,[251,293,294] with reports showing that these can be mobilised in response to challenge with IFNγ.[251] In this study whilst IFNγ was able to upregulate the surface expression levels of IL-13Rα2 in both HBFs and PBECs, it was only in the latter that this was found to be due to the presence of intracellular stores of IL-13Rα2.

Whilst there have been some reports of intracellular staining of IL-13Rα2 in fibroblasts these data are not conclusive. One study by Konstantinidis, Puddicombe *et al.*[252] looked at a range of cell types, including primary bronchial epithelial cells and fibroblasts, and, contrary to other reports, were unable to detect any extracellular IL-13Rα2. However, they did observe intracellular staining in each of the different cell types analysed. These data however contradict other existing publications, which have demonstrated extracellular staining on many of these cell types including epithelial cells and fibroblasts, which has also been observed in this chapter. In this study the localisation of IL-13Rα2 was determined by immunohistochemistry and flow

cytometry, however the only comparative staining showing the extracellular as well as intracellular IL-13Rα2 included in this study was for epithelial cells, where it is widely agreed that intracellular pools of IL-13Rα2 are present. This study also only analysed a small patient group ($n = 3$ asthmatic patients), rather than the systematic analysis of cells obtained from both healthy controls and asthmatic subjects presented herein, so the lack of extracellular staining on the epithelial cells could simply be due to these subjects being low expressers of IL-13Rα2. In the work presented within this chapter fibroblasts from a total of 9 subjects were analysed for the ability to rapidly mobilise IL-13Rα2 from intracellular stores after treatment with IFNγ. No increase in surface expression was observed in the fibroblasts until after 24 hours of treatment, which is slower than might be expected if there were intracellular pools of IL-13Rα2 present, and provides sufficient time for *de novo* protein synthesis of this receptor. This was also confirmed by treatment with CHX which prevented the increase in surface expression of IL-13Rα2 in the fibroblasts but not the epithelial cells.

The fact that both IL-4 and IL-13 can induce upregulation of IL-13Rα2 surface expression over 24 hours suggests that the surface expression levels of IL-13Rα2 may have been artificially raised by the addition of the pre-treatment step used in Figures 3.2 and 3.3, and that this might be the cause of the abrogation of eotaxin production observed in response to challenge with IL-4 or IL-13, as has previously been suggested.[165] However it was necessary to consider that the regulation may not be solely due to IL-13Rα2. Both IL-4Rα and IL-13Rα1 are key components of IL-4 and 13 signalling in human cells, although IL-4 can also signal via IL-4Rα and γc. Whilst many cells express both the type I (IL-4Rα:γc) and type II (IL-4Rα:IL-13Rα1) receptors this is not the case for all cells. The expression of IL-4Rα and IL-13Rα1 has previously been reported in primary human bronchial fibroblasts, however the presence of γc has been debated. The ability of HBFs to respond to IL-4 and IL-13 is dependent on the presence of the different receptor subunits, and it has been suggested that the overall profile of these receptors may be responsible for the differences in responsiveness observed

between subjects, rather than just IL-13Rα2 surface expression levels.[290] It was therefore, important to consider the roles of the other receptor subunits in the regulation of IL-4 and IL-13.

To address this, systematic characterisation of these receptor subunits was undertaken. The mRNA and surface expression levels of IL-4Rα, IL-13Rα1 and γc were analysed to establish whether the expression levels varied between subjects and if they were affected by IL-4 or IL-13 treatment.

It was hypothesised that, in contrast to what was observed with the IL-13Rα2 expression, the levels of the signalling receptors would not increase in response to either IL-4 or IL-13 as otherwise the level of signalling could rapidly become uncontrollable. If a change did occur in response to either cytokine it would be more likely that, as these are signalling receptors, their expression would be downregulated with treatment.

Whilst both IL-13Rα1 (Figure 3.7) and IL-4Rα (Figure 3.8) mRNA were detected in all subjects, the levels of expression were not affected by IL-4 or IL-13 treatment. γc mRNA expression, on the other hand, was not detected in any of the subjects studied. To confirm the accuracy of these data, positive controls were used in this assay. The stable production of IL-4Rα and IL-13Rα1 mRNA, regardless of treatment, was expected because if these levels were to increase in response to IL-4 or IL-13 treatment this could result in uncontrolled responses to these cytokines, which would have a damaging effect on the body.

As the expression of mRNA does not always correlate with changes in protein expression, the cells were also analysed for surface expression levels of each IL-4 or IL-13 receptor subunit by flow cytometry. Similarly to the mRNA, IL-13Rα1 was detected in every subject and was not affected by IL-13 treatment (Figure 3.9). This is consistent with previous reports and detection of IL-13Rα1 in these cells was expected as without this subunit the cells would not be able to respond to IL-13.

Surface expression of γc (Figure 3.10), analogous to that which was observed at the mRNA level, was not detectable in any of the subjects studied. Previous studies investigating expression of the IL-4 and IL-13 receptors have

demonstrated differing results with regards to γc, with several suggesting γc is not present on non-haematopoietic cells [233] or skin fibroblasts, [295] whilst another group [27] has demonstrated the presence of γc in bronchial fibroblasts. Whilst the former two studies corroborate with my data it is important to note that in the latter the γc mRNA was only detectable in either foetal or adult fibroblast cell lines, rather than primary cells, in the presence of cycloheximide, an inhibitor of protein synthesis. When these experiments were performed with primary cells obtained from cancer patients, constitutive mRNA expression, which was not dependent on cycloheximide, was observed; however, in this instance it was in human lung myofibroblasts rather than fibroblasts and this was reported to be in contrast to observations in normal cells. [27] These cancerous myofibroblasts may exhibit many disease-specific phenotypic differences to the primary human bronchial fibroblasts obtained from healthy or asthmatic volunteers that were used in this study, which could explain the presence of γc in these cells.

There were, however, differences observed at the surface expression level of IL-4Rα in response to both IL-4 and IL-13 stimulation. In this instance, whilst the mRNA levels remained unchanged, the surface expression of IL-4Rα was downregulated by treatment with either IL-4 or IL-13, whilst Interferon γ had no effect (Figure 3.11). This reduction in IL-4Rα expression occurred in a dose-dependent manner in response to IL-4 (Figure 3.12*a*), with 10 ng/ml reducing the expression by approximately $50 - 60\,\%$ after 24 hours. The surface expression of IL-4Rα decreased rapidly and plateaued within 1 hour of treatment with either IL-4 or IL-13 and was sustained over a 48 hour period (Figure 3.12*b*). No correlation was observed between the surface expression levels of IL-4Rα to the responsiveness of the HBFs to IL-4 or IL-13 either at baseline or after treatment with either cytokine.

The phenomenon of IL-4Rα internalisation with IL-4 or IL-13 stimulation has been debated in the literature. Some previous studies have seen IL-4Rα internalisation in the presence of IL-4 or IL-13 in both foetal lung fibroblast cell lines and T cells. This was found to occur very rapidly (within 15 minutes for IL-4, but slower for IL-13). In the experiments by Doucet, Brouty-Boyé *et*

al.[296] IL-4Rα was found in the early endosomes and they postulated that it might be either recycled back to the cell surface or degraded. In the washout experiments undertaken in this chapter the surface IL-4Rα is repopulated within three hours with fresh media, suggesting that the majority of the IL-4Rα is recycled back to the surface.

A study by Friedrich, Kammer *et al.*[297] found that IL-4Rα internalised with IL-4 but that this was not necessary for IL-4 signalling. They found that γc was needed for this to occur, despite the fact that the two receptor subunits internalised with different kinetics. Whilst γc was not found on the cell surface of the fibroblasts studied, IL-13Rα1 was observed, and it may be that this subunit is required for internalisation. IL-13Rα1 surface expression did not change after 24 hours of treatment with IL-13, however it may be that they are both internalised together and that IL-13Rα1 is recycled more rapidly to the surface. Alternatively the IL-13Rα1 subunit may be expressed in such high abundance that the difference observed due to internalisation with IL-4Rα does not cause a noticeable reduction in IL-13Rα1 surface expression.

It might also be that the IL-13Rα1 subunit is not needed here, as another study, which investigated an IL-4 toxin demonstrated that $\sim$ 60 % of the receptor internalised if only IL-4Rα was present and that this did not change if either IL-13Rα1 or γc were present.[298] These experiments were undertaken with CHO-KI cells, which do not normally express IL-4 or IL-13 receptors so the IL-4Rα, IL-13Rα1 and γc were incorporated by transfection. Whilst using CHO-KI cells provides the advantage of studying the effects of the individual receptor subunits independently, artificially adding these receptors may also lead to differing kinetics than those which would be observed in cells naturally expressing these receptors. Further experiments would therefore be needed in primary human bronchial fibroblasts, which naturally express high levels of IL-4Rα and IL-13Rα1, to determine the exact kinetics of internalisation and whether IL-13Rα1 is necessary for internalisation of IL-4Rα to occur.

It has also been reported that the expression of IL-4Rα increases in response to treatment with IFNγ.[299] This study, however, used an epithelial cell line, BEAS-2B, rather than fibroblasts and a very high dose (100 ng/ml) of IFNγ over a 48 hour period was required to see this effect. As IFNγ is involved in the switch to a Th1 phenotype it would be unexpected for treatment with this cytokine to result in upregulation of a Th2 receptor, unless this occurred only in an already Th1 polarised environment, where it may be involved in a feedback loop. A study by Huang, Xin *et al.*,[300] on the other hand, demonstrated in mice that IFNγ reduces the phosphorylation of STAT6, whilst no change was observed in levels of IL-4Rα, although in a previous study the same group found levels of IL-4Rα increased in response to IL-4 treatment in spleen and lymph cells from C57Bl/6 mice.[301] The differing results found in these studies may be due to the cell types analysed or the methods used. In the fibroblast studies reported in this chapter investigating the effect of IFNγ on IL-4Rα surface expression a standard dose of 10 ng/ml was used, and previous results from within our laboratory have found that treating fibroblasts with a higher dose of IFNγ results in significant cell death of both fibroblasts and epithelial cells (Andrews, unpublished observations).

A pretreatment step with IL-4 or IL-13 had been included in some experiments to modulate the responsiveness of these cells to IL-4 and IL-13. As treatment with IL-4 or IL-13 for more than 1 hour results in down-regulation of IL-4Rα, it was important to establish whether this reduction in surface expression of IL-4Rα could be responsible for the regulation of these cytokines. When a pretreatment step was included in an experiment, the cells were washed for 3 hours with fresh media before the addition of the subsequent treatment. The data presented herein, demonstrate that this three hour period is long enough for cells pretreated with IL-13 to repopulate their surface expression to similar levels observed on untreated cells. Whilst the surface expression levels did not completely recover within this time after IL-4 pretreatment, the responses to IL-4 or IL-13 treatment by HBFs were similar regardless of which pretreatment was used. If minor changes in the surface expression levels of IL-4Rα were important for regulating the

responsiveness to either cytokine it would be likely that differences would be observed here.

In contrast the levels of IL-13Rα2 were raised during treatment with both IL-13 and IL-4 and were unaffected by the washout step, suggesting that this is the mechanism of regulation of both IL-4 and IL-13 in HBFs. This, however, needed to be confirmed by the use of a neutralising antibody against IL-13Rα2.

Cells incubated with an IL-13Rα2 NAb after pretreatment with either cytokine and subsequently challenged with IL-4 or IL-13 for a further 24 hours produced similar levels of eotaxin (Figure 3.14) and IL-6 (Figure 3.15) to those which did not undergo the pretreatment step, whilst an isotype control had no effect. This demonstrates the specific involvement of IL-13Rα2 in this regulatory mechanism. This also highlights that IL-13Rα2 is able to regulate IL-4, even though IL-4 is not able to bind directly to this receptor. These data are consistent with work by Andrews, Nasir *et al.*, where the importance of IL-13Rα2 in the regulation of IL-4 in human bronchial fibroblasts was first noted. [165]

The neutralising antibody experiment, also demonstrates that the decrease in IL-4Rα surface expression does not affect the cells responsiveness to IL-4 or IL-13. The pretreatment step would have caused not only upregulation of IL-13Rα2 but also significant downregulation of IL-4Rα on the surface. A washout step was included, however as shown in Figure 3.12, the IL-4 pretreated cells would not have fully recovered their surface pools of IL-4Rα within this time, therefore if IL-4Rα was critical in the regulation of IL-4 or IL-13 signalling the IL-4 pretreated cells would have responded differently to the IL-13 pretreated cells, which was not the case.

However to confirm that the effect of the pretreatment step was the same as observed in naturally high and low expressers of IL-13Rα2 it was also necessary to test this at baseline. When the IL-13Rα2 NAb was added to the cells which were naturally high expressers their ability to produce eotaxin in response to IL-4 or IL-13 increased and was found to be similar to the naturally low expressers of IL-13Rα2.

3.4 Summary

In summary this work has highlighted the importance of IL-13Rα2 in the regulation of IL-4 and IL-13 mediated signalling in human bronchial fibroblasts. Whilst the IL-13Rα1 subunit is essential for IL-13 and IL-4 signalling in fibroblasts the expression levels appear to have little regulatory effect on these cells. Although rapid reduction in surface expression levels of IL-4Rα may have short-term regulatory effects, it is the IL-13Rα2 surface expression levels that appear to be most important for regulation of both IL-13 and IL-4 signalling.

As the importance of IL-13Rα2 as a regulator of IL-4 and IL-13 signalling has been demonstrated in this chapter, the next chapter will investigate the effects of modulating the levels of IL-13Rα2 in order to provide a model that can be used to determine how IL-13Rα2 affects the responsiveness of cells to IL-4 and IL-13. Whilst a pretreatment step was used to regulate the responsiveness of the HBFs to IL-13 and IL-4, by upregulating the surface expression levels of IL-13Rα2, this is not a clean system as it may have other unintended effects, such as upregulation of SOCS proteins, as well as the observed effects on IL-4Rα. Therefore the next chapter will attempt to establish a clean model whereby IL-13Rα2 levels can be specifically knocked down in HBFs using specific siRNA against IL-13Rα2.

Chapter 4

Development of a Model to Modulate Levels of IL-13Rα2

4.1 Introduction & Objectives

The importance of IL-13Rα2 for the regulation of both IL-4- and IL-13-mediated signalling in human bronchial fibroblasts was established in Chapter 3 and it was, therefore, of interest to understand more about this receptor.

In the previous chapter a neutralising antibody against IL-13Rα2 was exploited to reduce the levels of this receptor. However large quantities of the IL-13Rα2 NAb were required, making it quite expensive to use. This prompted further studies to develop a more effective model whereby IL-13Rα2 can be knocked down at the gene expression level in primary human bronchial fibroblasts to enable further characterisation of its actions. An alternative method of knocking down gene expression in cells is through the use of small interfering RNAs (siRNAs), which disrupt the expression of a specific gene of interest. [302–305]

siRNAs are 20-30 nucleotide sections of double stranded RNA (dsRNA), which have highly specific, generally inhibitory functions in a wide range of organisms and cell types, [302–305] including human bronchial fibroblasts. These are routinely used *in vitro* and have allowed targeted studying of gene knockdown in primary human cells after development.

The objectives of this study were to:

i) develop a model to modulate the levels of IL-13Rα2 in HBFs using siRNA against IL-13Rα2 in high expressing cells,

ii) confirm that modification of the surface expression of IL-13Rα2 affects the responsiveness of cells to both IL-13 and IL-4.

4.2 Results

4.2.1 siRNA Knockdown of IL-13Rα2

To study the knockdown efficiency of the siRNA fibroblasts from a subject previously shown to be a high expresser of IL-13Rα2 were selected. HBFs were seeded at 2×10^4 cells per well in 12 well plates and grown in the absence of antibiotics until $\sim 30 - 40\,\%$ confluence. The cells were subsequently incubated with either scrambled siRNA, as a control, or a pool of four siRNAs against IL-13Rα2 for 24 hours. Specific knockdown of IL-13Rα2 was analysed by RT-qPCR.

This initial experiment demonstrated approximately a 50% reduction in IL-13Rα2 mRNA due to treatment with specific siRNA ($p < 0.05$), in comparison to the control after 24 hours (Figure 4.1). Interestingly though, it was only the lower of the doses used which appeared to have an effect, with higher doses actually increasing expression of IL-13Rα2 mRNA. This was noted with both the IL-13Rα2 siRNA, as well as the scrambled control.

In addition, it was important to determine whether a knockdown of IL-13Rα2 surface expression was also observed, as expression on the surface of the cells is required for regulation of both IL-13 and IL-4. To this end, HBFs were seeded and treated with siRNA as previously described and IL-13Rα2 expression was analysed by flow cytometry after 48 hours.

Treatment with the siRNA did not cause a knockdown of IL-13Rα2 protein expression on the surface of the cells. In fact, a significant increase

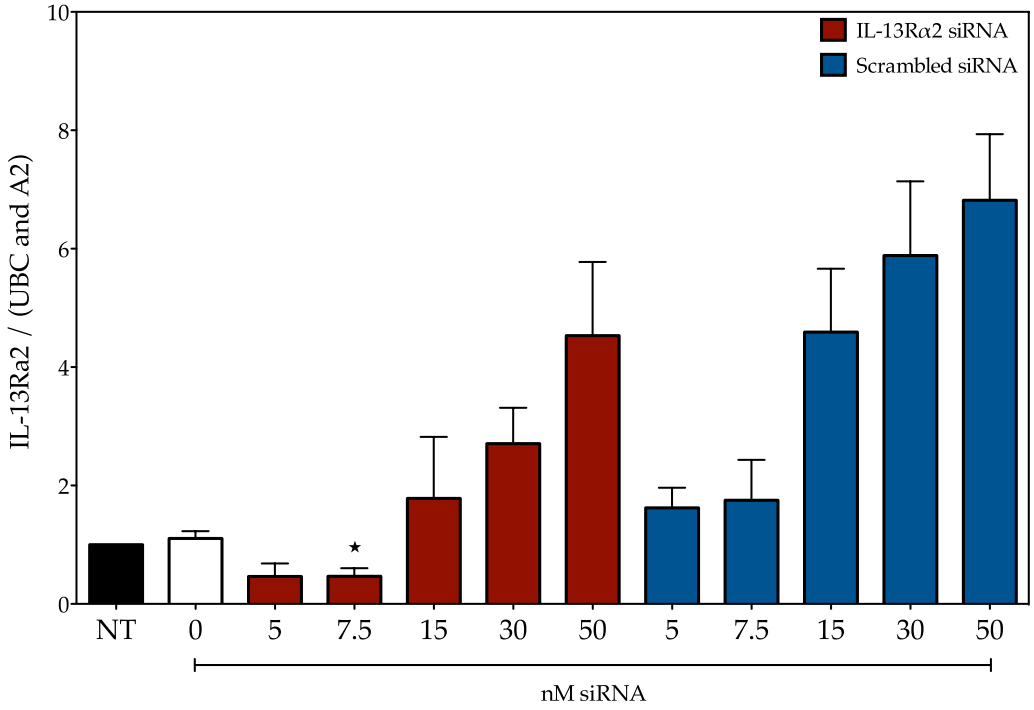

Figure 4.1: The effect of siRNA on IL-13Rα2 mRNA levels. Fibroblasts from a known high-expresser of IL-13Rα2 were treated with with fresh media (NT) or siRNA against IL-13Rα2 or a scrambled control for 24 hours in the absence of antibiotics. RNA was extracted using the Trizol method. PCR analysis compared IL-13Rα2 mRNA levels to the geometric mean of two housekeeping genes (UBC/A2). Data have been normalised to the average dCT of IL-13Rα2 in the unstimulated controls. $n = 3$ independent experiments. $\star = p < 0.05$

in IL-13Rα2 surface expression was observed after treatment with 15 nM scrambled siRNA compared to the no siRNA control (Figure 4.2). As this was the exact opposite effect to that which was anticipated, this implied that the cells were eliciting an alternative response to the presence of siRNA, resulting in an increase in IL-13Rα2 expression.

As higher concentrations of siRNA appeared to increase the levels of IL-13Rα2 in a dose dependent manner it was considered that lower doses of siRNA may actually be more useful.

