The Role of STAT6 in the Regulation of IL-4 and IL-13 Mediated Responses in Bronchial Epithelial Cells in Asthma

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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Doctor of Philosophy

THE ROLE OF STAT6 IN THE REGULATION OF IL-4 and IL-13 MEDIATED RESPONSES IN BRONCHIAL EPITHELIAL CELLS IN ASTHMA

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Asthma is associated with increased production of the Th2 cytokine, IL-4, which helps to orchestrate the asthmatic response through diverse actions on both inflammatory cells and the airway structural cells. As IL-4 mediates its effects through activation of the transcription factor STAT6, it was hypothesised that dysregulation of this signalling pathway has the potential to impinge on the structural cells as well as the inflammatory cells to contribute to disease pathology. Therefore, the aims of this study were: to characterise the expression of STAT6 in the bronchial epithelium of asthmatic and non-asthmatic subjects; to assess expression of STAT6 variants in bronchial epithelial cells; to investigate the effect of IL-4 on release of the profibrogenic growth factor TGFβ_2 from the H292 bronchial epithelial cell line and to establish the role of STAT6 in this response.

Immunohistochemistry applied to bronchial biopsies demonstrated the expression of STAT6 and one of its upstream activators JAK1 in the bronchial epithelium. In severe asthma, epithelial STAT6 expression was significantly increased when compared to with mild asthma (P=0.001) and normal controls (P=0.037). Furthermore, for the first time, this study identified expression of mRNA encoding a dominant negative isoform of STAT6 in primary bronchial epithelial cells, but no relationship between expression and disease status could be identified. As no studies have demonstrated a mechanism through which IL-4 might contribute airway wall remodelling, the effect of IL-4 on epithelial TGFβ_2 release and the involvement of STAT6 in this process was assessed. Treatment of H292 cells with 1.5nM IL-4 significantly increased TGFβ_2 release from 69$\pm$ 10 to 118$\pm$27 pg/4x10^5 cells (n=8, P=0.034) at 48 hours. Using TaqMan™ real-time PCR, increases in TGFβ mRNA production were not observed until 48 hours post treatment (from 0.817±0.12 pg/4x10^5 cells in untreated cells to 1.41±0.19 pg/4x10^5 cells following stimulation with 1.5nM IL-4, (n=5, P=0.013)). To investigate the involvement of TGFβ processing enzymes in this delayed response, the effects of a matrix metalloproteinase inhibitor (MMPI) on TGFβ_2 release and transcription was assessed. In the presence of this inhibitor, IL-4 failed to induce TGFβ_2 release or mRNA synthesis, however, the MMPI inhibitor alone promoted both TGFβ gene activation and protein release. To investigate the involvement of STAT6 in IL-4 induced TGFβ_2 release, cells were transfected with a dominant negative STAT6 mutant and a wild type STAT6 control plasmid. In untransfected cells, TGFβ_2 release was significantly increased from 80±7.88 pg/4x10^5 cells to 151±25.53 pg/4x10^5 cells (n=3, P=0.05) following IL-4 treatment for 48 hours. A similar increase was observed in cells transfected with wild type STAT6 (101.63±19.02 pg/4x10^5cells versus 188±39.7 pg/4x10^5cells (n=3 P=0.05) following IL-4 treatment). Transfection with dominant negative STAT6 abolished the IL-4 induced TGFβ_2 release consistent with a role for STAT6 in IL-4 mediated TGFβ_2 release.

In conclusion, this study has shown that STAT6 expression is increased in the bronchial epithelium of severe asthmatic subjects. A possible site of interaction between the Th2 cytokines and airway wall remodelling in asthma is further suggested by the novel finding that IL-4 induced TGFβ_2 release from bronchial epithelial cells in a STAT6 dependent manner. This effect appeared to be indirect and may involve the activity of an MMP. As TGFβ is considered to be a central co-ordinator of profibrotic responses, the ability of IL-4 to promote TGFβ release and the involvement of STAT6 in this response may be amplified in asthmatic subjects, where this transcription factor is expressed at increased levels.

Table of Contents

Table of Figures

Chapter 6

List of Tables

Chapter 2

The work contained within this thesis, except where specifically specified is entirely the work of the author. Work contained within this thesis is reproduced in part in the following publications.