To this end, HBFs were treated with siRNA as before, with doses ranging from 0.625 to 5 nM. A high dose of 30 nM siRNA was also included for

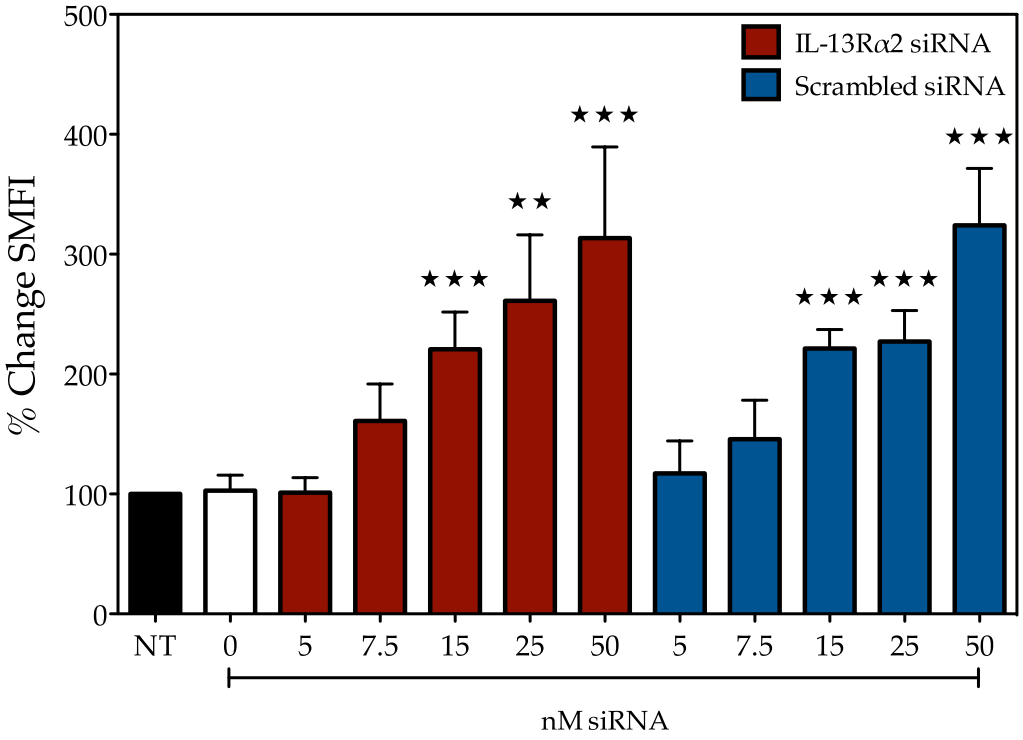

Figure 4.2: The effect of IL-13Rα2 siRNA on the surface expression of IL-13Rα2. Fibroblasts known to express high levels of IL-13Rα2 were grown in the absence of antibiotics for 24 hours before treatment with various doses of siRNA as indicated for 48 hours. A no transfection reagent control (NT) was also included. 1×10^5 cells/subject were incubated with an antibody against phycoerythrin-conjugated IL-13Rα2 or relevant isotype control before analysis on a FACSAria. Data were normalised to an isotype control and expressed as percentage change of specific mean fluorescent intensity (SMFI) $n = 5$ independent experiments. Data are mean +/− standard error. $\star\star = p < 0.01$ and $\star\star\star = p < 0.001$

comparison with the previous experiment. In this instance no decrease in surface expression of IL-13Rα2 was observed with the lower doses of IL-13Rα2 siRNA studied, but a significant increase in IL-13Rα2 expression was still seen with the higher dose of siRNA (Figure 4.3). No difference was observed between the scrambled and specific IL-13Rα2 siRNA added.

Although no decrease in surface expression level was observed with IL-13Rα2 siRNA treatment, it was of interest to establish whether this could

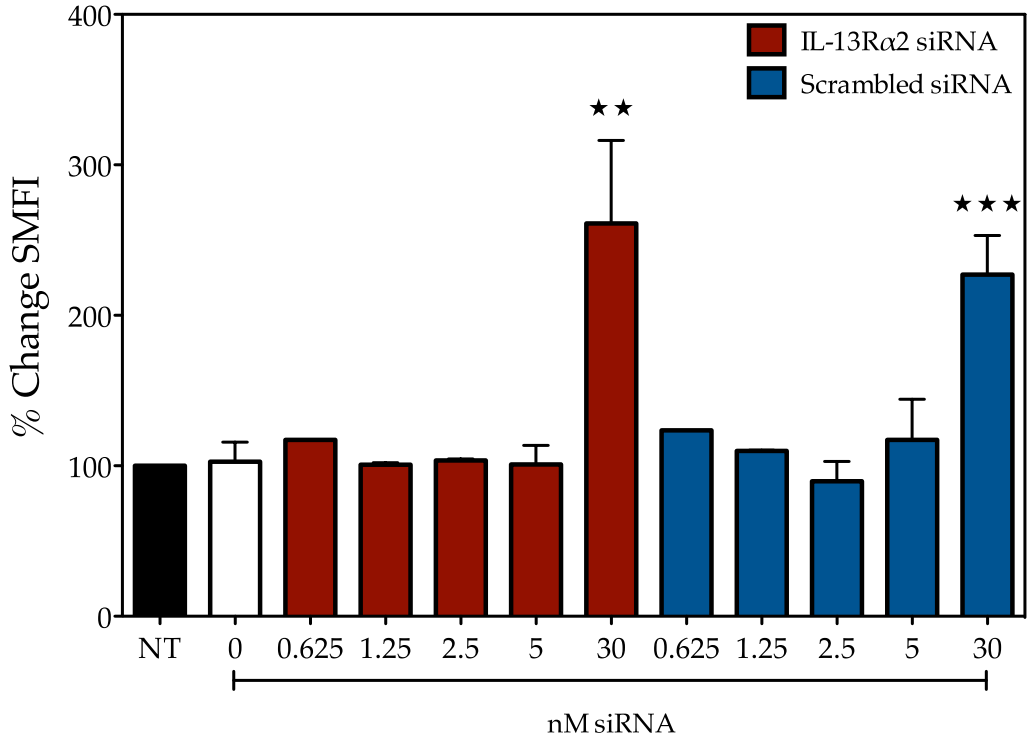

Figure 4.3: Characterisation of IL-13Rα2 surface expression in response to lower doses of siRNA. Human bronchial fibroblasts from a high expresser of IL-13Rα2 grown in the absence of antibiotics were treated with fresh media (NT) or varying concentrations of siRNA against either IL-13Rα2 or a scrambled control for 48 hours. The cells were trypsinised and incubated with an antibody against IL-13Rα2 or a matched IgG_1 isotype control. Surface expression of IL-13Rα2 was determined by flow cytometry. Data were normalised to an isotype control and expressed as percentage change of specific mean fluorescent intensity (SMFI) +/− standard error $n = 5$ independent experiments. $\star\star = p < 0.01$ and $\star\star\star = p < 0.001$

prevent an increase in IL-13Rα2 by treatment with IL-13. In Chapter 3, IL-13 was shown to increase the surface expression of IL-13Rα2 by fibroblasts over a 24 hour period. Therefore, a range of siRNA doses were studied with IL-13 treatment included.

Addition of specific siRNA against IL-13Rα2 did not prevent the increase in surface expression levels due to IL-13 treatment (Figure 4.4), which was significant in every instance ($p < 0.001$). The surface expression levels of IL-

13Rα2 in fact increased even higher with the top doses of siRNA in the presence of IL-13 (purple bars). This seemed to be the case more with scrambled siRNA than the specific IL-13Rα2 siRNA, although there was no significant difference between matched pairs.

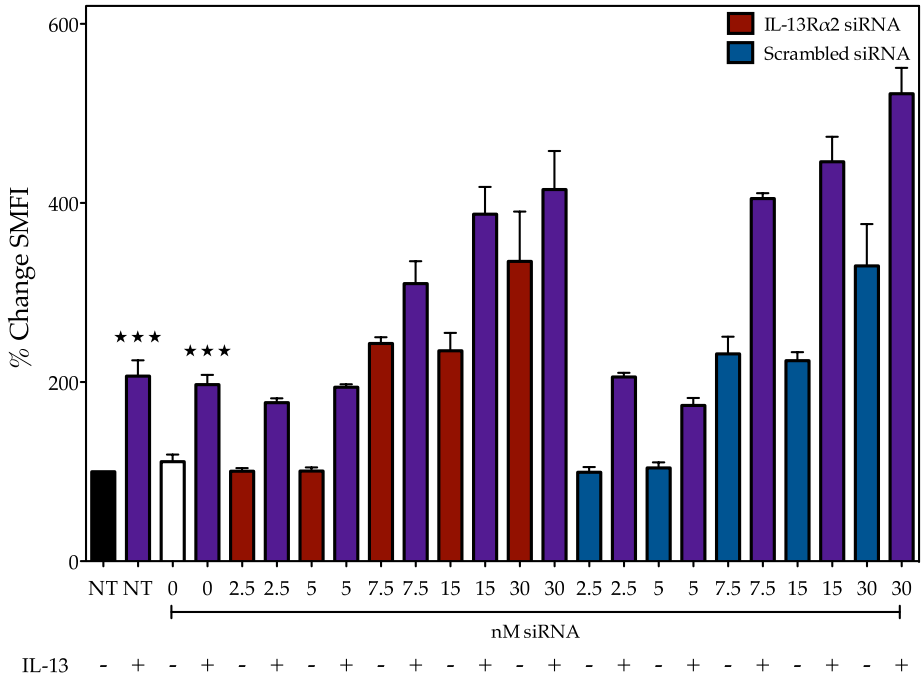

Figure 4.4: The influence of siRNA against IL-13Rα2 on IL-13-induced upregulation of IL-13Rα2. Fibroblasts were treated with various doses of siRNA against IL-13Rα2 or a scrambled control in the absence of antibiotics for 24 hours before dropwise addition of 10 ng/ml IL-13 to half of the wells for a further 24 hours. A no transfection reagent control was also included (NT). Cells were trypsinised and incubated with either an antibody against IL-13Rα2 or a relevant isotype control before analysis on a FACSAria. Data were normalised to an isotype and expressed as % change of specific mean fluorescent intensity (SMFI) +/− standard error. $n = 5$ independent experiments. $\star\star\star = p < 0.001$

4.2.2 siRNA Induced Interferon Response

One of the known pitfalls of using siRNA to knock down gene expression is the potential induction of an Interferon response due to the cells detecting

the siRNA as double stranded RNA (dsRNA). This is a well documented complication of siRNA treatment [306] that could be having an effect in these cells and may be affecting the ability of the cells to knock down IL-13Rα2.

In Chapter 3 a type II Interferon, IFNγ, was found to upregulate surface expression levels of IL-13Rα2, therefore it was hypothesised that the cells were detecting the siRNA as dsRNA resulting in the production of Interferon, which caused the cells to increase their expression of IL-13Rα2.

To test this hypothesis, cells were treated with polyinosinic:polycytidylic acid (Poly I:C), a strand of poly (inosine) annealed to a strand of poly (cytidine), which is frequently used *in vitro* as a synthetic analogue of dsRNA. Poly I:C has previously been shown to induce an Interferon response in human bronchial fibroblasts. [307] Therefore, to establish whether IL-13Rα2 mRNA levels were affected by dsRNA, fibroblast cDNA samples (kindly provided by Dr Nicole Bedke) from healthy and asthmatic patients treated for different times and different doses of Poly I:C and were then analysed for IL-13Rα2 mRNA expression by RT-qPCR.

IL-13Rα2 mRNA increased after 24 hours in both healthy and asthmatic subjects in response to Poly I:C in a dose dependent manner (Figure 4.5). This increase reached significance with 1 μg/ml Poly I:C in both the healthy and asthmatic subjects ($p < 0.05$ and $p < 0.001$ respectively) but the greatest increase was observed with 10 μg/ml Poly I:C ($p < 0.01$ and $p < 0.001$ respectively). This increase in IL-13Rα2 mRNA appeared to be time-dependent, reaching significance after 24 hours.

HBFs from healthy and asthmatic patients were subsequently treated with Poly I:C for 24 hours for analysis of changes in surface expression of IL-13Rα2 as previously described. In this instance, challenge with IL-13 was also included as a positive control.

Treatment with either Poly I:C or IL-13 resulted in a significant increase in IL-13Rα2 expression on the surface of the cells after 24 hours (Figure 4.6). This was the case with both healthy and asthmatic subjects and no difference was observed between the two.

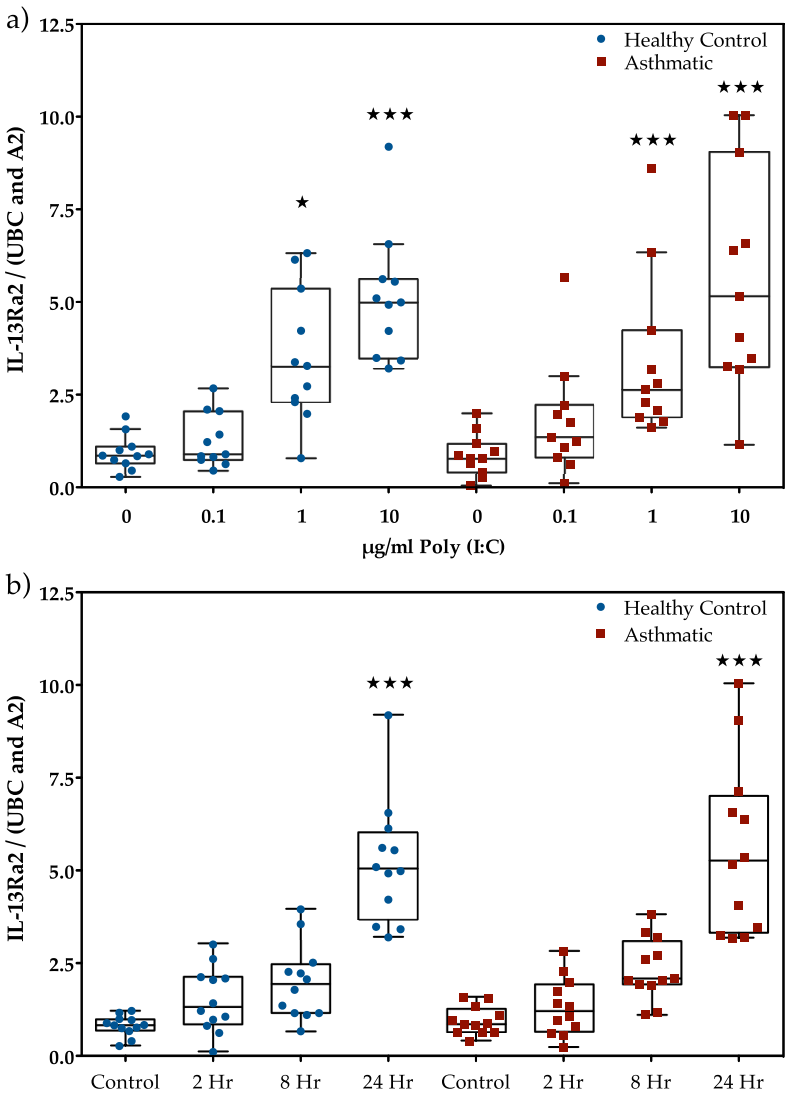

Figure 4.5: Dose and kinetic characterisation of IL-13Rα2 mRNA expression in response to Poly I:C treatment. HBFs were seeded at 1×10^5 cells/well on 6 well plates and grown until ~95 % confluent. Cells were serum starved for 24 hours before treatment with varying concentrations of Poly I:C for 24 hours. RNA was extracted using the Trizol method. PCR analysis compared IL-13Rα2 mRNA levels to the geometric mean of two housekeeping genes (Ubiquitin C and Phospholipase A2). Data have been normalised to the average dCT of IL-13Rα2 in the unstimulated controls. $n = 11$ healthy and 11 asthmatic subjects. Data are presented as median with standard Tukey whiskers. $\star = p < 0.05$ and $\star\star\star = p < 0.001$.

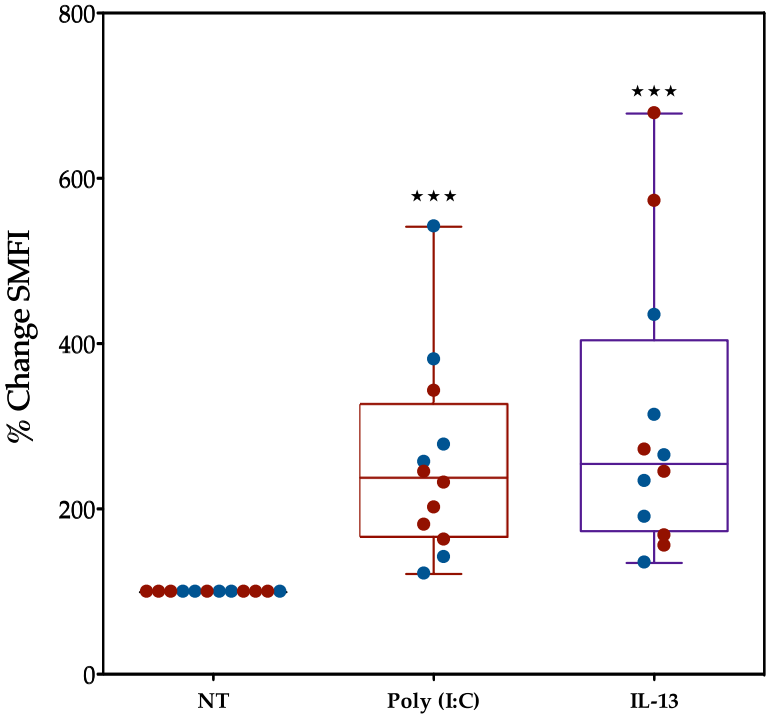

Figure 4.6: The effect of Poly I:C on IL-13Rα2 surface expression. Fibroblasts from healthy and asthmatic subjects were grown until ∼95 % confluent and serum starved for 24 hours before stimulation with either 10 μg/ml Poly I:C or 10 ng/ml IL-13 for 24 hours. Cells were trypsinised and incubated with either an antibody against IL-13Rα2 or a relevant isotype control before analysis on a FACSAria. Data were normalised to an isotype and expressed as % change of specific mean fluorescent intensity (SMFI). $n = 5$ healthy and 6 asthmatic subjects. Data are median +/− IQR with Tukey whiskers. $\star\star\star = p < 0.001$.

In the previous chapter, IL-13Rα2 surface expression levels were shown to regulate the cells responsiveness to IL-13 and IL-4. To confirm whether this Poly I:C induction of IL-13Rα2 surface expression translated to a functional response, the cells were pretreated with Poly I:C in a similar manner to the the IL-4 and IL-13 pretreatments used in the previous chapter. To this end, HBFs from healthy and asthmatic subjects were grown in 12 well plates until ∼95 % confluent. The cells were serum starved in the presence or absence of 10 μg/ml Poly I:C. As with previous pretreatment experiments, the cells were exposed to a 3 hour wash period, before subsequent treatment with IL-13. A neutralising antibody against IL-13Rα2 was also included in some of the treatments, where indicated. Conditioned media was collected after 24 hours for analysis of eotaxin production and the cells were fixed and stained with methylene blue for cell number determination.

As previously observed in Chapter 3, fibroblasts from all of the subjects produced eotaxin in response to IL-13 (Figure 4.7), however when the cells were pretreated with Poly I:C this production was significantly reduced ($p < 0.05$). This effect could be reversed by the addition of an IL-13Rα2 neutralising antibody, matching what was previously observed with IL-4 or IL-13 pretreatments.

As Poly I:C is known to induce production of Interferon (IFN) β in fibroblasts[307] it was therefore postulated that it is the IFNβ production in response to Poly I:C that induces increased IL-13Rα2 surface expression. To confirm this, the cDNA samples provided by Dr Bedke were also analysed for changes in IFNβ production in response to Poly I:C treatment.

The induction of IFNβ mRNA was found to occur very rapidly with the largest increase observed after 2 hours (Figure 4.8). This increase was found to be dose dependent, reaching significance after 1 μg/ml Poly I:C ($p < 0.01$) although a greater increase was observed with 10 μg/ml ($p < 0.001$). However, this production of IFNβ mRNA was diminished over time and after 24 hours was greatly reduced.

This rapid increase in IFNβ mRNA again indicated that this Interferon production by the fibroblasts may be the driving force behind the increased

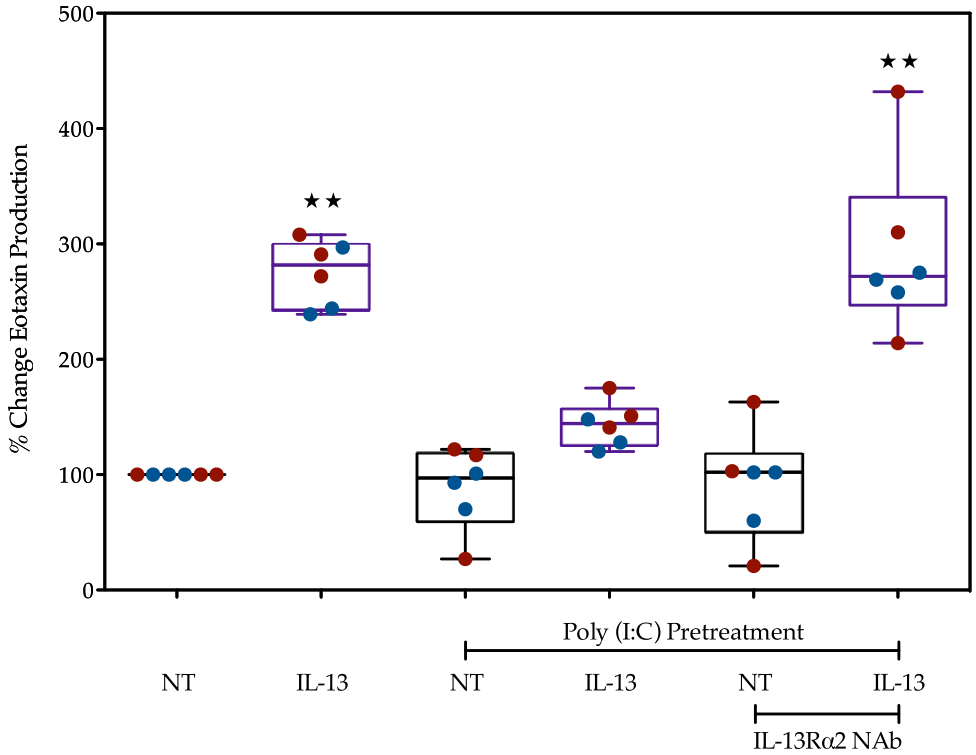

Figure 4.7: The effect of Poly I:C pretreatment on eotaxin production, and the consequences of treatment with an IL-13Rα2 NAb. Confluent HBFs were serum starved in the presence or absence of 10 μg/ml Poly I:C. The cells were washed for 3 hours before subsequent challenge with 10 ng/ml IL-13 for 24 hours. In some cases an IL-13Rα2 neutralising antibody (NAb) was added during the washout period and subsequent challenge as indicated. Eotaxin production was measured by ELISA of conditioned media and levels were normalised to cell number, determined by methylene blue assay. n = 3 healthy and 3 asthmatic subjects. Data expressed as median with standard Tukey whiskers. $\star\star$ = $p < 0.01$.

expression of IL-13Rα2. To test this, HBFs were treated with various doses of IFNβ for 24 hours before analysis of IL-13Rα2 surface expression by flow cytometry as previously described. Again, IL-13 was included as a positive control.

IFNβ (as previously observed with IFNγ, Figure 3.6) was found to increase the surface expression of IL-13Rα2 in a dose dependent manner (Figure 4.9).

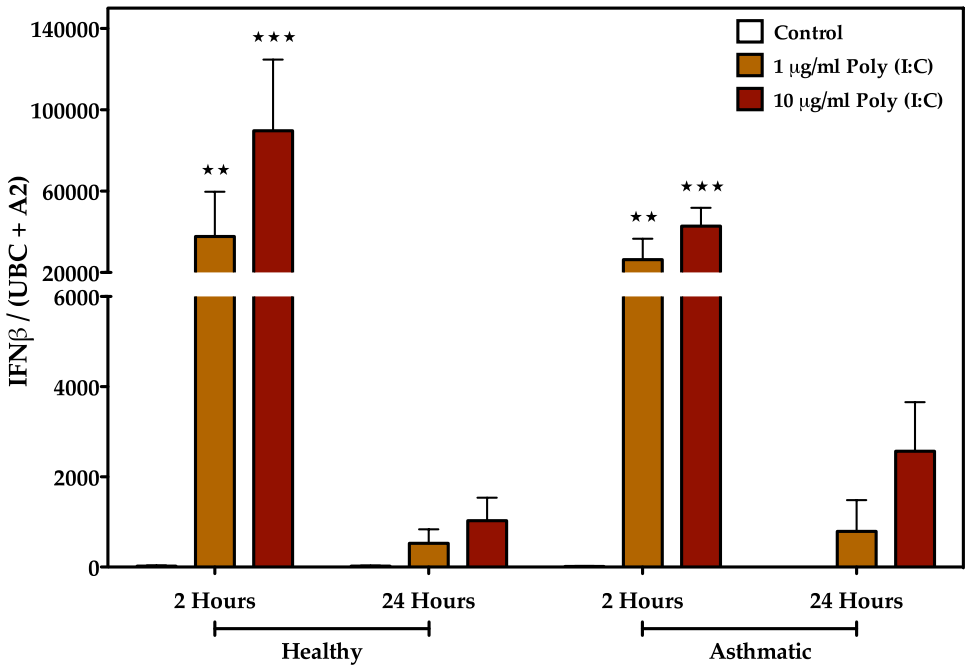

Figure 4.8: The impact of Poly I:C treatment on Interferon β mRNA production by bronchial fibroblasts. Fibroblasts from healthy and asthmatic patients were serum starved for 24 hours before exposure to 0-10 μg/ml Poly I:C for up to 24 hours. Total RNA was extracted using the Trizol method. RT-qPCR compared Interferon (IFN) β expression with the geometric mean of two housekeeping genes (Ubiquitin C and Phospholipase A2). Data were normalised to the average dCT of IFNβ in unstimulated controls. n = 6 healthy and 6 asthmatic subjects. Data are mean +/− SEM. $\star\star p < 0.01 \star\star\star \;=\; p < 0.001$.

The largest increase in surface expression occurred with the highest doses used, 50 and 100 IU IFNβ ($p < 0.001$), with 50 IU having a similar effect as 10 ng/ml IL-13. As expected, the increase in surface expression occurred later than the mRNA expression. The highest dose of IFNβ analysed did induce a statistically significant increase in surface expression of IL-13Rα2 after 8 hours, which is earlier to what has previously been observed with either IL-13, IFNγ or Poly I:C treatment, although the greatest increase was observed after 24 hours.

To further establish the role of IFNβ in the upregulation of IL-13Rα2 a neutralising antibody against the Interferon α/β receptor (IFN α/β R NAb)

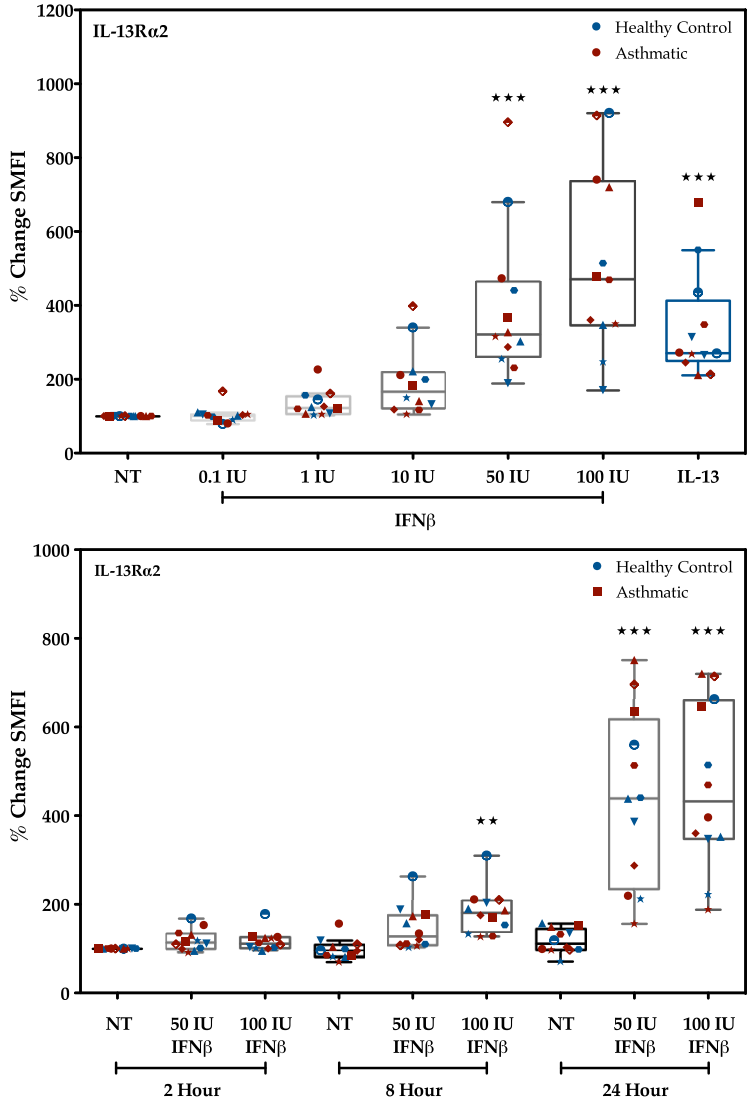

Figure 4.9: Dose and kinetic characterisation of IL-13Rα2 surface expression in response to IFNβ in human bronchial fibroblasts. Fibroblasts were seeded in 12 well plates and grown until ~95 % confluent. Cells were serum starved for 24 hours before treatment with 1 - 100 IU IFNβ, 10 ng/ml IL-13 or no treatment (NT). Cells were trypsinsed and incubated with an isotype control or antibodies against IL-13Rα2 and analysed for surface expression by flow cytometry. Data were normalised to an isotype control and expressed as specific mean fluorescent intensity (SMFI). n = 5 healthy and 7 asthmatic subjects Data are median +/− standard Tukey whiskers. $\star$ = $p < 0.05 \star\star$ = $p < 0.01 \star\star\star$ = $p < 0.001$.

was utilised. Both IFNα and IFNβ share a receptor consisting of IFNAR1 and IFNAR2, and this antibody is able to block access to the active site, preventing signalling.

HBFs were serum starved for 24 hours before a 3 hour pretreatment step with either the IFN α/β R NAb or a relevant isotype control. The cells were subsequently challenged with 10 μg/ml Poly I:C for 24 hours before analysis of IL-13Rα2 surface expression by flow cytometry.

The fibroblasts demonstrated a significant increase in surface expression of IL-13Rα2 in response to Poly I:C both in the presence and absence of the isotype control at a range of doses, with no difference observed when the isotype was included (Figure 4.10). However, when the IFN α/β R NAb was added a dose dependent attenuation of this increase in IL-13Rα2 surface expression was observed ($p < 0.001$). The highest dose used (10 μg/ml) had the greatest affect and it appeared as if this had not yet plateaued, however due to the cost implications higher concentrations were not tested.

When this experiment was repeated with IFNβ treatment, rather than Poly I:C, again a significant attenuation was observed in the presence of the IFN α/β R NAb, whilst the isotype control had no effect (Figure 4.11).

So, as these experiments demonstrated that challenge with dsRNA induces an IFNβ-mediated increase in IL-13Rα2 expression this may be the cause of the problems with the siRNA model.

Steroid Sensitivity

Steroids are regularly used in the treatment of the moderate to severe forms of asthma due to their immuno-suppressive and anti-inflammatory actions, which occur via suppression of inflammatory gene expression. Dexamethsone (Dex) is a synthetic glucocorticosteriod, which is often used in a laboratory setting.

It has previously been observed that dexamethasone treatment can suppress the IFNβ gene expression caused by challenge with Poly I:C. [308] It was therefore hypothesised that cells treated with both Dex and Poly I:C would

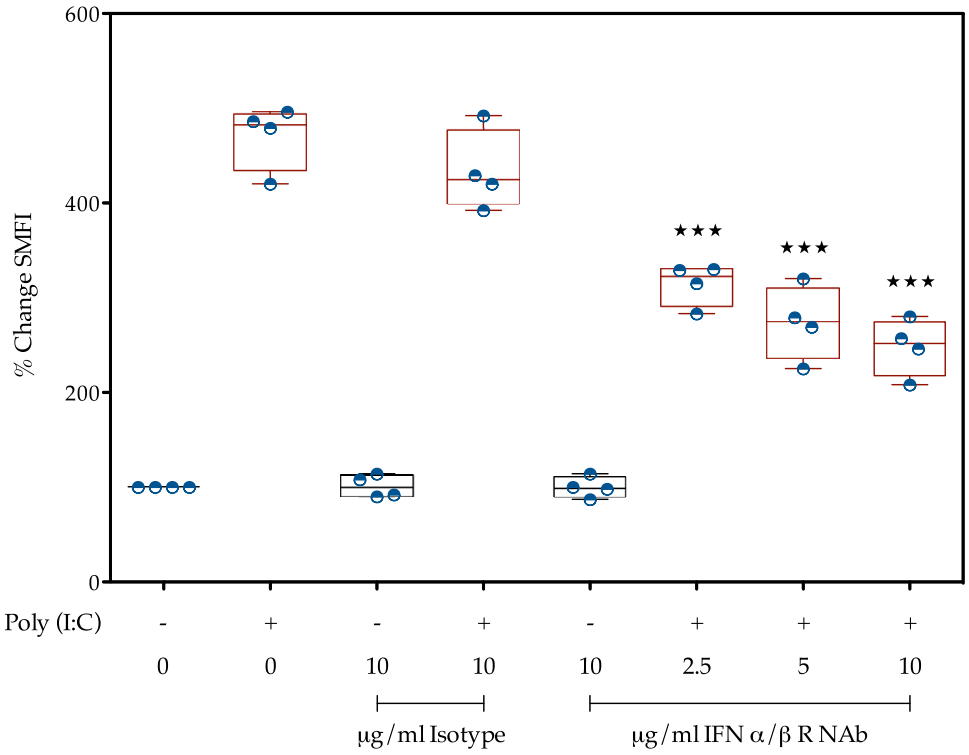

Figure 4.10: The effect of a neutralising antibody against IFN α/β R on Poly I:C induced upregulation of IL-13Rα2. HBFs grown in 12 well plates until ~95% confluent before 24 hours serum starvation. The cells were incubated in the presence or absence of either a neutralising antibody against the IFN α/β receptor (IFN α/β R NAb) or a relevant isotype control for 3 hours before subsequent challenge with 10 μg/ml Poly I:C for 24 hours. Surface expression of IL-13Rα2 was analysed by flow cytometry. Data were normalised to an isotype control and expressed as percentage change specific mean fluorescent intensity (SMFI) n = 4. Data presented as median with standard Tukey whiskers. $\star\star\star = p < 0.001$.

not increase surface expression of IL-13Rα2, but that the expression of IL-13Rα2 on cells treated with dexamethasone and IFNβ would still increase, demonstrating that direct stimulation with IFNβ, rather than IFNβ-induced gene expression, is required for this upregulation.

HBFs were treated with Poly I:C or IFNβ for 24 hours in the presence or absence of Dex and surface expression of IL-13Rα2 was analysed by flow cytometry.

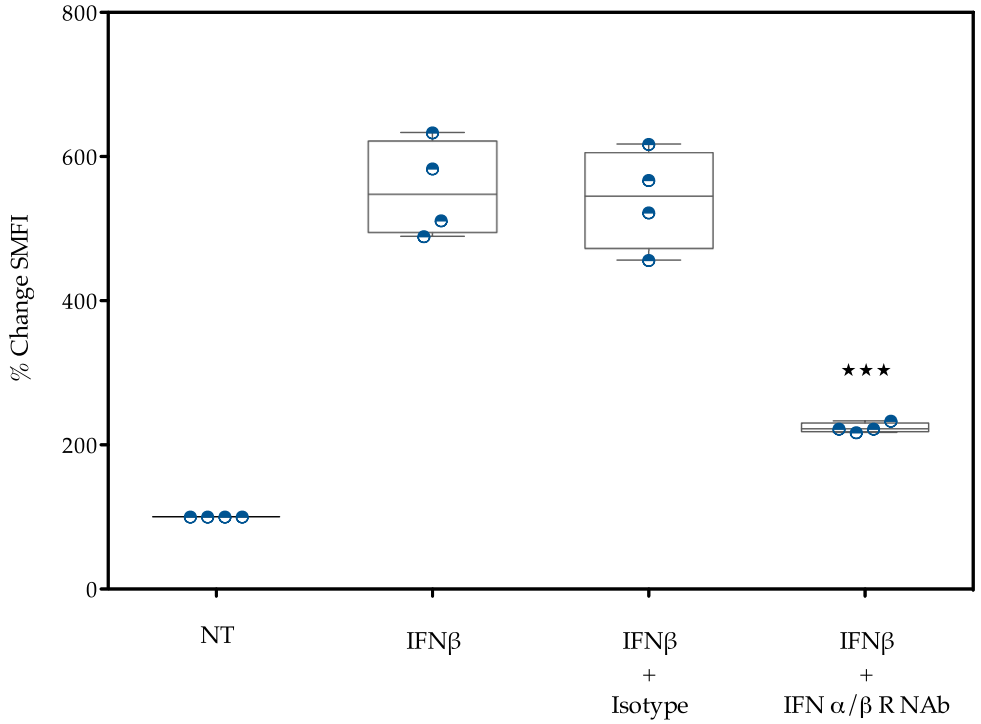

Figure 4.11: The impact of an IFN α/β R NAb on IFNβ-induced IL-13Rα2 upregulation. Fibroblasts were grown until ∼95 % confluent before being serum starved for 24 hours. A neutralising antibody (NAb) against the IFN α/β receptor (10 μg/ml) or a relevant isotype control (10 μg/ml) was added for 3 hours where indicated before challenge with 100 IU IFNβ for a further 24 hours. Surface expression of IL-13Rα2 was analysed by flow cytometry. Data were normalised to an isotype control and expressed as percentage change specific mean fluorescent intensity (SMFI) n = 4. Data presented as median with standard Tukey whiskers. $\star\star\star$ = $p < 0.001$.

As before, cells treated with either Poly I:C or IFNβ demonstrated significantly higher levels of expression of IL-13Rα2 than those which were not exposed to any treatment ($p < 0.01$ in both instances) (Figure 4.12). Addition of Dex in the absence of any other treatment resulted in a slight reduction in IL-13Rα2 levels. When cells were treated with Poly I:C in the presence of Dex the increase in IL-13Rα2 expression previously observed was suppressed. With IFNβ treatment in the presence of Dex, a significant increase in surface expression was observed in comparison to cells treated with

Dex alone ($p < 0.05$), however this was still much reduced in comparison to IFNβ treatment alone.

Whilst this small increase was observed with IFNβ in the presence of Dex, compared with Dex treatment alone, it is clear that the addition of steroids has a suppressive effect on IL-13Rα2 expression.

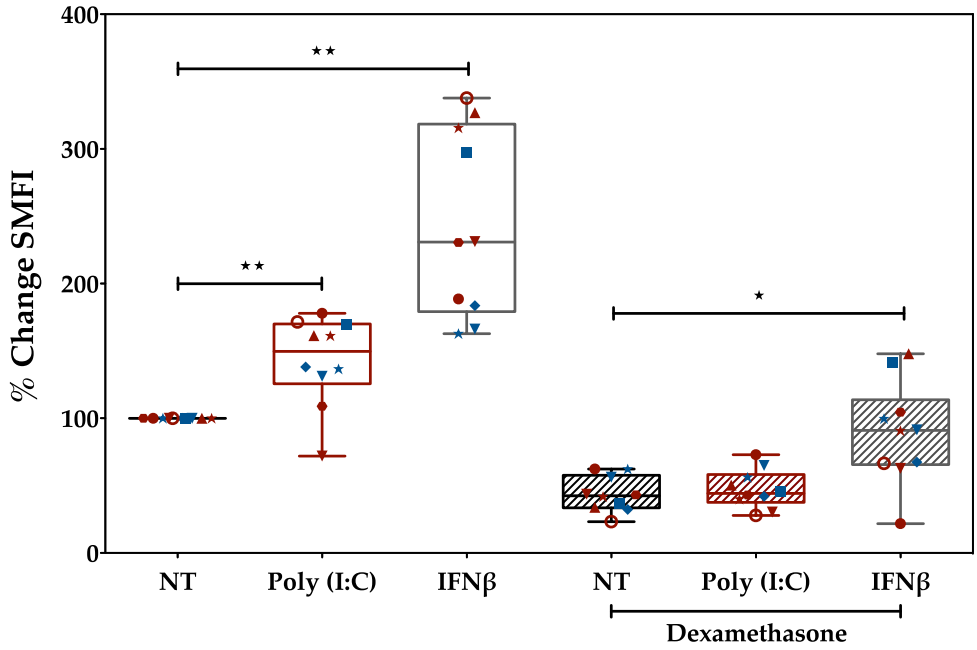

Figure 4.12: Attenuation of Poly I:C and IFNβ induced IL-13Rα2 upregulation by dexamethasone. Fibroblasts were seeded in 12 well plates and grown until confluent before being serum starved for 24 hours. Cells were challenged with 10 μg/ml Poly I:C or 50 IU IFNβ in the presence or absence of 50 nM dexamethasone for a further 24 hours. Surface expression of IL-13Rα2 was analysed by flow cytometry. Data were normalised to an isotype control and expressed as percentage change specific mean fluorescent intensity (SMFI) n = 4 healthy and 6 asthmatic subjects. Data presented as median with standard Tukey whiskers. $\star$ = $p < 0.05$ $\star\star$ = $p < 0.01$.

4.3 Discussion

As the mechanism by which IL-13Rα2 regulates IL-4 is unclear, because it is unable bind directly, it was therefore of interest to establish a model whereby levels of IL-13Rα2 could be specifically modulated. To this end this chapter aimed to develop a knockdown model using specific siRNA against IL-13Rα2.

Initial experiments were undertaken to characterise an siRNA knockdown model to selectively regulate the surface levels IL-13Rα2. Whilst this appeared to have some effect at the mRNA level (Figure 4.1) there was no decrease in surface expression of IL-13Rα2 (Figure 4.2 and 4.3).

The IL-13Rα2 siRNA not only failed to induce a specific knockdown of surface IL-13Rα2 but it was also unable to prevent an IL-13-mediated increase in IL-13Rα2 surface expression, regardless of the dose used (Figure 4.4). This was therefore found not to be a satisfactory model.

The lack of knockdown in response to siRNA and the fact that higher doses actually increased expression of IL-13Rα2 mRNA and surface expression suggested that the cells may be eliciting an Interferon response to the siRNA.

Interferon production is a potential side effect of treating cells with siRNA as it can be detected by pattern recognition receptors, such as Toll-Like Receptor (TLR) 3, which detects double stranded RNA. In Chapter 3 IFNγ was found to upregulate IL-13Rα2 surface expression, and it was therefore hypothesised that any Interferon production in response to siRNA treatment may have actually upregulated IL-13Rα2 levels.

To test this, fibroblasts were treated with a synthetic analogue of dsRNA, Poly I:C. IL-13Rα2 surface expression was upregulated in a time and dose dependent manner in response to Poly I:C. There was a similar increase in IL-13Rα2 in response to IL-13, with no difference found between healthy and asthmatic subjects with either treatment, corresponding to that which was observed in Chapter 3. This upregulation was shown to have a functional consequence on the cells, in a similar manner to IL-4 or IL-13 pretreatment,

where cells pretreated with Poly I:C demonstrated a reduced responsiveness to IL-13.