Full Publications

Mullings,R.E., Wilson, S. J., Puddicombe, S. M., Lordan, J. L., Bucchieri, F., Djukanovic R., Howarth, P., Harper, S.T., Holgate, S.T. and Davies, D. E. Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium 2001. *Journal of Allergy and Clinical Immunology*; **108**: 832-8

Abstracts

Mullings,R.E., Powell, R., Holgate, S.T. and Davies, D.E. STAT6 Regulates Transforming Growth Factor β2 Release From IL-4 Stimulated Epithelial Cells. (Abstract). S*ubmitted for American Thoracic Society international conference 2002.*

Mullings, R.E., JL Lordan, F Bucchieri, SM Puddicombe, R Djukanović, S Harper, ST Holgate and DE Davies. Dominant Negative STAT6 as a Potential Regulator of IL-4 and IL-13 Responsiveness in Bronchial Epithelium. (Abstract). (2001). *American Journal of Respiratory and Critical Care Medicine*. **Vol 163, No 5,** *Abstract Issue.*

Mullings, R. E., Wilson, S. J., Djukanovic R., Howarth, P., Harper, S.T., Holgate, S.T. and Davies, D. E. Increased STAT 6 Expression in Bronchial Epithelium of Severe Asthmatic Subjects. (Abstract). (2000). *American Journal of Respiratory and Critical Care Medicine*. **Vol 161, No 3,** *Abstract Issue.*

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Abbreviations

Ab	antibody
AEC	Aminoethylcarbozole
AHR	airway hyperreactivity
APS	Ammonium persulphate
BAL	bronchoalveolar fluid
BCL-6	B cell lymphoma gene-6
BEGM	bronchial epithelium growth medium
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CK	cytokeratin
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	dexoyribonucleic acid
cDNA	complementary DNA
ECL+	enhanced chemiluminescence plus
ECM	extracellular matrix
ECP	eosinophil cationic protein
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbant assay
ET-1	endothelin 1
FCεRI	high affinity IgE receptor
FBS	foetal bovine serum
FEV1	forced expiratory volume one
GAS	gamma interferon activation site
GMCSF	granulocyte macrophage colony stimulating factor
GMA	glycolmethacrylate
HBSS	Hanks balanced salt solution
HRP	horseradish peroxidase
15-HETE	15-hydroxyeicosatetranoic acid
ICAM	Intercellular adhesion molecule
IgE	immunoglobulin E
IFNγ	interferon gamma

IGF	insulin like growth factor
IL-	interleukin
IL-4Rα	interleukin 4 receptor alpha chain
IL-13Rα	interleukin 13 receptor alpha chain
iNOS	inducible nitric oxide synthase
IRS	insulin receptor substrate
ITIM	immunoreceptor tyrosine based inhibitory motifs
JAK	Janus kinase
LAP	latency associated peptide
LTBP	latent TGFβ binding protein
LTB4	leukotriene B4
LTC4	leukotriene C4
MBP	major basic protein
MEM	minimal essential medium
MHC	major histocompatability complex
MMPs	matrix metalloproteinases
MMPI	matrix metalloproteinase inhibitor
MUC	mucin
NC	nitrocellulose
NCI-H292	National Cancer Institute H292
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGD 2	prostaglandin D 2
PI_3K	phosphatidyl inositol triphosphate kinase
PVDF	polyvinylidene difluoride (hydrophobic membrane)
PY	phosphotyrosine
PY STAT6	tyrosine phosphorylated STAT6
RANTES	regulated on activation normal T cell expressed and secreted
RNA	ribonucleic acid
mRNA	messenger RNA
RT	reverse transcription
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SE	standard error

SH2 domain	*src* homology 2 domain
SHIP	SH2-containing inositol 5'-phosphatase
SHP	SH2-containing tyrosine phosphatases
SOCS	suppressors of cytokine signalling
STAT	signal transducer and activator of transcription
Strept-ABC	streptavidin biotin-peroxidase
TBE	Tris/boric acid/EDTA
TBS	Tris buffered saline
TGFβ	transforming growth factor beta
TGFα	transforming growth factor alpha
Th	T helper lymphocyte
TIMPs	tissue inhibitors of metalloproteinases
TMB	tetra-methyl benzidine
TNFα	tumour necrosis factor alpha
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	very late antigen