Fibroblasts have previously been shown to produce IFNβ in response to Poly I:C [307] and this was confirmed initially by measuring IFNβ mRNA, which was found to significantly increase within 2 hours of Poly I:C treatment. Challenging fibroblasts with IFNβ directly also significantly increased surface expression levels of IL-13Rα2 in a dose-dependent manner, over 24 hours.

When a neutralising antibody against IFN α/β R was added both the Poly I:C and IFNβ-mediated increase in IL-13Rα2 surface expression could be attenuated, confirming that the Poly I:C induction of IL-13Rα2 is IFNβ-mediated.

This was not a complete attenuation, as there was still an increase in surface expression compared to the baseline, although when a range of doses of the NAb were used prior to Poly I:C pretreatment, it was found that higher doses could elicit a greater effect. Unfortunately it was not possible to test greater doses as it was cost prohibitive but it would be of interest to determine whether this could be further attenuated or if another mediator is involved in the upregulation of IL-13Rα2. The fact that a similar response was found with both the Poly I:C and IFNβ treatment suggests that it is simply inefficiency of the antibody, but this would need to be confirmed.

Neither IFNλ nor IFNγ can signal through the IFN α/β R, so it may be that the fibroblasts also produce one of these Interferons in response to the Poly I:C treatment, and that this is able to upregulate surface expression of IL-13Rα2. Further research would be required to establish the full range of mediators produced by these cells in response to dsRNA stimulation.

As will be discussed further in Chapter 6 these results have implications in respiratory viruses, which are one of the leading causes of asthma exacerbations. [118]

Subsequent experiments investigated the effect of steroids on IL-13Rα2. Here it was found that the addition of Dex was able to attenuate the Poly I:C-mediated induction of IL-13Rα2 surface expression, however challenge with IFNβ could partially overcome the Dex treatment. This suggests that whilst Poly I:C treatment could normally induce IFNβ production, which could in turn increase the IL-13Rα2 surface expression, this was prevented with by the addition of steroids. Whilst direct treatment with IFNβ was still able to increase surface expression of IL-13Rα2 in the presence of Dex, this was substantially reduced in comparison to cells treated in the absence of steroids. However, in these experiments only one dose of Dex was tested and it would be important to further characterise this steroid suppressive effect by investigating multiple concentrations of this drug.

The fact that the baseline levels of IL-13Rα2 decreased in response to Dex suggests that the regulation of this receptor is steroid-sensitive. Whilst there was insufficient time to study this further it would be of interest to greater understand the regulation mechanisms controlling IL-13Rα2 surface expression. This is especially true for understanding the role of steroids as these are regularly used as a therapy for moderate and severe forms of asthma. These drugs elicit anti-inflammatory effects and have been found to reduce levels of IL-13 production. [309,310] Therefore, if these also downregulate the levels of IL-13Rα2 any IL-4 or IL-13 produced could have a greater effect than in the absence of steroids. It would also be of interest to determine whether Dex can suppress IL-13 induced IL-13Rα2 surface expression.

4.4 Summary

In the current chapter attempts were made to develop a model to study the effects of IL-13Rα2. When siRNA knockdown was tested it was found that the cells detected siRNA as dsRNA, which induced an Interferon response.

Whilst not ideal, this work has highlighted an increase in IL-13Rα2 expression due to this Interferon response, which occurred when the fibroblasts were stimulated with dsRNA.

Unfortunately, it was therefore not possible to develop a model for knocking down IL-13Rα2 in primary human bronchial fibroblasts using siRNA due to this production of Interferon. However, as the importance of IL-13Rα2 as a regulator of IL-4 and IL-13 signalling has been highlighted in Chapter 3 an alternative model is still required to determine how IL-13Rα2 is able to regulate the effects of IL-4 despite being unable to bind to it directly.

As mentioned previously, it has been suggested that IL-13Rα2 may be able to instigate an IL-4 mediated signal via STAT3 in glioblastoma cells, however this has not yet been studied in non-malignant cells or asthma.

Therefore the next chapter will attempt to validate an overexpression model of IL-13Rα2, which is already in use in our laboratory. This model will then be used to establish whether IL-13Rα2 is able to instigate a signal in response to IL-4.

Chapter 5

Regulation of IL-4 & IL-13 by IL-13Rα2

5.1 Introduction & Objectives

Having established in Chapter 3 the importance of IL-13Rα2 in the regulation of both IL-4 and IL-13 it was of interest to try to establish the mechanism by which this occurs. In Chapter 4 attempts were made to develop a model whereby IL-13Rα2 levels could be reduced by siRNA to complement experiments using the IL-13Rα2 neutralising antibody. As this proved unsuccessful due to the induction of an Interferon response by the fibroblasts in response to the siRNA another model was required to investigate the mechanisms of action of IL-13Rα2 in a clean system. An alternative method for studying a receptor is overexpression in cells which normally only express very low levels. An overexpression model of IL-13Rα2 in BEAS-2B cells has previously been developed in our laboratory and the preliminary experiments of this Chapter will look to validate this model.

IL-13Rα2 was originally considered a non-signalling decoy receptor due to its short intracellular tail, which, unlike the IL-4 and IL-13 signalling receptors, is unable to instigate a STAT6 signal. However, recent studies have proposed several, as yet unconfirmed signalling mechanisms,[261,264,280] one of which could explain how IL-13Rα2 is able to regulate IL-4, despite being unable to bind to it directly.[243]

In studies investigating the differences between glioblastoma cells and normal astrocytes it has been suggested that IL-13Rα2 may instigate a signal via activation of the transcription factor STAT3. [249,280] Glioblastoma cells are unable to induce IL-4 or IL-13 mediated STAT6 signals and express high levels of IL-13Rα2 in comparison to normal astrocytes which are IL-4 and IL-13 responsive and do not express this regulatory receptor. [247,249,277] The mechanism of STAT3 activation is currently undetermined and direct binding of STAT3 to IL-4Rα does not appear to be required. [280]

STAT3 signalling is classically associated with the IL-6-type family of cytokines, which signal via the gp130 receptor. [311] However it has been shown more recently to act as a signalling molecule for various Interferons and growth factors. [312]

Similarly to STAT6 signalling, STAT3 is activated by JAK, which causes phosphorylation of tyrosine 705 near the carboxy-terminus, resulting in homodimerisation. The STAT3 dimer translocates to the nucleus where it binds to DNA and activates transcription of a range of genes. [313] The genes activated by STAT3 vary by cell type and often have seemingly contradictory effects. [312]

STAT3 elicits anti-apoptotic and pro-proliferative actions, which are commonly observed with cytokines in the IL-6 family, in B and T lymphocytes. However, the opposite effect is seen in monocytes, where STAT3 prevents growth and activates terminal differentiation. [312,313]

STAT3 also exhibits pro- and anti-inflammatory effects. Various models of gastric inflammation and cancers have observed constitutive STAT3 activation resulting in the release of pro-inflammatory cytokines and chemokines. [314] However, the reverse has also been observed with STAT3 seeming to negatively regulate inflammation in macrophages, and knockout studies have demonstrated chronic enterocolitis, an inflammatory condition of the bowel, in STAT3 deficient mice. [315]

The mechanism by which these differing responses to one signal transducer occur is currently unknown, although it is of particular interest in

diseases such as cancer and autoimmune conditions where excessive proliferation and/or inflammation occurs.

Many of the studies which have investigated STAT3, and in particular the potential of this signal occurring via IL-13Rα2, have done so in a malignant setting as STAT3 has been found to be constitutively activated in various cancers,[316–318] however this has not yet been considered to have implications in non-malignant conditions such as asthma.

As both inflammation and fibrosis are hallmarks of asthma it would be of great interest to determine whether IL-4 is able to induce STAT3 activation via IL-13Rα2 in primary human bronchial fibroblasts.

The objectives of this study were to:

i) validate an overexpression model of IL-13Rα2,

ii) establish whether IL-4 or IL-13 induce STAT3 phosphorylation in HBFs,

iii) determine whether this STAT3 signal is mediated by IL-13Rα2.

5.2 Results

5.2.1 Validation of an IL-13Rα2 Overexpression Model

As the attempts to knockdown IL-13Rα2 surface expression in Chapter 4 were unsuccessful, an over expression model of IL-13Rα2 was instead validated. In this model, established by Dr Allison-Lynn Andrews, BEAS-2B cells, which normally only express low levels of IL-13Rα2 on the surface were stably transfected with a pcDNA3.1 vector containing cDNA for full length IL-13Rα2 (B2B-FL) or a mock transfected sequence (B2B). The cells which had accepted the plasmid were selected via a geneticin selection method.

BEAS-2B cells are a bronchial epithelial cell line which has been transformed using an adenovirus12-SV40 virus hybrid. In Chapter 3, it was

established that there are some key differences in the regulation of IL-13Rα2 in fibroblasts and epithelial cells, such as pooling of IL-13Rα2 within intracellular stores in epithelial cells but not fibroblasts. Therefore this is not an ideal model for studying the regulation of IL-4 by this receptor in fibroblasts. However, previous attempts by Dr Andrews to establish similar models in a variety of lung fibroblast cell lines, such as MRC-5 cells proved unsuccessful. Therefore, as no alternative model was available, preliminary experiments were undertaken using these cells as this provided a clean system in which to study the regulation of IL-4 by IL-13Rα2.

Initially, expression of IL-13Rα2 was confirmed in B2B-FL cells. To this end, surface expression levels of IL-13Rα2 on both B2B and B2B-FL cells were analysed by flow cytometry at baseline. To determine the responsiveness of the cells to IL-4 and IL-13 STAT6 phosphorylation was studied via western blotting as these cells do not produce eotaxin in response to challenge with these cytokines.

When surface expression levels of IL-13Rα2 were analysed by flow cytometry, a clear shift in fluorescence was observed in the B2B-FL cells compared with the B2B cells, demonstrating substantial IL-13Rα2 expression on the cell surface (Figure 5.1*a*).

B2B and B2B-FL cells which were treated with either IL-4 or IL-13 also exhibited differing functional responses, with a strong phospho-STAT6 signal visible in response to treatment with either cytokine in the B2B cells but only very minimal STAT6 phosphorylation was observed in the B2B-FL cells (Figure 5.1*b*). These data are consistent with previous reports that STAT6 phosphorylation is substantially reduced in the presence of high levels of IL-13Rα2. [165]

As this model has been shown to behave as we would functionally expect, it can therefore be utilized to provide greater understanding of the regulatory methods of IL-13Rα2.

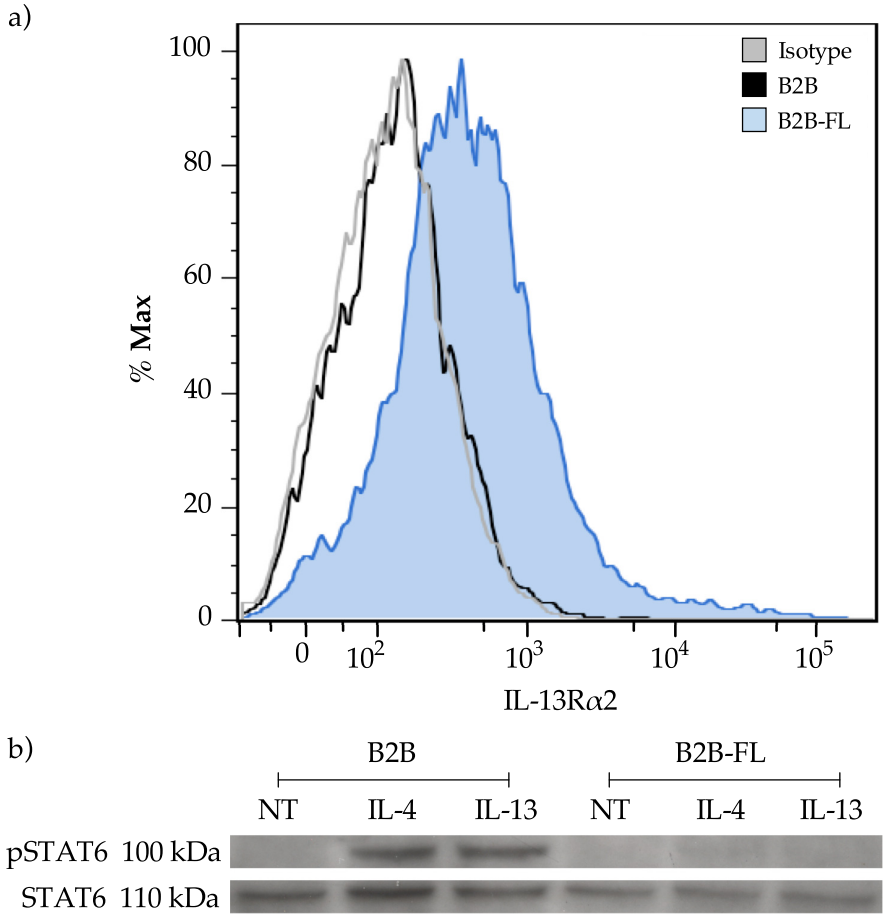

Figure 5.1: Validation of an IL-13Rα2 overexpression model in BEAS-2B cells. BEAS-2B cells, which had previously been stably transfected with either full length IL-13Rα2 (B2B-FL) or a mock control (B2B) were grown in 12 well plates until ∼95 % confluent. The cells were serum starved for 24 hours before a) flow cytometric analysis of surface expression of IL-13Rα2 and b) treatment with either serum free medium (NT), IL-4 or IL-13 for 1 hour and analysis of STAT6 phosphorylation by western blotting. Representative of $n = 3$ independent experiments.

5.2.2 STAT3 Signalling in an Overexpression Model of IL-13Rα2

IL-4 has been shown to induce an IL-13Rα2-mediated STAT3 signal in glioblastoma cells,[249,280] therefore, to confirm this, initial experiments were undertaken utilising the B2B model of IL-13Rα2 overexpression.

B2B and B2B-FL cells were seeded in 6 well plates at a density of 5×10^5 cells per well and grown until ∼95 % confluent. The cells were serum starved for 24 hours before challenge with IL-4 or IL-13 for 1 hour and analysis of STAT3 activation by western blotting.

The B2B-FL cells, expressing high levels of IL-13Rα2 on the cell surface, demonstrated a marked increase in phosphorylation of STAT3 in response to treatment with IL-4 (Figure 5.2), which was not found in the B2B cells ($p < 0.001$). A smaller apparent increase was also observed after challenge with IL-13, however this was not found to be significant.

5.2.3 STAT3 Signalling in Primary Human Bronchial Fibroblasts

Whilst the B2B data in Figure 5.2 corroborate the work in glioblastoma cells by Rahaman *et al.*,[249,280] it was important to determine whether this could be replicated in primary human bronchial fibroblasts, which naturally express IL-13Rα2.

Initial experiments were therefore undertaken to establish whether treatment with either IL-4 or IL-13 resulted in an increase in STAT3 phosphorylation in HBFs. Fibroblasts were grown on 12 well plates until ∼95 % confluent. The cells were serum starved for 24 hours and either lysed immediately (baseline) or treated either in the absence (T60) or presence of 10 ng/ml IL-4 or IL-13 for 1 hour. As before, the cell lysates were analysed by western blotting for phosphorylated STAT3, with total STAT3 protein acting as a loading control.

In these cells, high basal activation of STAT3 was observed in the absence of any stimulus (Figure 5.3), which was significantly reduced by exchanging the media ($p < 0.001$). Because the baseline STAT3 activation was so high, a significant decrease in STAT3 was observed in every treatment group. Whilst there did appear to be a trend towards an increase with IL-4 or IL-13 treatment when compared to T60, where basal phosphorylation was still observed, this was not found to be significant.

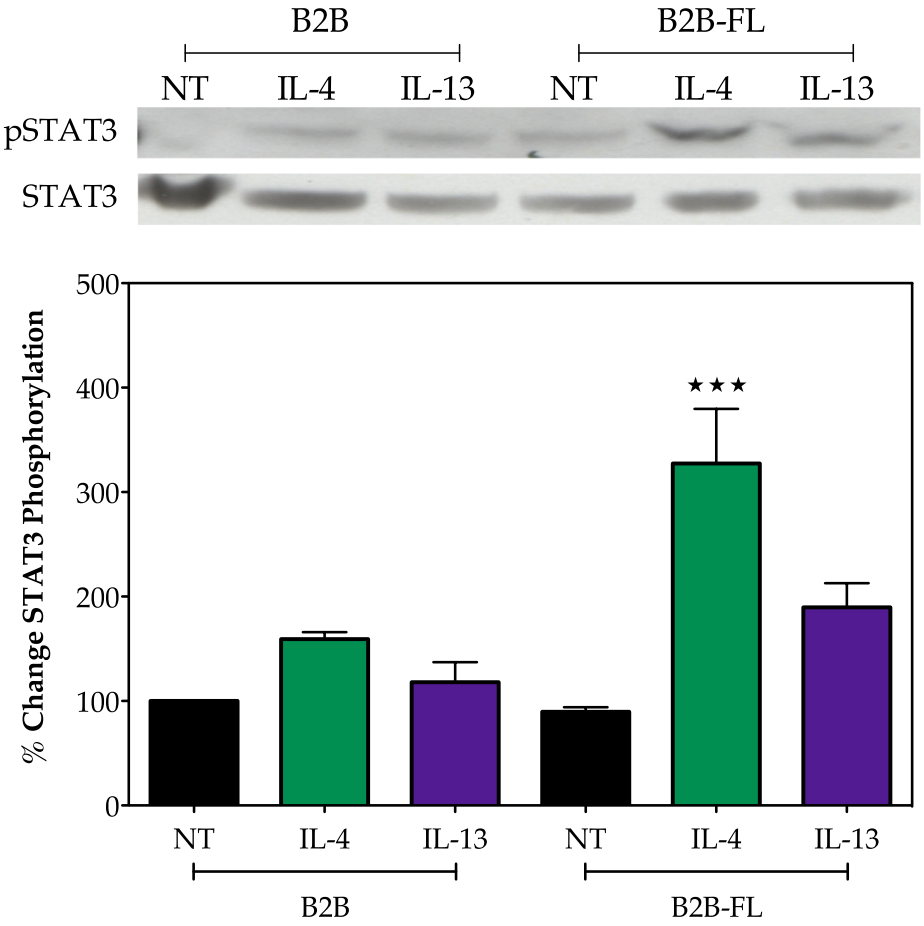

Figure 5.2: STAT3 phosphorylation by BEAS-2B expressing IL-13Rα2. Phosphorylated STAT3 was detected in western blots of lysates from BEAS-2B cells expressing full length IL-13Rα2 (B2B-FL) but not those which had been mock transfected (B2B). STAT3 phosphorylation was normalised to the total STAT3 and presented as % change in peak optical density (OD). $n = 4$ independent experiments. $\star\star\star = p < 0.001$

As experiments in glioblastoma cells have suggested that the STAT3 signal is initiated via IL-13Rα2 [280] it was of interest to determine whether increasing the surface expression of IL-13Rα2 resulted in a greater signal, which would be more clearly visible above the background phosphorylation that was observed in Figure 5.3, even when the medium was exchanged for an hour.

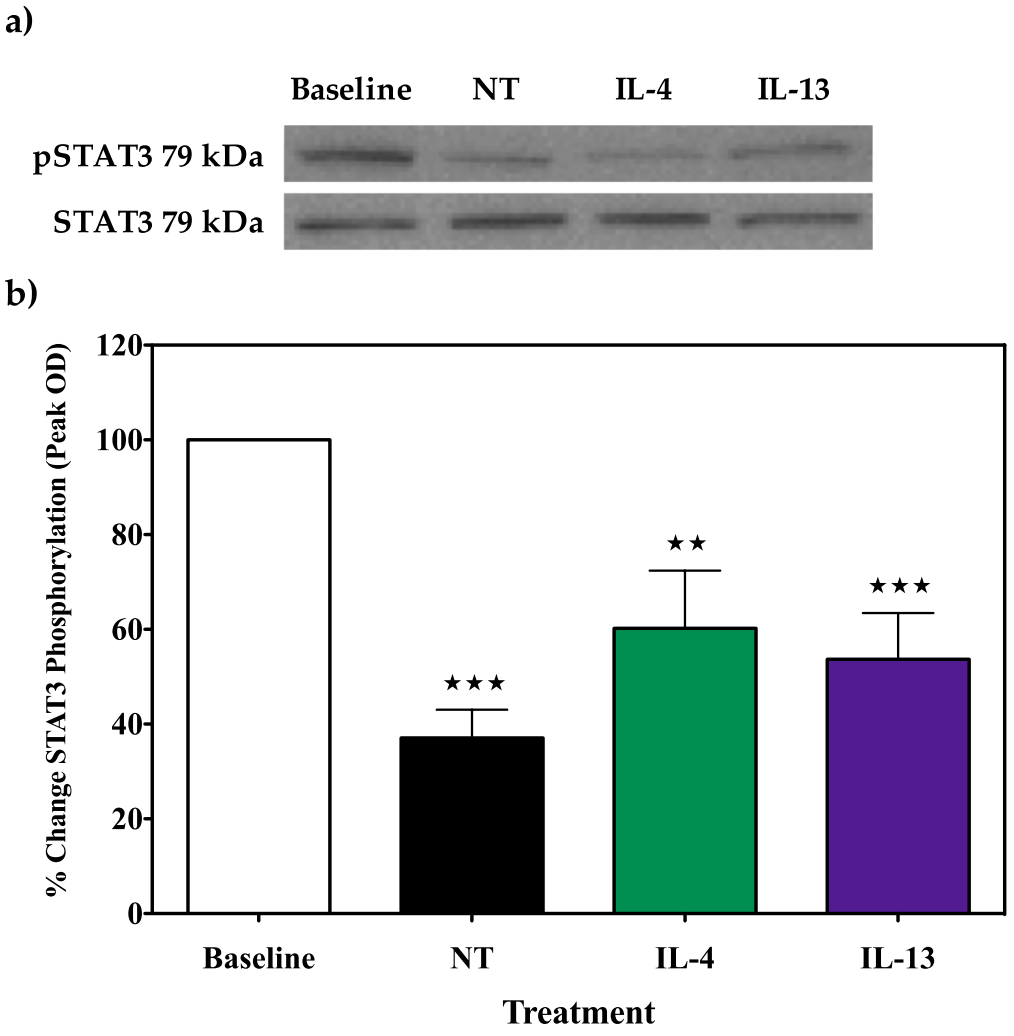

Figure 5.3: STAT3 phosphorylation by bronchial fibroblasts. Phosphorylated STAT3 was detected in western blots of lysates from fibroblasts serum starved for 24 hours and either lysed immediately (baseline) using Sample Buffer, or the media was exchanged for 1 hour in the absence (T60) or presence of 10 ng/ml IL-4 or IL-13. Data were normalised to the pan STAT3 levels as a loading control, quantified by densitometry and expressed as percentage change in peak optical density (OD). $n = 10$ subjects +/− SEM. $\star\star$ = $p < 0.01$ $\star\star\star$ = $p < 0.001$.

The experiments performed in Chapter 3 demonstrated substantial differential expression of IL-13Rα2 on the surface of bronchial fibroblasts obtained from different subjects, with often only relatively low expression levels on the surface of the cells at baseline (Figure 3.5). To study the link between IL-13Rα2 and STAT3, subjects naturally expressing higher levels of IL-13Rα2 were selected and pretreated with IL-4 or IL-13 for 24 hours to further increase the surface expression of this receptor. The cells were exposed to a 3 hour washout period to remove any traces of the pretreatment before challenge in the absence (T60) or presence of IL-4 or IL-13 for one hour and analysed for changes in STAT3 phosphorylation by western blotting. In this instance STAT6 phosphorylation was also analysed as an indirect measure of the increase in the levels of IL-13Rα2. [165]

As found in previous experiments, high levels of STAT3 phosphorylation were observed at baseline, resulting in a significant decrease in activation in every instance when the media was exchanged (Figure 5.4*a*). When comparing the STAT3 phosphorylation levels between T60 and cells treated for 1 hour with either IL-4 or IL-13 there was again a trend towards an increase but this was once more not found to be significant.

In comparison, both IL-4 and 13 induced activation of STAT6 (Figure 5.4*c*) as anticipated, which was attenuated by the addition of the pretreatment step (Figure 5.4*d*), suggesting that the pretreatment resulted in increased expression of IL-13Rα2 as observed in Figure 5.1.

Whilst addition of the pretreatment with IL-4 or IL-13 for 24 hours appeared to increase surface expression of IL-13Rα2, it did not result in increased STAT3 activation and even appeared to attenuate the trend previously observed (Figure 5.4*b*).

Whilst neither of these initial experiments in HBFs demonstrated a significant increase in STAT3 phosphorylation, these studies into the potential role of IL-13Rα2 in STAT3 signalling were complicated by endogenous activation of STAT3 observed in the absence of any treatment (Figure 5.3 and Figure 5.4) in this model. This high background phosphorylation suggested an autocrine signalling molecule was activating STAT3 in these cells.

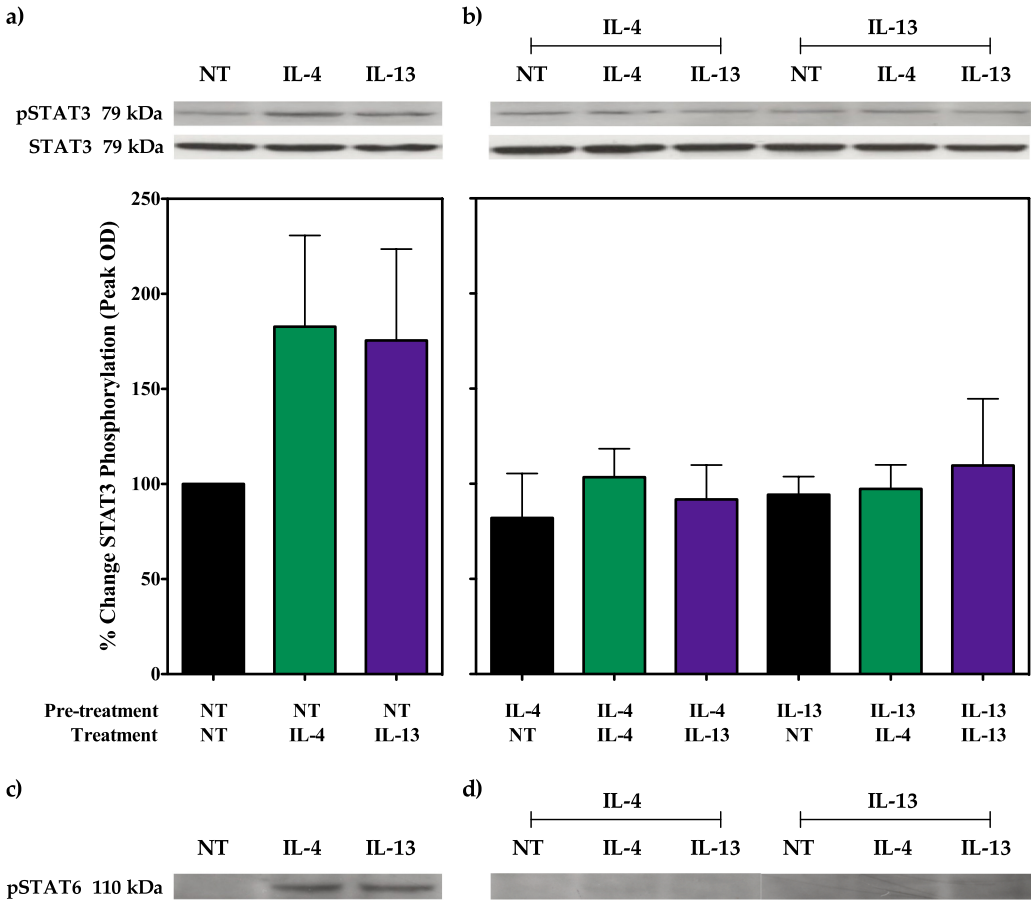

Figure 5.4: The effect of IL-4 or IL-13 pretreatment on STAT3 and STAT6 phosphorylation. Fibroblasts were seeded on 12 well plates and when ∼95 % confluent were serum starved with UltraCulture in the absence (NT) or presence of 10 ng/ml IL-4 or IL-13 (pretreatment). After 24 hours the media was exchanged for one hour with either 10 ng/ml IL-4, IL-13 or no treatment (T60). Cell lysates were obtained from solubilisation in Sample Buffer and analysed by Western blotting for STAT3 and STAT6 phosphorylation, which were normalised to a pan-STAT3 control by densitometry. Data is expressed as percentage change in peak optical density (OD). High baseline activation of STAT3 was observed, which showed a trend towards an increase in response to IL-4 or IL-13 (a), however when a the cells were pretreated with IL-4 or IL-13 this trend was no longer observed (b). In the absence of any pretreatment, IL-4 and IL-13 induced phosphorylation of STAT6 (c), which was attenuated by pretreatment with either cytokine (d). $n = 5$ subjects +/− SEM.

Therefore, it was necessary to determine what might be driving this STAT3 phosphorylation, to see if background activation could be removed, enabling us to determine whether IL-4 or IL-13 affect STAT3 phosphorylation.

Previous studies have shown that the IL-6 signalling pathway activates STAT3.[319] IL-6 is a key pro-inflammatory cytokine associated with airway remodelling in asthma and is dysregulated in interstitial lung disease, where it results in the associated downstream STAT3-mediated responses becoming pro-survival, rather than pro-apoptotic as seen in healthy peripheral lung fibroblasts.[58] Therefore, to determine if IL-6 could be the cause of this basal activation of STAT3 it was necessary to determine if HBFs from both healthy and asthmatic subjects endogenously produce IL-6, and if variation exists between patient groups.

HBFs from healthy and asthmatic subjects seeded at 5×10^4 cells per well on 12 well plates, and when ∼95 % confluent were serum starved for 24 hours before collection of conditioned media. Levels of IL-6 production by HBFs from healthy and asthmatic subjects were analysed by ELISA.

Although no difference was found between HBFs from healthy and asthmatic subjects, in the absence of any external stimuli high basal expression of IL-6 was observed after 24 hours (Figure 5.5), which could account for the basal STAT3 phosphorylation. However, to establish a causal relationship between endogenous IL-6 and STAT3 phosphorylation, augmented activation of STAT3 would need to be demonstrated by IL-6 treatment and endogenous STAT3 phosphorylation would need to be knocked down with the use of a specific inhibitor against IL-6.

To this end, an experiment to demonstrate STAT3 phosphorylation by IL-6 was undertaken. HBFs grown from healthy or asthmatic subjects were grown on 12 well plates (seeded at 5×10^4 cells per well) and once ∼95 % confluent were treated either in the absence (T60) or presence of IL-6 at 1 and 10 ng/ml for 1 hour. STAT3 phosphorylation was compared to basal STAT3 activation (baseline) by western blotting of cell lysates. Protein bands were normalised to pan-STAT3 to act as a loading control.

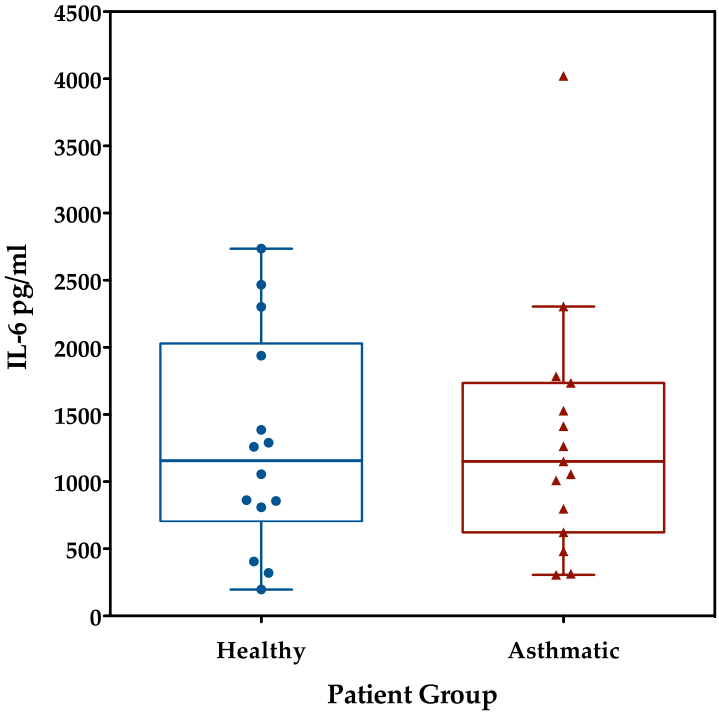

Figure 5.5: Basal IL-6 production by fibroblasts from healthy and asthmatic patients. To determine whether HBFs derived from asthmatics differentially express IL-6, levels of this cytokine from unstimulated HBFs derived from 14 healthy and 15 asthmatic subjects were measured in conditioned media by ELISA. Data are presented as median with standard tukey whiskers.

STAT3 activation due to IL-6 was shown to behave in a dose-dependent manner (Figure 5.6). Treatment with 10 ng/ml IL-6 was sufficient to induce STAT3 phosphorylation to a greater extent than observed at baseline after the initial 24 hour serum starvation period, whilst 1 ng/ml IL-6 instigated STAT3 phosphorylation to approximately 87 % of the baseline level (Figure 5.6).

A further experiment was subsequently undertaken to confirm that IL-6 was responsible for this observed baseline activation of STAT3. A neutralising antibody which blocks the IL-6R (IL-6R NAb), and therefore prevents IL-6 signalling, was added as indicated in Figure 5.7 to confluent fibroblasts from healthy and asthmatic subjects.

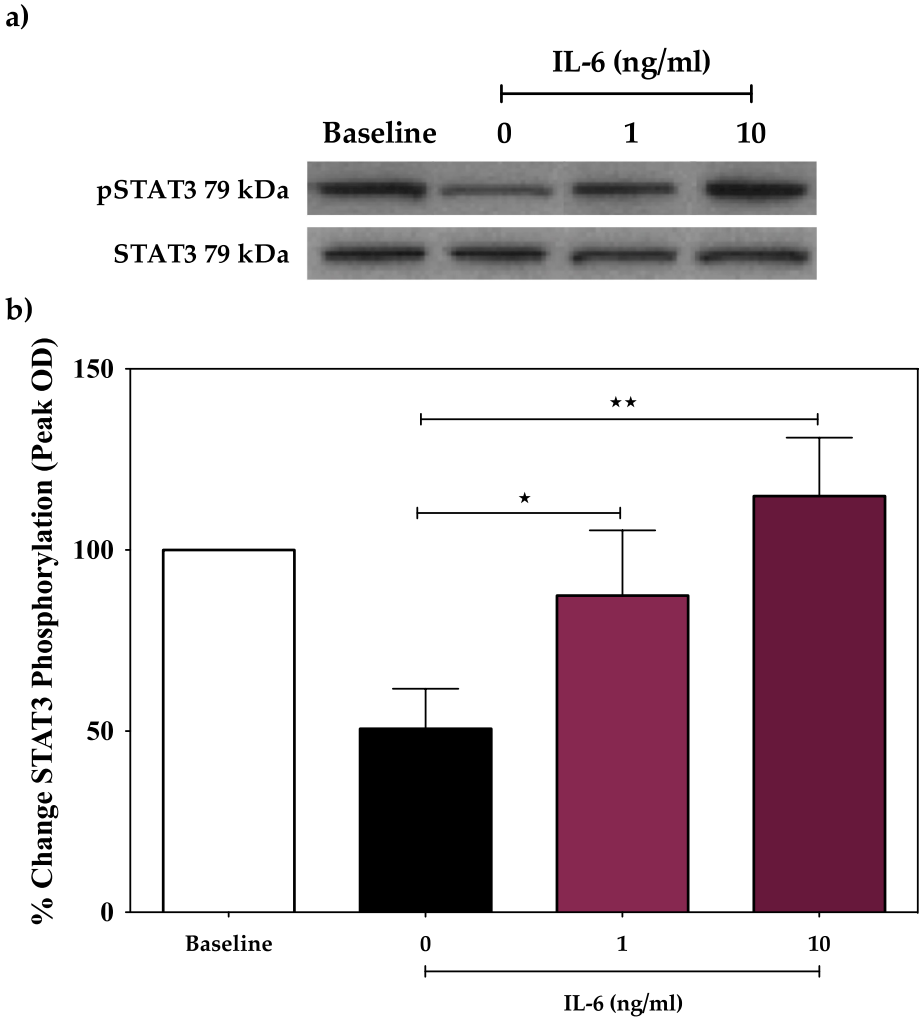

Figure 5.6: STAT3 Phosphorylation by IL-6. Confluent HBFs were serum starved overnight and harvested directly (baseline) or the media changed and incubated for a further hour in the absence (T60) or presence of IL-6. Cells were solubilised in sample buffer before western blotting and probing with a phospho-STAT3 antibody. Bands were analysed by densitometry (BioRad) and normalised for loading using a pan STAT3 antibody. Data are mean +/- SEM. $n = 6$.

Addition of the IL-6R NAb resulted in the attenuation of the baseline STAT3 activation by between 50 and 64 % respectively at the lowest and highest doses used, with all doses found to cause a statistically significant

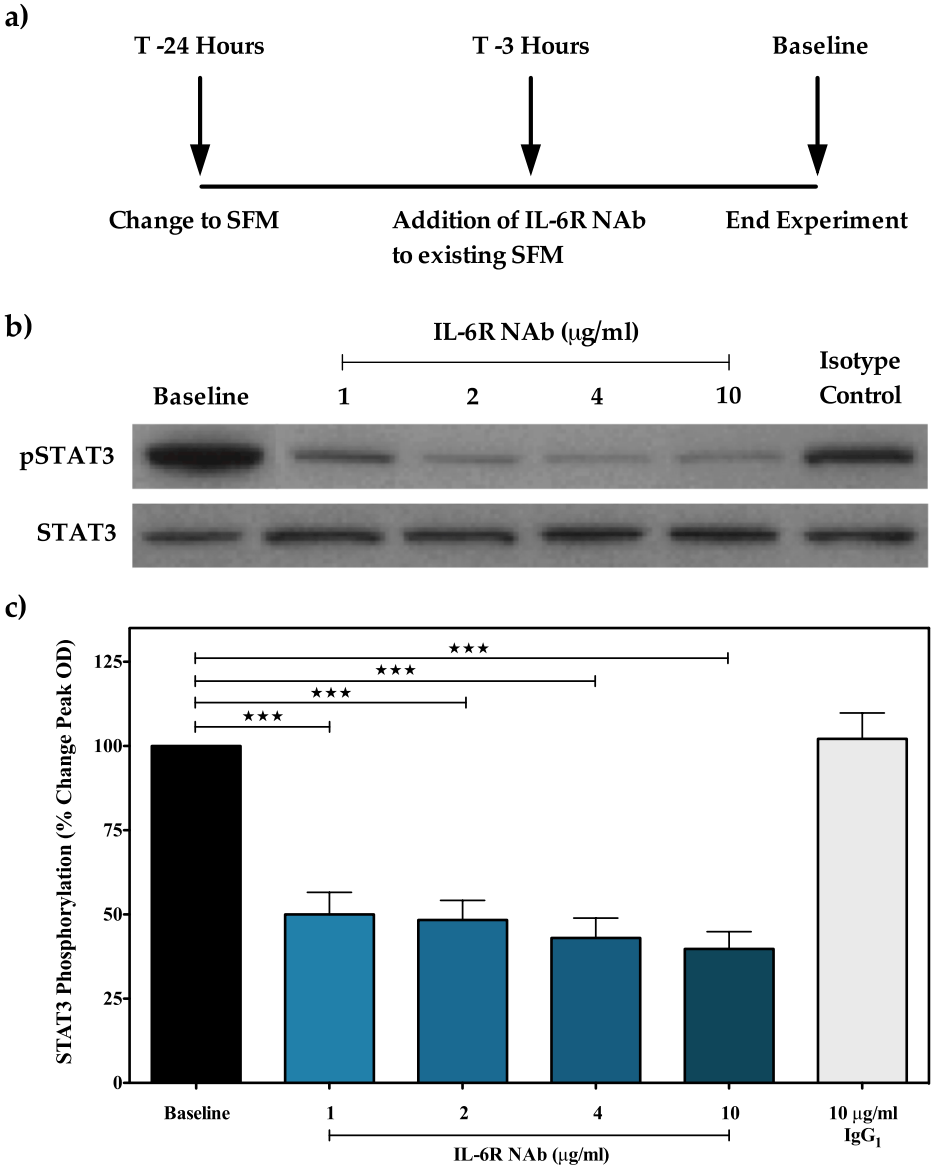

Figure 5.7: The effect of IL-6R neutralising antibody on STAT3 phosphorylation. To assess whether basal STAT3 phosphorylation was due to endogenous IL-6, HBFs were treated as described in the schematic diagram and STAT3 phosphorylation was measured by western blotting (normalised to pan-STAT3 for loading). In the presence of IL-6R NAb, STAT3 phosphorylation was significantly reduced. Data are mean +/− SEM. $n = 6$ healthy and 8 asthmatic subjects.

reduction in STAT3 phosphorylation ($p < 0.001$ for every dose of IL-6R NAb) (Figure 5.7).

As this IL-6R NAb was able to reduce the background phosphorylation, the initial experiment studying the effects of IL-4 and IL-13 on STAT3 activation in HBFs (Figure 5.3) was repeated in the presence or absence of the neutralising antibody as indicated (Figure 5.8*a*).

As before, in the absence of the IL-6R NAb the cells demonstrated relatively high levels of basal activation of STAT3, which did not significantly increase with treatment (Figure 5.8*b*). When the IL-6R NAb was included however, the basal phosphorylation was reduced and a significant increase in activation of STAT3 was then observed in response to IL-4 ($p < 0.01$). No significant change was seen in response to IL-13 treatment (Figure 5.8*c*).

Although addition of the IL-6R NAb highlighted a difference in phosphorylation between IL-4 and IL-13 treatment, the question remains that if high basal activation of this transcription factor is observed does a small increase in phosphorylation have an effect? Whilst it is possible that it simply requires a threshold be reached before activating further gene transcription, the fact that the cells were producing high levels of IL-6 in the absence of any stimulus it was considered that actually the cells may be in a more activated state than would normally be observed *in vivo*.

Matrigel is a basement membrane matrix which is used to better emulate the natural environment for fibroblasts within a tissue culture setting, and can therefore be used to establish whether this high basal IL-6 production is an artefact of standard tissue culture. This gel contains solubilised extracellular matrix proteins, such as collagen IV, laminin and proteoglycans, as well as various growth factors, and can be used either as a coating on which the cells grow or a thick gel with cells embedded within.

Initial experiments were performed to characterise the effects of growing fibroblasts either on (2D culture) or within (3D culture) Matrigel, in comparison to tissue-culture coated plastic. HBFs were serum starved for 24 hours prior to analysis of basal IL-6 production by ELISA of conditioned media.