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

1.1.1 *Incidence of Asthma*

The prevalence of asthma is increasing, predominantly in countries that offer more affluent lifestyles for example the UK, America, New Zealand and Australia. In contrast the symptoms of asthma are more infrequent in Eastern Europe [1]. Whilst asthma and atopy are strongly familial and several genetic polymorphisms have been shown to predispose an individual to atopy and asthma, the increase in asthma prevalence has occurred at a greater rate than can be accounted for by new genetic changes. Thus, this increase has been linked to changes in the environment, involving damaging matter such as increased pollution generated by motor vehicles, increased ozone, as well as changes in diet. These factors have the potential to stress airways, making the consequences of pre-existing genetic susceptibilities more apparent [2].

1.1.2 *Characteristics of Asthma*

Asthma is essentially a chronic inflammatory disorder superimposed on remodelled airways, which gives rise to reversible airflow obstruction, caused primarily by constriction of hyperreactive airways, both spontaneously and in response to stimuli which are not in themselves noxious. The disease manifests itself as recurrent episodes of wheezing, breathlessness, chest tightness and cough. Whilst these acute attacks are reversible, the underlying pathological changes in the airways are not, and may progress over time, leading to a progressive and more rapid decline in lung function when compared with the general population [3].

The pathological changes in the asthmatic airways show distinct characteristics and many of the changes described below are present even in the mildest form of the disease. In 1960, Dunnill published a detailed description of the pathological changes in the lungs of subjects who had died in *status asthmaticus*. The lungs of these subjects were found to be grossly distended with the presence of mucus plugs in the conducting airways. The airway walls of the subjects were thickened due to increased smooth muscle mass, the presence of oedema and vasodilation of pulmonary blood vessels. There was also a

substantial degree of epithelial shedding with areas of regeneration and the basement membrane of the airway wall was strikingly thicker, with a collagenous appearance, than in non-asthmatic subjects [4].

The advent of fibre optic bronchoscopy in the last twenty years has allowed detailed studies to be performed on living subjects and has confirmed many of the findings obtained from *post mortem* studies. Sampling methods include bronchoalveolar lavage (BAL) with physiological saline, brushing to obtain the superficial epithelial layer, and endobronchial biopsy to sample deeper layers of the mucosa. Studies using this technique have identified a dense inflammatory infiltrate in the mucosa of asthmatic subjects and morphological changes in the airway structural cells. They have also begun to allow study of the cellular and molecular mechanisms necessary for inflammation to persist, in the hope of identifying abnormalities, which may in the future serve as targets for pharmacological intervention [5 6].

1.1.3 *Pathology of Asthma*

Asthma is complex disease and is now regarded as having two components: chronic airways inflammation mediated by haematopoietic cells and structural changes in the airways themselves. Whether one is a consequence of the other remains to be determined. The majority of subjects with asthma also exhibit acute immediate hypersensitivity to common inhaled proteins, known as allergens. These subjects are termed atopic asthmatics and the basis of their airways inflammation is thought to be due a heightened response to common aero-allergens, such as house dust mite, pollen and feathers. However, in a large number of subjects with asthma, there appears to be no identifiable allergen to exacerbate the disease. In these subjects the cause of airways inflammation and remodelling is unknown, and it has been hypothesised that dysregulation of inflammatory processes or of the subjects own structural cells in the airway may be the cause. Asthma is a chronic, often life long, disease and it can fluctuate in severity over time. In both atopic and non-atopic asthmatics a number of stimuli can provoke an asthma 'attack'. These include exercise, cold air, viral infection and in allergic asthma, the allergen itself can also provoke an attack. Whilst the cause of asthma is unknown, in many cases the mechanisms of inflammation observed in the airways are very similar between both atopic and non-atopic asthmatics [7].

1.2 AIRWAYS INFLAMMATION IN ASTHMA

Airways inflammation is a highly complex multifactorial process involving a number of cell types and the release of many cytokines and pro-inflammatory mediators. Complex interactions between the cells and mediators contribute to the symptoms of asthma, as well as propagating the inflammatory response in the airways. One of the most striking features in the conducting airways of asthmatics is the presence of a dense mucosal infiltrate consisting of mast cells, eosinophils, macrophages and lymphocytes [8]. The interactions between these cells are described briefly below and are highlighted in figure 1.1.