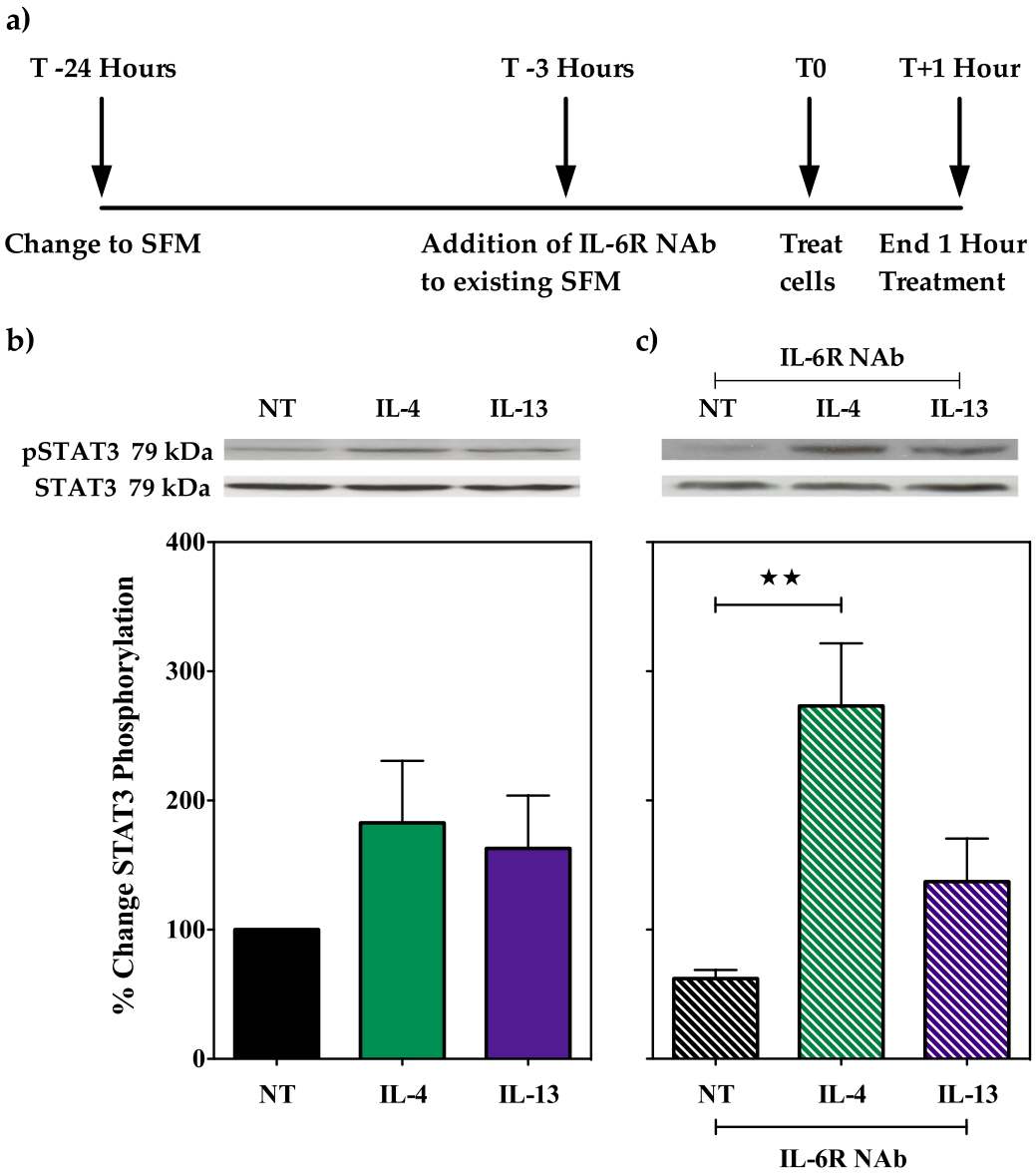

Figure 5.8: The effect of an IL-6R NAb on IL-4 and IL-13–mediated STAT3 phosphorylation. Fibroblasts were pretreated with or without an IL-6R neutralising antibody (NAb) before stimulation with 10 ng/ml IL-4 or IL-13 for 1 hour as described in the schematic diagram (a). Phosphorylation of STAT3 was determined by western blotting and normalised to total STAT3 protein. Data are mean +/− SEM. $n = 14$

As found in Figure 5.5, at baseline the HBFs produced approximately 1000 pg/ml IL-6 over a 24 hour period (Figure 5.9), however the cells grown either in 2D culture on Matrigel or 3D culture embedded within Matrigel produced substantially less IL-6 than those on tissue-culture plastic. This decrease was significant when the cells were grown in 3D culture ($p < 0.05$). In the 2D Matrigel system, only a trend towards significance was observed, which may be due to the limited number of subjects studied in this preliminary experiment.

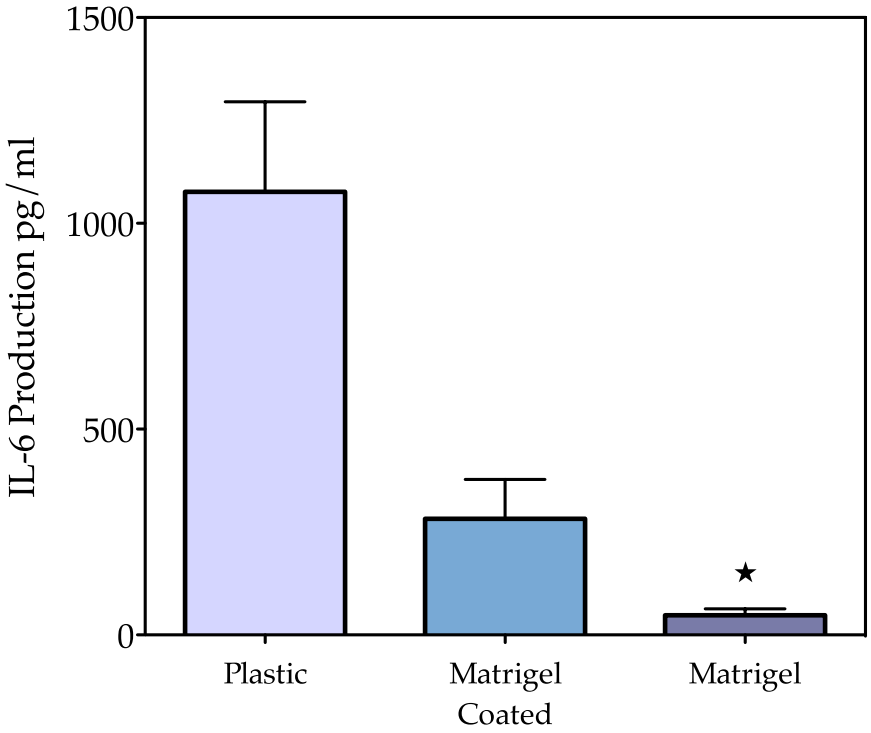

Figure 5.9: Basal IL-6 production by fibroblasts in various culture conditions. HBFs were grown either directly on tissue-culture plastic, on a well coated with Matrigel or embedded within Matrigel. The cells were serum starved for 24 hours before analysis of IL-6 production by ELISA of the conditioned media. Data are mean +/− SEM. $n = 3$ healthy subjects.

The smaller reduction in IL-6 production observed with the cells grown on Matrigel could also be related to the experimental design. One of the issues found with the 2D Matrigel culture was the tendency of uneven coating of

the well by the gel, therefore some of the cells may still have been growing either partially or fully on tissue-culture plastic.

The 3D culture of cells within Matrigel also provides complications as, to date, no reliable method for extracting cells from the gel at the end of the experiment has been established. Matrigel is a thermo-responsive matrix, which forms a gel at 37°C but liquifies at colder temperatures (∼4°C). Therefore, it was considered that cooling the matrix could allow recovery of the cells. Preliminary experiments were undertaken where cells were grown in Matrigel or on tissue-culture plastic and treated for 1 hour with IL-4, IL-13 or fresh media. At the end of the experiment, ice cold PBS containing phosphatase and protease inhibitors was added to the cells to liquify the Matrigel. The liquid Matrigel:PBS mix was transferred to a fresh tube and stored on ice and this process was repeated until all of the Matrigel had been collected. The cell:Matrigel:PBS solution was centrifuged at 300 g for 5 minutes to pellet the cells, before the addition of sample buffer.

To confirm the presence of protein in samples obtained from each compartment an SDS-PAGE gel was run. Fairly consistent recovery of protein was observed between the tissue-culture plastic and Matrigel-extracted samples (Figure 5.10), although there was more higher molecular weight protein present in the latter. Presumably this protein is due to carry-over of the matrix proteins, although no cell counting experiments have been performed at this stage to ascertain if more cells were recovered in these samples.

Subsequently these samples were analysed for levels of STAT3 activation by western blotting. When the cells were grown on tissue-culture plastic basal phosphorylation of STAT3 was observed in both the presence and absence of IL-4 and IL-13 (Figure 5.11). However, this endogenous activation of STAT3 was markedly reduced in the cells which were embedded within the Matrigel. As with the cells grown on tissue-culture plastic, no difference was observed with treatment, however this is possibly due to the inability of the cytokine to penetrate into the gel, and therefore the mechanics of this would need to be considered for future experiments. To overcome this, the cells may need to be challenged for longer to allow the cytokine to penetrate.

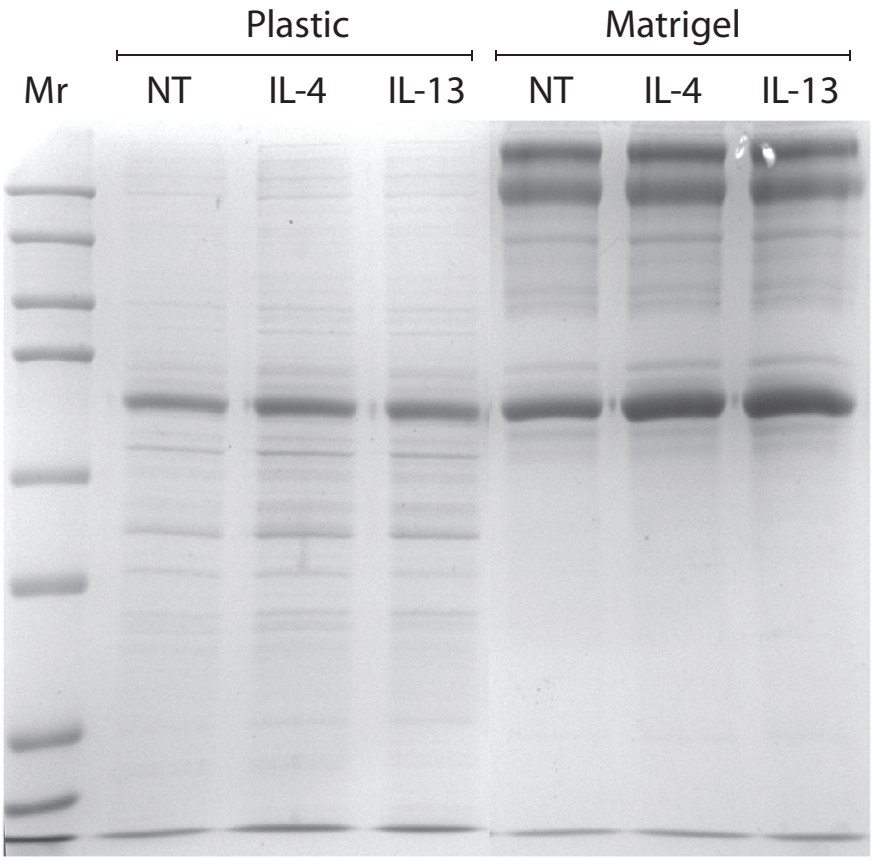

Figure 5.10: Protein extracted from fibroblasts grown within Matrigel. Fibroblasts were grown either directly on tissue-culture plastic or embedded within Matrigel. The cells were serum starved for 24 hours before treatment with IL-4, IL-13 or no treatment (NT) for 1 hour. The cells grown on plastic were lysed by direct addition of sample buffer. The Matrigel-embedded cells were recovered by addition of chilled PBS before pelleting and lysing the cells in sample buffer. Protein content was visualised by Coomassie blue stain. Mr = molecular weight marker. Representative blot of $n = 3$ healthy subjects.

Alternatively, the cells could be grown embedded within Matrigel and then extracted immediately prior to stimulation with IL-4 and IL-13, however the cooling of the cells to extract them from the matrix could induce a response.

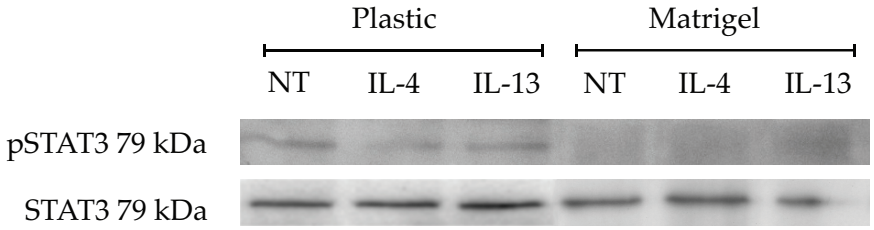

Figure 5.11: STAT3 phosphoylation by fibroblasts in various culture conditions. To assess whether basal STAT3 phosphorylation was observed in different culture conditions, fibroblasts were grown either directly on tissue-culture plastic or embedded within Matrigel. The cells were serum starved for 24 hours before media exchange in the presence of IL-4, IL-13 or no treatment (NT) for 1 hour. STAT3 phosphorylation was measured by western blotting (normalised to pan-STAT3 for loading). Representative blot of $n = 3$ healthy subjects.

5.3 Discussion

As IL-13Rα2, which has been classically considered to act as a "decoy receptor", has recently been suggested to be able to initiate an intracellular signal [249, 280] this chapter focussed on attempting to elicit whether IL-13Rα2 can mediate STAT3 phosphorylation via IL-4.

In Chapter 4 attempts were made to develop a knockdown model of IL-13Rα2. As these experiments using siRNA provided an unsatisfactory model alternatives were sought. Another method of studying IL-13Rα2 is via an overexpression model. As there was already such a model established within our laboratories, initial experiments focused on validating this, and demonstrated not only high levels of IL-13Rα2 on the surface of the transfected cells, but also that these cells behaved as expected, with reduced responsiveness to both IL-4 and IL-13.

The overexpression model of IL-13Rα2 validated in Section 5.2.1 was subsequently used to investigate the role of IL-13Rα2 in STAT3 signalling to determine whether IL-4 could mediate a STAT3 signal and establish if this required IL-13Rα2 on the cell surface.

Initial experiments demonstrated a significant increase in STAT3 phosphorylation in the B2B-FL cells, which had been transfected with full length IL-13Rα2. This was not observed in the B2B cells, which only contained a mock sequence, suggesting that IL-13Rα2 is able to induce an IL-4-mediated STAT3 signal in these cells.

Whilst these preliminary experiments showed similar results to that which has been observed in glioblastoma by Rahaman *et al.*[249,280] these were performed in an epithelial cell line. This is not an ideal model as in Chapter 3 fibroblasts were shown to behave differently to epithelial cells with regards to IL-13Rα2, however previous attempts by Dr Andrews to develop this overexpression model in fibroblast cell lines have been unsuccessful.

Initial attempts to determine if IL-13Rα2 allows IL-4 signalling through STAT3 activation in primary human bronchial fibroblasts were complicated by the constitutive STAT3 phosphorylation by HBFs cultured for 24 hours in serum free medium, with the removal of the conditioned medium leading to a reduction in STAT3 phosphorylation, regardless of whether IL-4 or IL-13 treatment was included.

No significant increase in STAT3 phosphorylation was observed in response to either IL-4 and IL-13 compared with 1 hour of serum free medium. This was even the case when the cells were pretreated with either cytokine to upregulate the levels of IL-13Rα2, although this experiment may have been complicated by also upregulating other genes such as SOCS proteins. Therefore subsequent experiments set out to establish what caused the basal STAT3 phosphorylation with the aim of minimising this artefact.

The reduction observed after exchanging the media suggested the production of an autocrine mediator by the cells, released over a 24 hour period, which constitutively activated STAT3. However, this mediator appeared to be removed when the media was exchanged for one hour as the STAT3 phosphorylation decreased, regardless of whether IL-4 or IL-13 were present. Therefore it was necessary to determine what this mediator was and what was causing its production.

As previous work has demonstrated that STAT3 is phosphorylated by IL-6, experiments were performed to establish whether HBFs from healthy and asthmatic patients release IL-6 at baseline. Substantial constitutive release of IL-6 was observed in both patient groups in the absence of any treatment, with no significant difference between HBFs obtained from healthy or asthmatic subjects.

This phenomenon has previously been observed by Gomes, Mathur *et al.* who demonstrated that IL-6 is endogenously secreted by a lung-derived fibroblast cell line in their studies investigating the effects of eosinophil-fibroblast co-cultures. [320]

Whilst no difference in basal IL-6 production was observed in this experimental setting between healthy and asthmatic subjects, the cytokine milieux within the lungs of subjects with and without asthma will vary greatly. For instance, there is reported to be higher levels of TGFβ present in the asthmatic lung. [135,321] TGFβ is a pro-fibrotic mediator associated with airway remodelling, that is produced by fibroblasts, myofibroblasts, epithelial and inflammatory cells in excess in asthma patients, in comparison to healthy controls. [100,135,321–323] In the study by Gomes, Mathur *et al.* they observed that the level of IL-6 produced by fibroblasts is augmented with TGFβ treatment. [320] I have also undertaken preliminary experiments and found that treatment of fibroblasts obtained from asthmatics but not healthy control subjects with TGFβ results in a substantial increase in the production of IL-6 (data not shown), suggesting there may be differences between the asthmatic and healthy lung not readily demonstrated by the experiments presented herein. Further work, however, would be required to fully characterise these disease-related differences.

Subsequent studies investigating the effects of adding IL-6 to the cells found that 10 ng/ml IL-6 significantly increased STAT3 phosphorylation, as expected based on previous reports in various cell types. [319] However, this was not sufficient to confirm that IL-6 was the only cause of this endogenous STAT3 phosphorylation so an IL-6R NAb was added, which resulted in attenuation of STAT3 phosphorylation in a dose dependent manner. In this

study four different concentrations of the IL-6R NAb were used, the highest of which resulted in a ∼64 % reduction in STAT3 phosphorylation. Whilst this did not provide complete inhibition of the endogenous STAT3 activation and addition of more antibody may have resulted in a greater reduction, the scale of this diminution of phosphorylation compared with the cost was not deemed viable.

Therefore, this dose was used in subsequent experiments to block the endogenous STAT3 phosphorylation within these cells and it was found that treatment of human bronchial fibroblasts with IL-4 in the presence of this IL-6R NAb resulted in a significant increase in STAT3 phosphorylation. Interestingly, as with the B2B-FL cells, a smaller but non-significant increase was also observed with IL-13 treatment. It is unclear whether this increase is sufficient to affect downstream signalling and gene transcription.

There are several potential explanations for the differences observed here between the IL-4 and IL-13 treatment. One explanation could simply be that as IL-13 is able to bind to IL-13Rα2 with high affinity that it may just be sequestered by this receptor, reducing the availability for signalling. As IL-4 is unable to bind to IL-13Rα2 directly this sequestration would not occur for this cytokine. Alternatively if IL-4Rα is also required for the activation of a STAT3 signal then the differing binding affinities of IL-4 and IL-13 for IL-4Rα may come into play.

Whilst in an overexpression system of IL-13Rα2 STAT3 phosphorylation increased in response to challenge with IL-4, further work is required to confirm if this is mediated by IL-13Rα2 in bronchial fibroblasts. Ideally this would have been confirmed using an IL-13Rα2 knockout system in primary human bronchial fibroblasts to see if this abrogated the STAT3 phosphorylation observed in response to IL-4 treatment, however due to complications with the siRNA model this was not possible. In the absence of this, an IL-13Rα2 NAb, which has previously been shown to have an effect on anti-inflammatory responses to IL-4 and IL-13, could be used to determine if this IL-4-induced phosphorylation of STAT3 is mediated by IL-13Rα2.

Based on the assumption that this IL-4 induced STAT3 signal is IL-13Rα2 mediated the obvious question is what is the relevance of this phosphorylation *in vivo*? Given the basal activation of STAT3 due to the endogenous production of IL-6 observed in the early experiments it could have been assumed that any changes in STAT3 phosphorylation would not be biologically significant. However, the basal IL-6 production appears to be an artefact from the *in vitro* culture of these fibroblasts and although they were serum starved prior to treatment they did not become completely quiescent. One method to further quiesce the cells is by growing the cells in Matrigel, which better mimics the *in vivo* environment where fibroblasts are not in an activated state. Preliminary experiments found that under these conditions the basal production of IL-6 by fibroblasts is substantially reduced in comparison to fibroblasts grown on tissue culture plastic. Therefore, one might hypothesise that within their normal environment, where the cells are not producing substantial amounts of IL-6, and any that is produced does not persist in the same manner as in a tissue culture well, much smaller increases in STAT3 are likely to be observed and that these will have an effect on the cells.

This experiment was expanded to analyse the STAT3 phosphorylation in these different culture conditions. The retrieval of cells from Matrigel has not been well characterised, therefore in this pilot experiment the gel was cooled with ice-cold PBS in order to liquify the matrix. The efficacy of this method was confirmed by running an SDS-PAGE gel, which was stained with coomassie brilliant blue dye, before undertaking western blotting on these samples.

In the cells obtained from within the Matrigel, a reduction in basal phosphorylation of STAT3 was observed, again suggesting that this endogenous activation is likely to be an experimental artefact. However, the treatment with either IL-4 or IL-13 failed to have an effect on these cells. This treatment was added after the gel had already formed, and thus it is not clear whether the cytokine was able to enter this matrix. Therefore, an essential next step is to establish a method whereby cells can be stimulated with a cytokine in this system. It may be that this is not possible, in which case the

Matrigel coating may be the best available alternative. If IL-4-mediated STAT3 phosphorylation was found in this system, this experiment could then be expanded with studies of gene activation to determine the effects of this STAT3-activation.

Another technical issue discovered during this experiment was the fact that upon removal of the Matrigel, a layer of cells was also found attached to the base of the well. Although all of the cells were seeded together with the Matrigel, some of these fibroblasts migrated to the tissue-culture plastic underneath. In future experiments, to prevent this from occurring, the base of the well could be lined with a reagent such as agar or gelatin prior to addition of the Matrigel:cells solution.

These Matrigel experiments have highlighted one of the issues of *in vitro* experiments. Whilst this system allows us to investigate the effects of specific cytokines in simplified systems, the effects of the culture conditioned must also be considered. In this instance, the high basal production of IL-6 by the fibroblasts grown on tissue-culture plastic, which was lost when the cells were embedded within Matrigel and therefore quiesced, suggests that the fibroblasts are receiving an activation signal by the tissue-culture plastic. Similar responses have been observed with hepatic stellate cells, which become myofibroblastic on tissue-culture plastic but quiescent in Matrigel. [324] This has implications in not only these specific experiments, but also the use of tissue-culture plastic as a growth support in fibroblast experiments. Whilst collagen coating is also regularly used, experiments performed by Dr Andrews found that similar levels of IL-6 were produced both at baseline and after 24 hours serum starvation on collagen-coated and uncoated wells. This suggests that a better model for fibroblast-based *in vitro* experiments might be to use the Matrigel system. However, before this can be done routinely the technical methodology would need to be improved to allow the current range of experiments to be undertaken. In particular, methods of treating cells within Matrigel, and extraction of cells embedded within this matrix would need to be established.

Previous work has suggested that the STAT3 mediated responses differ in interstitial lung disease patients compared with healthy subjects. [58] Here Moodley, Misso *et al.* demonstrate a switch to a pro-survival phenotype resulting in fibrosis. [58] In idiopathic pulmonary fibrosis these differences have been attributed to IL-6 and in particular a shift in IL-6 signalling from pro-apoptotic, as observed in healthy controls, to pro-survival has been observed. [58] However, if IL-13Rα2 is able to instigate a STAT3 signal via IL-4 it would be of great interest to determine whether this affects cell proliferation in the asthmatic lung where remodelling in the form of fibrosis is also observed.

It could be hypothesised that in healthy patients STAT3 prevents proliferation, and that this is dysregulated in asthma, which may contribute to the subepithelial fibrosis observed in asthmatic lung remodelling. This is another interesting area for future work.

Several studies have also highlighted the role of STAT3 in the activation of angiogenesis in cancer, however to date this has not been studied in asthma. [325] As angiogenesis is often observed in asthmatic airway remodelling the potential involvement of both IL-4 and STAT3 in this angiogenesis would need to be investigated further.

As IL-6-mediated activation of STAT3 has also been found to elicit both pro- and anti-inflammatory effects it would also be of interest to investigate this further. IL-4 is involved in Th2-mediated inflammation, which is often considered a hallmark of asthma. Therefore, if IL-4 is able to mediate an erroneous STAT3 signal via IL-13Rα2 this could impact the chronic inflammation observed in asthma. [314]

5.4 Summary

In summary, an overexpression model of IL-13Rα2 demonstrated the ability of IL-4 to induce STAT3 phosphorylation and that this requires IL-13Rα2. Initial experiments to confirm this in primary human bronchial fibroblasts

were complicated by the presence of endogenous IL-6 production, but when this was neutralised, IL-4-mediated activation of STAT3 was observed. Further experiments using a neutralising antibody against IL-13Rα2 would be required to confirm this is IL-13Rα2-mediated.

Whilst no difference was found between healthy and asthmatic subjects, further work is required to understand the signalling consequences of this IL-4-mediated STAT3 activation. Initially this would involve confirmation of nuclear localisation of the phosphorylated STAT3 by immunostaining, followed by analysis of STAT3-induced gene expression using a focussed micro-array.

Chapter 6

Final Discussion

6.1 Overview

Both IL-4 and IL-13 can be regulated by IL-13Rα2, however very little is known about this receptor. In particular the mechanism by which IL-13Rα2 can attenuate the effects of IL-4, when the two are unable to bind, and the importance of the other IL-4 and IL-13 receptors in the regulation of these cytokines has been uncertain.

The key findings of this project are that long-term upregulation of IL-13Rα2 surface expression occurs in response to stimulation by a range of both Th2 and Th1 mediators, including IL-4, IL-13 and IFNβ. In fibroblasts this requires *de novo* protein synthesis as these cells do not contain intracellular stores of this receptor, a phenomenon previously observed in epithelial cells. Whilst a transient reduction in IL-4Rα was found in response to IL-4 or IL-13, thereby reducing the ability to bind ligand and transduce a signal, it is the IL-13Rα2 subunit that appears to be responsible for the longer-term regulation of these two, key Th2 cytokines.

Investigations into the mechanism by which IL-13Rα2 can regulate IL-4 in the absence of their direct association identified that IL-4 can induce a STAT3 signal in the presence of IL-13Rα2, although further work is required to establish the signalling consequences of this phosphorylation on gene expression and cell function.

6.2 Regulation of IL-4 & IL-13 Receptor Surface Expression

The initial aim of this project was to characterise the expression and regulation of the different IL-4 and IL-13 receptors, and determine their roles in the variation in responsiveness observed between subjects to IL-4 and IL-13. To this end, in Chapter 3 IL-13Rα2, IL-4Rα, IL-13Rα1 and γc were systematically examined from gene to surface expression in HBFs from a range of healthy and asthmatic subjects.

The Interleukin 4 and 13 receptor system appears to be dynamic, with surface expression of IL-4Rα rapidly depleting in response to stimulation by either ligand, whilst IL-13Rα2 surface expression increased much more slowly and appears to be less dynamic once it reaches the cell surface. Whilst the internalisation of the IL-4Rα receptor may help to regulate IL-4 and IL-13 in the short term, it appears that IL-13Rα2 is important for the longer term regulation of these Th2 cytokines.

Interestingly though, the regulation of IL-13Rα2 does not appear to occur simply in response to IL-4 and IL-13. Instead there appears to be much broader regulation by other cytokines. Previously, upregulation of surface IL-13Rα2 from intracellular stores has been observed in response to IFNγ in epithelial cells.[251] In this project this work has been expanded and it was found that not only IFNγ, but also IFNβ and dsRNA are able to upregulate IL-13Rα2 surface expression on human bronchial fibroblasts, and that this occurs despite the lack of intracellular pools in these cells. Instead, in these cells addition of any one of these stimuli results in *de novo* protein synthesis.

In epithelial cells, where IL-13Rα2 can be upregulated from intracellular pools, an increase in expression is observed hours before that of fibroblasts, which require *de novo* synthesis. This slower upregulation in fibroblasts may be part of the longer-term modulation of these cytokines, with a reduction in IL-4Rα regulating short term effects.

The internalisation of IL-4Rα in this system appears to be transient, with restoration of surface expression occurring shortly after the ligand is removed, suggesting that this receptor is not being degraded. This internalisation of IL-4Rα in response to ligand binding is a common phenomenon observed with receptors to prevent overstimulation. [326,327] In the IL-2 receptor system, which shares a common γc subunit with the type I IL-4 receptor, binding of IL-2 leads to the endocytosis of IL-2Rβ:γc, which is then targeted for degradation. [328] With the IL-4 receptor system neither IL-4Rα not IL-4 appear to be broken down after internalisation. [297] Both IL-2Rβ and γc contain sequence elements in their cytoplasmic domains which target these receptor subunits for degradation. [329] No such sequences, however, have been found on IL-4Rα. [330–333]

IL-4Rα internalisation also does not appear to be necessary for induction of an IL-4 or IL-13 signal, [297] and therefore would be assumed to play a role in the regulation of these ligands by reducing the amount of the receptor available on the surface. In this study the regulation of IL-4 and IL-13 was studied after 24 hours where addition of an IL-13Rα2 NAb restored responsiveness to IL-4 and IL-13, as measured by eotaxin production. However, whilst this longer-term regulation appears to be dependent on IL-13Rα2, it is likely that IL-4Rα internalisation is responsible for any short-term regulation of responses to these cytokines.

The ability of fibroblasts to upregulate IL-13Rα2 in response to not only Th2, but also Th1 cytokines and stimuli, and subsequently regulate Th2 responses suggests this receptor is important in the Th1/Th2 balance. However, whilst the role of IL-13Rα2 in regulating inflammatory gene expression has been demonstrated through suppression of both eotaxin and IL-6 production, it is not clear whether this receptor is also involved in upregulating the expression of other genes, or if this alternative gene activation is important for the regulatory mechanism of IL-13Rα2.

6.3 Can IL-13Rα2 Instigate a Signal?

One of the main questions regarding IL-13Rα2 has been how does IL-13Rα2 regulate IL-4, despite being unable to bind directly to it? Whilst IL-13 responsiveness could be regulated by IL-13Rα2-mediated sequestration of the ligand, in recent years several groups have proposed that in fact both IL-4 and IL-13 may induce a signal via IL-13Rα2, which may be involved in this regulatory mechanism.

In Chapter 5, IL-4 was found to induce STAT3 phosphorylation in the presence of IL-13Rα2 in both an overexpression model and primary human bronchial fibroblasts. However, due to the time constraints of this project further interrogation of this pathway is required to understand the role of this STAT3 activation.

STAT3 is a transcription factor normally associated with anti-apoptotic, pro-proliferative actions, although this appears to elicit pleiotropic effects with the activation of both pro- and anti-inflammatory genes depending on the cell type. Whilst no disease-related differences were observed in the activation of STAT3, it is unclear whether greater activation of STAT3 would be found in asthmatic subjects compared with healthy controls *in vivo*, due to the increased levels of IL-6 in the asthmatic lung.[334] This endogenous IL-6 in the asthmatic lung could negate the requirement for an IL-4-mediated STAT3 signal, or this may act in an additive manner. However, STAT3 signalling has been found to exhibit pleiotropic, and often opposing effects, where sometimes different signalling consequences are observed depending on the disease state. One such example is found with IL-6-mediated STAT3 signalling, which in the healthy lung is pro-apoptotic, however in idiopathic pulmonary fibrosis (IPF) this switches to become pro-fibrotic.[58,335] As fibrosis and remodelling are often observed in the asthmatic lung, it could be that this is caused by an increase in anti-apoptotic, pro-fibrotic signals in response to STAT3 activation.

In the brain, the role of IL-4 appears to be linked to reducing proliferation and arresting growth.[268–273] However, in glioblastoma, the induction of

STAT3 activation by IL-4 via IL-13Rα2 results in increased transcription of anti-apoptotic genes, with IL-13Rα2 seeming to induce a survival signal in these cells.[249,280] In the lung, proliferation of fibroblasts is a hallmark of the remodelling observed in asthma. Therefore, it is hypothesised that the IL-4-mediated induction of STAT3 in fibroblasts might lead to anti-apoptotic effects in these cells by activation of the BCL2 family.

IL-13Rα2 has previously been found to regulate both IL-4 and IL-13, however the data presented in this thesis suggest that the regulation mechanism may be different for each of these cytokines, with IL-4 able to instigate a strong STAT3 signal, which was not observed with IL-13. With IL-13, on the other hand, it might be that IL-13Rα2 simply acts as a decoy receptor for this cytokine. Alternatively, as has been recently suggested, AP-1 may also be involved in at least part of the regulation of IL-13.

A variety of possible mechanisms have been proposed for the regulation of IL-4 and IL-13 by IL-13Rα2 (Figure 6.1), each of which will be discussed below.

IL-13Rα2 has previously been considered as a "decoy receptor" for IL-13 as it binds this ligand with high affinity (Figure 6.1*a*). The consequence of this may simply be sequestration of IL-13, thus reducing the amount of this cytokine available for signalling. Alternatively, binding of IL-13 to IL-13Rα2 may be sufficient to induce a signal. This latter option has been generally considered unlikely due to IL-13Rα2 having short intracellular tail, containing only 17 amino acid residues and lacking any obvious signalling motifs,[241] suggesting that another protein would be required to allow signalling to occur. However, it has been suggested recently that IL-13Rα2 may induce an intracellular signal in response to IL-13.[261–265] These studies have identified an alternative signalling molecule that may be involved, an AP-1 variant consisting of c-Jun and Fra-1, which activates the *TGFB1* promoter.[262,336] This appears to be specifically in response to IL-13, and not IL-4, which is unable to bind directly to this receptor. As IL-13Rα2 has a short intracellular tail and is not known to bind to IL-13Rα1, it is not currently clear how this activation might occur. The investigations by Fichtner Feigl *et al.* have

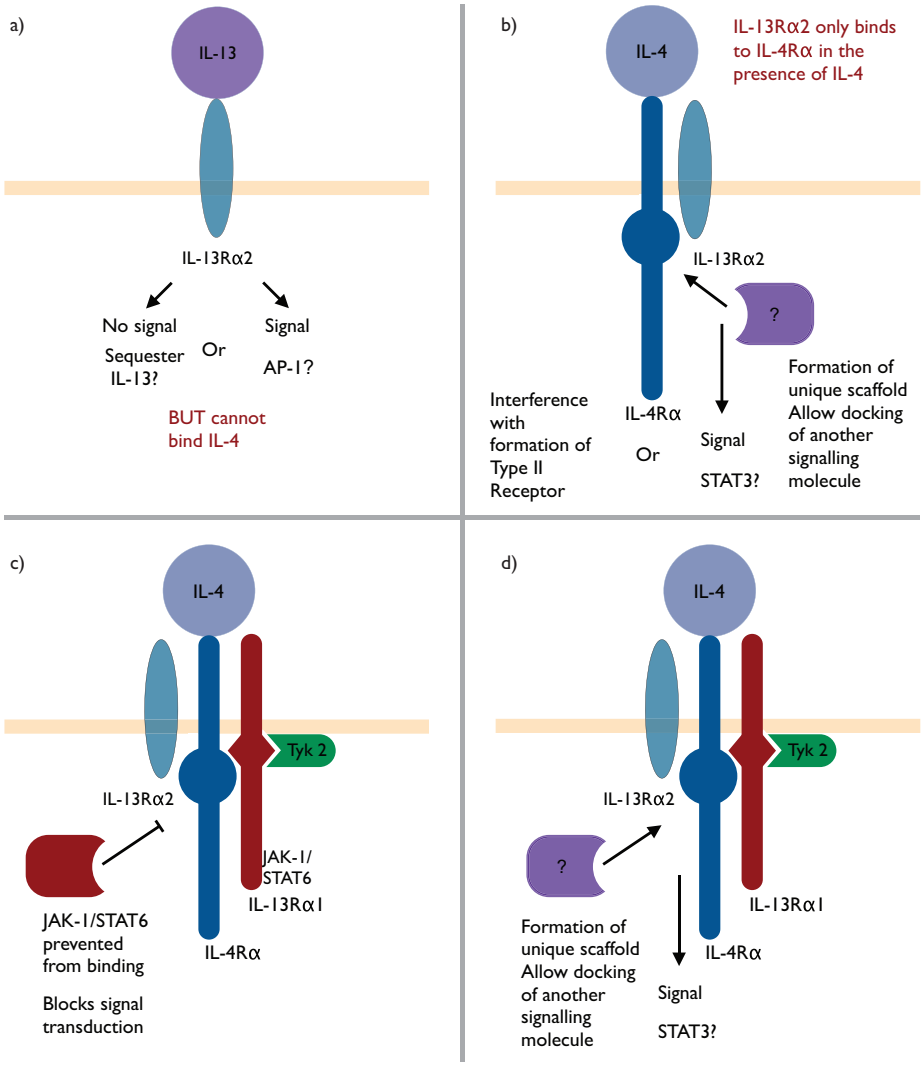

Figure 6.1: The possible mechanisms of IL-13Rα2 signalling. IL-13Rα2 may attenuate IL-4 and IL-13 in a variety of ways. a) Whilst previous studies have suggested that IL-13Rα2 can block IL-13 signalling by acting as a decoy this is an unlikely mechanism for controlling IL-4-mediated responses as it is unable to bind to this receptor.[165] IL-13Rα2 can, however, bind to IL-4Rα, which could b) prevent recruitment of IL-13Rα1 or γc, which may allow activation of an alternative signalling pathway. Binding of IL-13Rα2 to the type II receptor may c) block activation of JAK-1 or STAT6 or d) could again form a unique scaffold, which might allow the docking of an alternative signalling molecule. IL-13Rα2 could also instigate a signal on its own via either STAT3 or AP-1.[261,264,280]

mainly used experimental models of Crohn's disease, a fibrotic condition of the intestines, and bleomycin-induced pulmonary fibrosis in mice, therefore, it would be important to establish if this signal also occurs in the human lung. The activation of AP-1 by IL-13 via IL-13Rα2 could be investigated using the overexpression model of this receptor and western blotting for c-Jun phosphorylation. If this model demonstrated the induction of phosphorylated c-Jun in response to IL-13, this could then be confirmed in primary bronchial fibroblasts in the presence and absence of a neutralising antibody against IL-13Rα2. However, despite these suggestions of IL-13-mediated signalling via IL-13Rα2, it is important to note that the soluble form of this receptor, which lacks the intracellular tail, can ameliorate the effects of IL-13. As this is the case, it is unclear whether the regulation of IL-13 occurs mainly, or entirely via sequestration and, if a signal such as AP-1 activation does occur, whether this specifically is a disease-related effect.

Previous studies have shown IL-13 to elicit pro-fibrotic effects.[337] Therefore, if IL-13 can mediate activation of the *TGFB1* promoter via IL-13Rα2, this could play a role in airway fibrosis. TGFβ has already been implicated as an important contributor of airway fibrosis, and the presence of high quantities of IL-13 in the lungs of "Th2 high" asthmatics could therefore be partially responsible for the remodelling observed in the asthmatic lung.

Whilst sequestration or direct signalling via only IL-13Rα2 could be involved in the regulation of IL-13, it is not likely to be the case for IL-4, as this cytokine is unable to bind directly to IL-13Rα2.[165,249] Therefore, any regulation of IL-4 by IL-13Rα2 would require the presence of an IL-4 binding protein. Andrews, Nasir *et al.* have previously demonstrated that a physical interaction occurs between the intracellular tail of IL-13Rα2 and IL-4Rα in the presence of IL-4.[165] As the extracellular domain of IL-13Rα2 cannot bind IL-4 directly[165] this could be essential for the regulation of IL-4 by IL-13Rα2. This association between IL-13Rα2 and IL-4Rα could prevent the recruitment of IL-13Rα1 or γc (Figure 6.1*b*), thus blocking the heterodimerisation required for STAT6 signalling with these receptors. This physical interaction between IL-4Rα and IL-13Rα2 might also result in the

formation of a unique scaffold allowing the mediation of alternative signals such as the activation of STAT3.

Alternatively, IL-13Rα2 may bind to the IL-4Rα subunit of the type II receptor heterodimer (Figure 6.1*c*). As the intracellular portion of IL-13Rα2 appears to be necessary for the regulation of IL-4, which cannot be controlled by the soluble form of this receptor,[165] this suggests that this region of IL-13Rα2 could prevent the normal recruitment of the intracellular signalling proteins. Whilst the intracellular portion of IL-13Rα2 is much shorter than the IL-4/IL-13 signalling receptors, it may be able to block the JAK1 binding site, Box 1, on IL-4Rα which is membrane proximal.

Alternatively, the presence of the IL-13Rα2 tail may form a unique scaffold which could allow the association of an alternative signalling molecule, that is then able to regulate IL-4 (Figure 6.1*d*). It is this latter scenario which may be the case for initiation of a STAT3 signal in response to IL-4. This interaction between IL-4Rα and IL-13Rα2 appears to only occur in the presence of IL-4,[165,249] and therefore it remains unclear if this is involved in IL-13Rα2-mediated regulation of IL-13. To establish whether IL-13Rα2 binds to IL-4:IL-4Rα alone or a larger receptor complex, IL-4Rα could be immunoprecipitated and the presence of IL-13Rα2 can be confirmed. This could then be expanded to see whether anything else co-precipitates with this complex, such as IL-13Rα1 or an alternative signalling molecule.

The activation of alternative signalling molecules could be initially studied using a phosphotyrosine antibody screen (antibody micro-array) of cells treated with IL-4 or IL-13. For this, the overexpression model could be compared with primary bronchial fibroblasts from naturally low and high expressers of IL-13Rα2. Any novel phosphorylation changes could then be further analysed for nuclear localisation of the activated transcription factors by immunocytochemistry and induction of gene expression using focussed micro-arrays. Of particular interest would be a specific micro-array investigating STAT3-regulated genes to establish whether activation of STAT3 by IL-4 induces anti-apoptotic effects.