1.2.1 *T lymphocytes in Asthma*

Airways inflammation is orchestrated by $CD4^+$ T lymphocytes through the release of small soluble glycoproteins termed cytokines, which interact with a number of inflammatory cells either recruiting them to the site of inflammation or initiating mediator release to sustain the appropriate environment for inflammation to persist. T helper lymphocytes can be grouped into two subtypes, T helper 2 (Th2) and T helper 1 (Th1) based on the array of cytokines that they release. Th1 cells produce tumour necrosis factor α (TNF-α), interferon γ (IFN-γ) and interleukin-2 (IL-2) whereas Th2 cells secrete the Th2 cytokines IL-4, IL-5, IL-3, IL-9 and IL-13. The cytokines produced from the two subsets cross-regulate each other during commitment of the common Th0 precursor. For example, IL-4 will inhibit the production of Th1 cells and IFN-γ will inhibit the production of Th2 cells [9 10]. It is the Th2 phenotype which is associated with allergy and asthma. This is based on several findings: T cell clones prepared from the blood of patients sensitive to house dust mite mainly produce IL-4 and only low levels of IFN-γ after antigen challenge [10], levels of the Th1 cytokine, IL-12, are much lower in the bronchial mucosa of asthmatics than levels of the Th2 cytokine, IL-13, [11] and BAL fluid T cells obtained from human asthmatic airways express high levels of the pro-inflammatory cytokines IL-3, IL-13, GM-CSF and IL-4 [12]. Furthermore, animal models have shown that transfer of Th2 lymphocytes into naïve animals prior to allergen challenge causes accumulation of eosinophils, mucus hypersecretion and airway hyperreactivity [13 14]. It is not only exposure to allergen which can initiate the development of a Th2 phenotype. There are various other stimuli, for example diesel particles, that can alter the equilibrium between Th1 and Th2 cells in favour of a Th2