Although IL-4 and IL-13 elicit many overlapping roles, these cytokines also exhibit some distinct differences. Previously the differences between IL-4 and IL-13 have been attributed to the differential expression of the type I (IL-4Rα:γc) and type II (IL-4Rα:IL-13Rα1) receptors on various cell types. However, if the regulation of these two cytokines by IL-13Rα2 differs, it might be that this is also important.

Several other factors may also affect the signalling pathways of IL-4 and IL-13. Two of the best known groups of proteins for preventing JAK/STAT signalling are the Suppressor of Cytokine Signalling (SOCS) and the Protein Inhibitor of Activated STAT (PIAS) family molecules.

Both IL-4 and IL-13 have been shown to upregulate expression of SOCS-1 and SOCS-3 in a variety of cell types.[338–340] The induction of SOCS-1 mRNA occurs rapidly, peaking after 1 hour.[338] In comparison, in this project the upregulation of IL-13Rα2 mRNA was not found until 8 hours. Whilst none of these studies have analysed upregulation of SOCS-1 protein expression it could be that this occurs more rapidly than IL-13Rα2, and therefore, in conjunction with IL-4Rα, these proteins may be involved in the short-term regulation of these cytokines, prior to upregulation of IL-13Rα2. In addition, in this study, an IL-13Rα2 neutralising antibody restored the responses of these cells to IL-4 and IL-13 in the presence of high levels of IL-13Rα2, again suggesting the role of SOCS proteins may be limited to this short-term regulation.

Whilst overexpression SOCS-1 has been shown to suppress the activation of STAT6-responsive genes by both IL-4 and IL-13, the regulation of these cytokines by SOCS-3 and SOCS-5 has been debated, with one study showing that although transient overexpression of SOCS-3 had an effect, this was lost when the cells were stably transfected with this inhibitor.[341,342] More recently, overexpression models using point mutations in the regions of SOCS-1 and SOCS-3 proposed to be necessary for this regulatory activity showed that the STAT6-mediated effects of IL-4 and IL-13 were suppressed in the presence of wildtype SOCS-1 or SOCS-3, but this regulation was lost when the mutated forms were present.[343] However, these studies have all used

overexpression models, which are driven by strong CMV promotors and, therefore, expression levels of these inhibitory proteins may not relate to what is found *in vivo*. These studies have also been undertaken in various cell lines, such as 293T human embryonic kidney cells or M12.4.1 B cell lymphoma cells. The natural expression levels of IL-13Rα2 are unknown in these cells, and therefore the regulation of IL-4 and IL-13 may differ between these cell types. As this system appears to be highly complex it would be important to establish the role of these SOCS proteins in both the short and long-term regulation of IL-4 and IL-13 in primary human fibroblasts, using both overexpression and knock-down models.

Interestingly, like IL-13Rα2, SOCS proteins also appear to be induced by Th1 cytokines, such as IFNγ.[342,344] Further to this, SOCS-1 appears to be important in the regulation of IFNγ as mice deficient in SOCS-1 die within 3 weeks of birth due to excessive production and response to IFNγ.[345–348] It is, therefore, possible that at least a portion of the regulation of IL-4 and IL-13 by these SOCS proteins is due to the regulation of IL-13Rα2 expression by IFNγ. This suggests that the regulation of the Th1 and Th2 cytokines is highly complex and may involve a range of inhibitors, each of which plays a role in regulating not only the the overall responsiveness to these cytokines, but also the magnitude of this response.

6.4 The Role of IL-13Rα2 in Asthma

In a subgroup of asthmatics, the Th2 phenotype is thought to be especially important and recently IL-13, and proteins induced by IL-13 such as periostin, have been proposed as biomarkers for this phenotype.[200–203] These "Th2 high" patients demonstrate Th2-type inflammation with a predominance of Th2 cytokines, such as IL-4, IL-5 and IL-13, and eosinophilia.

In the fibroblasts analysed, there was heterogeneity in the IL-13Rα2 surface expression, both in the absence and presence of IL-4 and IL-13. Therefore, regardless of the level of expression of these cytokines in the lung at baseline, differences are likely to be observed in the responsiveness

of subjects to IL-4 and IL-13 *in vivo*. Whilst it might be assumed that fibroblasts obtained from asthmatic subjects would express lower levels of IL-13Rα2, this was not found to be the case, with no difference in IL-13Rα2 surface expression observed between disease states in this study. However, it is not clear whether IL-13Rα2 expression levels is consistent between cell types, and therefore, the higher expression of IL-13Rα2 on fibroblasts by one subject, might be compensated for by lower expression on other cells, such as epithelial cells.

As there is much heterogeneity in the asthma phenotype, the "Th2 high" and "Th2 low" subgroups must instead be considered. With IL-13Rα2 acting as a regulator for IL-13, biomarkers such as periostin, which is induced by IL-13, might be more accurate indicators of the "Th2 high" phenotype as subjects expressing high levels of IL-13 levels as well as high IL-13Rα2 expression would likely express lower levels of periostin than those with lower levels of IL-13Rα2.

In the "Th2 high" group, it could be predicted that the higher levels of IL-4 and IL-13 would result in increased expression of IL-13Rα2. However, these patients also express high levels of IL-13-induced proteins, such as periostin, suggesting that this may not be the case. This system appears to be highly complex, and one aspect that must be considered in this scenario is the effect of genetics on the regulation of these cytokines by IL-13Rα2, for instance the rs20541 variant of IL-13 cannot be regulated as effectively by IL-13Rα2 as the wildtype form.[281] This polymophism, which is more common in asthmatic subjects,[147] mediates increased responsiveness to IL-13 and therefore could result in greater expression of periostin. In patients expressing this mutation, even if high levels of IL-13Rα2 were present, the effectiveness of this would be reduced, which may explain some of the differences between these "Th2 high" and "Th2 low" subjects.

Asthma is characterised by periods of worsening symptoms, called exacerbations. Asthmatic patients exhibit increased susceptibility to RV infections, which are one of the leading causes of exacerbations,[349–351] as the viral infection persists in the lower aiways.[352] In these patients "minor" infections

can impact on morbidity and mortality. Current asthma therapy is ineffective at preventing these exacerbations and existing treatments for viral infection have only limited effects. Therefore understanding the differences between asthmatic and healthy individuals response to viral infection could provide new insight for asthma therapy.

The Th1/Th2 balance is regulated *in vivo* by the expression levels of IFNγ and IL-4. This balance between Th1 and Th2 states has been considered an important aspect of both the antiviral response and allergic inflammation and asthma. With IL-13Rα2 able to modulate the responses of both IL-4 and IL-13, which is important for the regulation of the Th2 response, it is likely that expression of this receptor plays a key role in switching from a Th2 bias to a Th1 environment. As this Th1 phenotype is associated with an innate immune response to bacteria and viruses, [16] increasing levels of IL-13Rα2 would enable switching the bias towards this, favouring an antiviral response, whilst also reducing Th2-mediated inflammation.

In Chapter 4 the attempts to develop a knockdown model of IL-13Rα2 in primary human bronchial fibroblasts using siRNA highlighted the induction of an Interferon response by these cells. As a part of this study it was found that IL-13Rα2 surface expression is upregulated in response to both dsRNA and IFNβ.

In both bronchial epithelial cells and fibroblasts, the innate immune response to dsRNA involves the production of type I (IFNα and IFNβ) and III (IFNλ) Interferons. [353] Addition of Poly I:C, a synthetic analogue of dsRNA, which is often used in an experimental setting to mimic the replication stage of the viral lifecycle, where epithelial cells release viral dsRNA, resulted in production of IFNβ by the fibroblasts, which in turn induced the upregulation of IL-13Rα2. Direct stimulation with either IFNβ or IFNγ was also found to increase surface expression of IL-13Rα2.

During viral replication double stranded RNA (dsRNA) is formed and this molecular pattern is detected by cells via RIG-like helicases and Toll-Like Receptor 3 (TLR3). The response to viral insult differs between epithelial cells and fibroblasts, which act in a coordinated manner to fight infection. In

epithelial cells the Interferon response to virus results in rapid apoptosis of infected cells, followed by rapid phagocytosis preventing further viral replication. These cells also release Interferons and pro-inflammatory mediators, which aids the orchestration of an innate immune response. [354,355] Both virus infection and stimulation with dsRNA induce an Interferon response in epithelial cells. In fibroblasts, on the other hand, IFNβ is only observed in response dsRNA, with no significant increase in Interferon production detected post infection with human rhinovirus (RV). [307]

The other major difference between epithelial cells and fibroblasts with regards to the Interferon response is the location of TLR3 receptors. In epithelial cells these are found intracellularly whereas fibroblasts express TLR3 receptors on their cell surface. [356] In epithelial cells, the intracellular location of TLR3 allows an autocrine response to viral replication occurring within the cell. [356] The presence of TLR3 on the fibroblast surface, on the other hand, enables these cells to produce a potent antiviral response to dsRNA, which is released by the epithelial cells infected with virus, allowing the induction of a robust antiviral response before the virus enters the cell. [307]

As IL-13Rα2 surface expression upregulated by dsRNA stimulation suppresses the fibroblasts responses to IL-13, this suggests that during viral replication, fibroblasts exposed to dsRNA and IFNβ would use this mechanism to downregulate the Th2 response, promoting a Th1 phenotype. *In vivo* IFNγ and IL-4 are classically considered the key mediators for switching the functionally plastic T helper cells between the Th1 and Th2 states, however these data suggest that this regulation is in fact much broader and more complex than previously thought. It is therefore hypothesised that the increased expression of IL-13Rα2 in response to a range of Th2 and Th1 stimuli aids the phenotypic switch to a Th1 response, which is required to fight the virus.

Recently it has been demonstrated that bronchial epithelial cells obtained from asthmatics are unable to produce a sufficient Interferon response to virus infection, with deficient induction of IFNα, IFNβ and IFNλ. [357–359] In the work presented in Chapter 4, similar responses were observed by fibroblasts

obtained from either healthy or asthmatic patients in response to challenge with IFNβ or dsRNA. This is similar to what was observed by Bedke, Haitchi *et al.*, who, in addition, found that fibroblasts only respond to the dsRNA signal, and not to direct viral infection when mounting an IFNβ response. [307] The fact that the fibroblasts produce IFNβ in similar quantities in response to dsRNA regardless of disease status suggests the deficiency observed by Wark, Johnston *et al.* [357] and Contoli, Message *et al.* [358] may be specific to the epithelial cells and alveolar macrophages, both of which are involved in the first line response to viral infection. However, this will still have a knock-on effect on the upregulation of IL-13Rα2.

Recent studies have investigated the use of IFNγ or IFNβ as a therapy for asthma, due to the deficiency observed in the production of these cytokines. In asthmatic patients, the deficient production of Interferons may result in a less robust switch to an anti-viral, Th1 response by not properly downregulating the Th2 phenotype. Therefore, providing one or more of these Interferons directly is thought to promote the Th1 environment. Whilst IFNγ is able to do this directly, by acting on naïve or Th2 polarised T cells, based on the ability of both IFNβ and IFNγ to upregulate IL-13Rα2, one of the actions of this novel therapeutic may also be increasing the expression of this receptor, thus favouring a Th1 bias.

Inhaled or oral steroids are commonly used in asthma, either as maintenance therapy or specific treatment after an exacerbation. In Chapter 4, IL-13Rα2 surface expression was found to be attenuated by steroids. Whilst this appeared to reduce basal expression of IL-13Rα2, this was particularly evident when fibroblasts were treated with a stimuli which can normally increase surface expression levels of this receptor, such as dsRNA, where Dexamethasone was found to completely abrogate this upregulation in response to treatment. These data suggest that whilst a non-asthmatic person exposed to virus would upregulate their expression of IL-13Rα2 on bronchial fibroblasts to help fight the infection, an asthmatic patient taking steroids would not see this same induction of IL-13Rα2. This, combined with the

deficient production of Interferons by asthmatic subjects, would potentially reduce their ability to switch to a Th1 environment.

Whilst it could be considered that the steroid-dependent expression of IL-13Rα2 would have negative implications in the response to viral infections for asthmatic patients taking corticosteroid therapy, there has been some indication that IL-13 itself is also regulated by steroids, resulting in lower expression of this cytokine,[309,310] and suppressing IL-13-induced gene expression. In recent years it has been established that patients who are "Th2 low" appear to respond less well to steroid therapy than their "Th2 high" counterparts.[203] In the "Th2 low" group it could be predicted that these patients normally express high levels of IL-13Rα2, which suppresses the effects of any IL-13 or IL-4 present. However, it is not clear whether the reduced expression of IL-13Rα2 would allow any IL-13 present to elicit a greater effect, therefore reducing the benefits of this steroid therapy, or whether in these "Th2 low" patients IL-13 is not a driving force of their asthma symptoms.

6.5 Conclusions

The ability of IL-13Rα2 to regulate the actions of both IL-13 and IL-4, and thus negatively regulate some of the inflammatory responses observed in asthma has led to the view that a small-molecule mimetic of this receptor could act as a therapy for asthma. However, in recent years the discovery that this receptor may induce a signal in response to either IL-4 or IL-13 has highlighted that IL-13Rα2 cannot be considered in such simplistic terms as "a good guy" in the asthmatic lung. The two proposed mechanisms for IL-13Rα2 signalling: activation of AP-1 leading to activation of the *TGFB1* promoter, thus inducing profibrotic effects, and STAT3 phosphorylation, which is known to induce anti-apoptotic proteins, and therefore could maintain fibroblast survival, both have the potential to induce fibrosis. Parallels can be drawn between these possible roles of IL-13Rα2 and those of TGFβ. Whilst TGFβ exhibits anti-inflammatory effects, this cytokine plays

a negative role in asthma, where high levels are expressed, due to its ability to promote fibrosis by activating fibroblasts. Whilst the suppressive effects of IL-13Rα2 on inflammatory gene expression have been well documented, the full effects of this receptor are unclear, and therefore global analysis of gene expression in response to IL-4 and IL-13 in both naturally high and low expressers of this receptor is required to clarify how IL-13Rα2 is eliciting its effects. The potential for IL-13Rα2 to drive a profibrotic response in the face of a viral infection, where large quantities of IL-4 and IL-13 may still be present highlights the urgency of determining which genes are regulated by this receptor.

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Appendix A

Reagents, Media & Buffers

A.1 Cell Culture Media

A.1.1 DMEM Complete

Dulbecco's Modified Eagles Medium (DMEM)

10% (v/v) Heat-inactivated foetal bovine serum (FBS)

50 IU/ml Penicillin

50 μg/ml Streptomycin

2 mM L-glutamine

1 mM Sodium pyruvate

1 mM Nonessential amino acids (MEM)

A.1.2 Serum Free Medium (SFM)

UltraCulture

50 IU/ml Penicillin

50 μg/ml Streptomycin

2 mM L-glutamine

A.1.3 siRNA Media

Dulbecco's Modified Eagles Medium (DMEM)

10% (v/v) Heat-inactivated foetal bovine serum (FBS)

2 mM L-glutamine

1 mM Sodium pyruvate

1 mM Nonessential amino acids (MEM)

A.2 PCR Primers

Human γc	
Sense Primer	CGACAATTCTGACGCCCAAT
Anti-sense Primer	GAACACAAAACACTGAACCTCTG
Human IL-4Rα	
Sense Primer	CCCAGCGAGCATGTGAAAC
Anti-sense Primer	GCATAGGTGAGATGATTATACAGGTA
Human IL-13Rα1	
Sense Primer	AATGGTCAAGGATAATGCAGGAA
Anti-sense Primer	GGTCATCATTGTGGAAGGAGAG
Human IL-13Rα2	
Sense Primer	AAACAACAAATGAAACCCGACAA
Anti-sense Primer	GTCTTCACCTTCCCAGCATTG
Human IFN-β	
Sense Primer	TTACTTCATTAACAGACTTACAGGT
Anti-sense Primer	TACATTAGCCATCAGTCACTTAAAC

A.3 SDS-PAGE Buffers

A.3.1 Reducing Sample Buffer

62.5 mM Tris-HCl pH 6.8

10% Glycerol

2% Sodium Dodecyl Sulphate

5% β-mercaptoethanol

0.01% Bromophenol Blue

A.3.2 Separation Gel

10% (w/v) Acrylamide/0.8% Bis-acrylamide

1.5 M Tris-HCl pH 8.8

20% (w/v) Sodium Dodecyl Sulphate (SDS)

10% (w/v) Ammonium persulphate (APS)

1% (v/v) TEMED

A.3.3 Stacking Gel

3.75% (v/v) Acrylamide/0.8% Bis-acrylamide

0.5 M Tris-HCl pH 6.8

20% (w/v) SDS

10% (w/v) APS

1% (v/v) TEMED

A.3.4 Tris-Glycine-SDS Running Buffer pH 8.3

25 mM Tris

192 mM Glycine

0.1% (w/v) SDS

A.3.5 Coomassie Brilliant Blue Stain

0.01% Coomassie Brilliant Blue R

45% (v/v) Methanol

10% (v/v) Glacial Acetic Acid

A.3.6 Gel Destain

25% (v/v) Methanol

10% (v/v) Glacial Acetic Acid

A.4 Western Blotting Buffers

A.4.1 Transfer Buffer pH 8.0-8.3

25 mM Tris

192 mM Glycine

20% (v/v) Methanol

A.4.2 Tris Buffered Saline (TBS)

137 mM Sodium Chloride

50 mM Tris

Adjust to pH 7.6 with HCl

A.4.3 Blocking/Antibody Buffer

0.1% (v/v) Tween 20, 5% Dried milk powder in TBS

A.4.4 Wash Buffer

0.1% (v/v) Tween 20 in TBS

A.4.5 Stripping Buffer

100 mM β-mercaptoethanol

2% (w/v) SDS

62.5 mM Tris pH 6.7

A.5 Flow Cytometry Buffers

A.5.1 Dulbecco's Phosphate Buffered Saline (DPBS)

137 mM NaCl

2.7 mM KCl

8.1 mM Na_2HPO_4

1.5 mM KH_2PO_4

pH 7.2 – 7.4

0.2 μm sterile filtered

A.5.2 FACS Buffer

0.5% Bovine Serum Albumin (BSA) Fraction V, 2 mM EDTA in DPBS

A.6 ELISA Buffers

A.6.1 Dulbecco's Phosphate Buffered Saline (DPBS)

137 mM NaCl

2.7 mM KCl

8.1 mM Na_2HPO_4

1.5 mM KH_2PO_4

pH 7.2 – 7.4

0.2 μm sterile filtered

A.6.2 Wash Buffer

0.05% (v/v) Tween 20 in DPBS

A.6.3 Reagent Diluent

1% (w/v) Bovine Serum Albumin (BSA) Fraction V in DPBS

A.7 Methylene Blue Buffers

A.7.1 Formal Saline

4% (v/v) Formaldehyde in 0.9% (v/v) saline solution

A.7.2 Methylene Blue

1% (v/v) Methylene blue in 10 mM borate buffer pH 8.5

A.7.3 Elution Buffer

1:1 Ethanol: 0.1% HCl solution

Appendix B

Publication List

B.1 Publications

Campbell-Harding, G; Holgate, ST; Davies, DE; Andrews, A-L (2012) Expression of IL-13Rα2 in Human Bronchial Fibroblasts Requires *de novo* Protein Synthesis (Manuscript in preparation).

Campbell-Harding, G; Sawkins, H; Bedke, N; Holgate, ST; Davies, DE; Andrews, A-L (2012) The Innate Anti-Viral Response Upregulates Interleukin 13 Receptor α 2 in Bronchial Fibroblasts (Manuscript submitted).

B.2 Oral Presentations

Campbell-Harding, G; Holgate, ST; Davies, DE; Andrews, A-L (2010) Potential involvement of STAT3 in IL-4 mediated signalling in bronchial fibroblasts. BALR Summer Meeting, Swansea, UK

B.3 Poster Discussions

Campbell-Harding, G; Sawkins, H; Bedke, N; Holgate, ST; Davies, DE; Andrews, A-L (2012) The Innate Anti-Viral Response Upregulates IL-13Rα2 In Bronchial Fibroblasts. American Thoracic Society International Meeting, San Francisco, USA. Abstract accepted.

B.4 Poster Presentations

Campbell-Harding, G; Holgate, ST; Davies, DE; Andrews, A-L (2012) Expression Of Interleukin 13 Receptor α 2 In Human Bronchial Fibroblasts Requires De Novo Protein Synthesis. American Thoracic Society International Meeting, San Francisco, USA. Abstract accepted.

Campbell-Harding, G; Holgate, ST; Davies, DE; Andrews, A-L (2011) The Role Of Signal Transducer And Activator Of Transcription (STAT) 3 In Interleukin-4 Signalling In Primary Fibroblasts. American Thoracic Society International Meeting, Denver, USA. Meeting reported in American Journal of Respiratory and Critical Care Medicine.

Campbell-Harding, G; Bondanese, V-P; Holgate, ST; Davies, DE; Andrews, A-L (2009) Endogenous IL-6 phosphorylates STAT3 in primary bronchial fibroblasts. American Thoracic Society International Meeting, San Diego, USA. Meeting reported in American Journal of Respiratory and Critical Care Medicine.

Campbell-Harding, G; Bondanese, V-P; Holgate, ST; Davies, DE; Andrews, A-L (2009) Endogenous IL-6 Induces STAT3 Phosphorylation in Primary Bronchial Fibroblasts. Biochemical Society Biochemical Basis of Respiratory Disease, Loughborough, UK. Meeting reported in Biochemical Society Transactions.