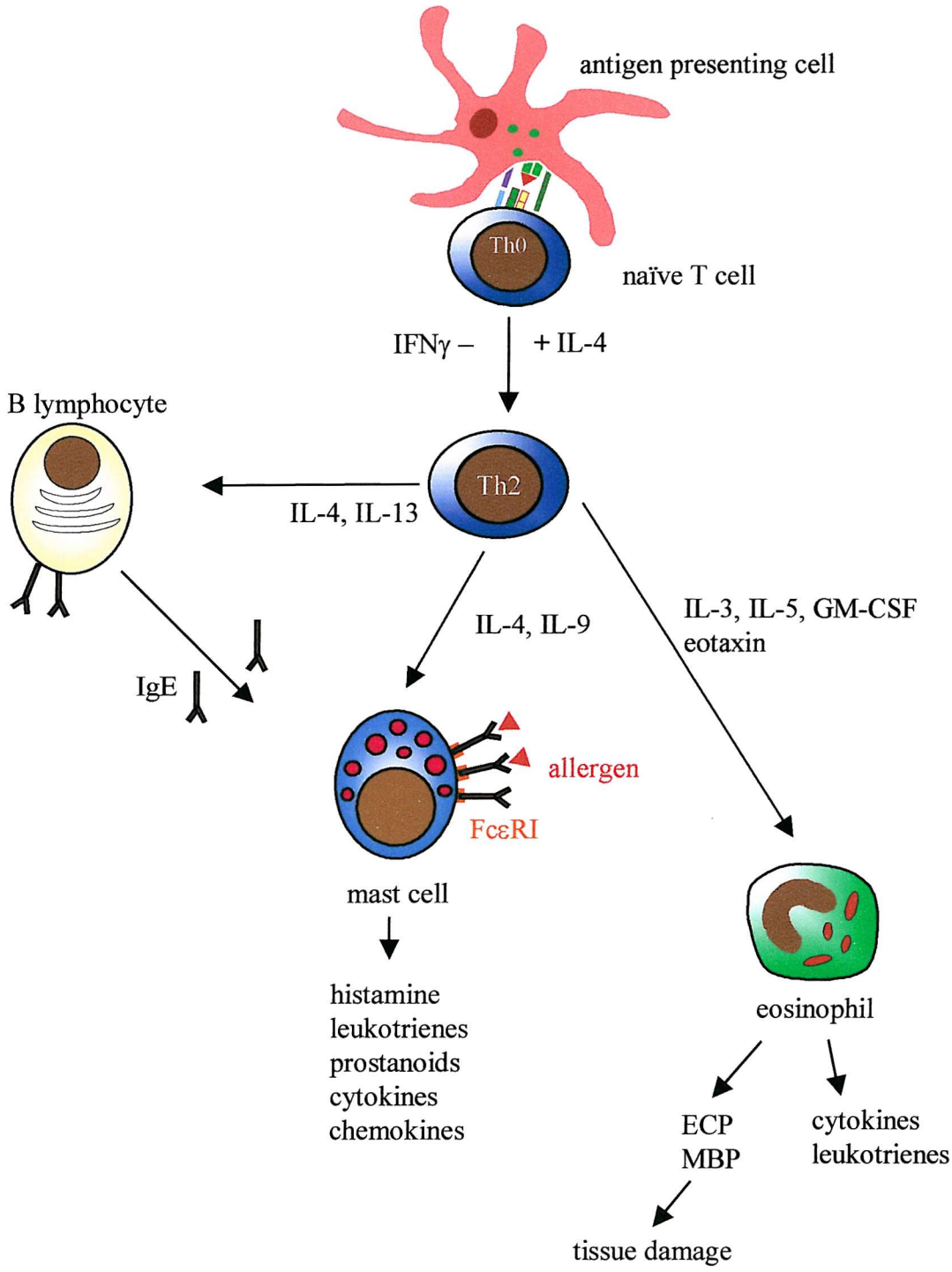


Figure 1.1 Development of an Allergic Inflammatory Response.
Antigen is taken up and processed by profession antigen presenting cells. Fragments of antigen are then displayed to naïve T cells at the cell surface in the cleft of and MHC class II molecule. Interactions between the naïve T cell and antigen presenting cell, in the presence of IL-4, causes differentiation of the T cell to a Th2 phenotype. Th2 cells release cytokines and mediators which can recruit eosinophils and mast cells. IL-4 and IL-13 act on B lymphocytes to induce antigen specific IgE production which in turn bind to high affinity receptors on mast cells. On subsequent exposure to allergen, crosslinking of IgE bound to its receptors results in mast cell activation.

phenotype [15]. Factors such as these may help explain the presence of asthma in subjects where there is no identifiable allergen.

1.2.2 *Dendritic Cells*

In the case of allergic asthma, the inflammatory response is initiated when antigen presenting cells in the airway identify inhaled allergen. The major antigen presenting cells in the lung are believed to be dendritic cells [5]. These cells reside within the bronchial epithelium and are suitably placed to take up the antigen and process it such that it can be displayed at the cell surface in the cleft of a major histocompatability complex (MHC) class II molecule. The mature dendritic cell then migrates to local lymph nodes when the antigen can be displayed to naïve T lymphocytes. Under appropriate conditions, and the presence of IL-4, the naïve T cell differentiates into a Th2 type lymphocyte and, as described above, it is this cell type which orchestrates the inflammatory response in asthma [16].

1.2.3 *B Lymphocytes and the Generation of IgE*

One of the main risk factors for the development of asthma is atopy, which involves the generation of specific Immunoglobulin E (IgE) against innocuous substances. Atopic asthmatic subjects have elevated levels of allergen-specific IgE in their serum [17] and as described in section 1.2.4, these antibodies have the ability to propagate the inflammatory response by binding to their high and low affinity receptors which are expressed on certain cell types. Whilst IgE is primarily associated with the atopic form of asthma, studies have revealed that non atopic asthmatics also have significantly higher IgE levels in their serum compared to normal controls, but these levels were still within the defined 'normal' range [7].

As shown in figure 1.2, the generation of IgE occurs when B-lymphocytes present processed allergen in MHC class II molecules to primed Th2 cells with receptors specific for the allergen. Once the Th2 cells are primed they are able to deliver several molecular signals that favour class switching of B-lymphocytes to the production of IgE. Ligation of the T cell receptor in this way stimulates the expression of CD40 ligand on the T cells and this in turn interacts with CD40 on the B cells. Engagement of CD40 in turn results in expression of a secondary co-stimulatory molecule on the B cell, B7. B7 engages CD28 on the Th2 lymphocyte and this event induces high rate secretion of IL-4 which

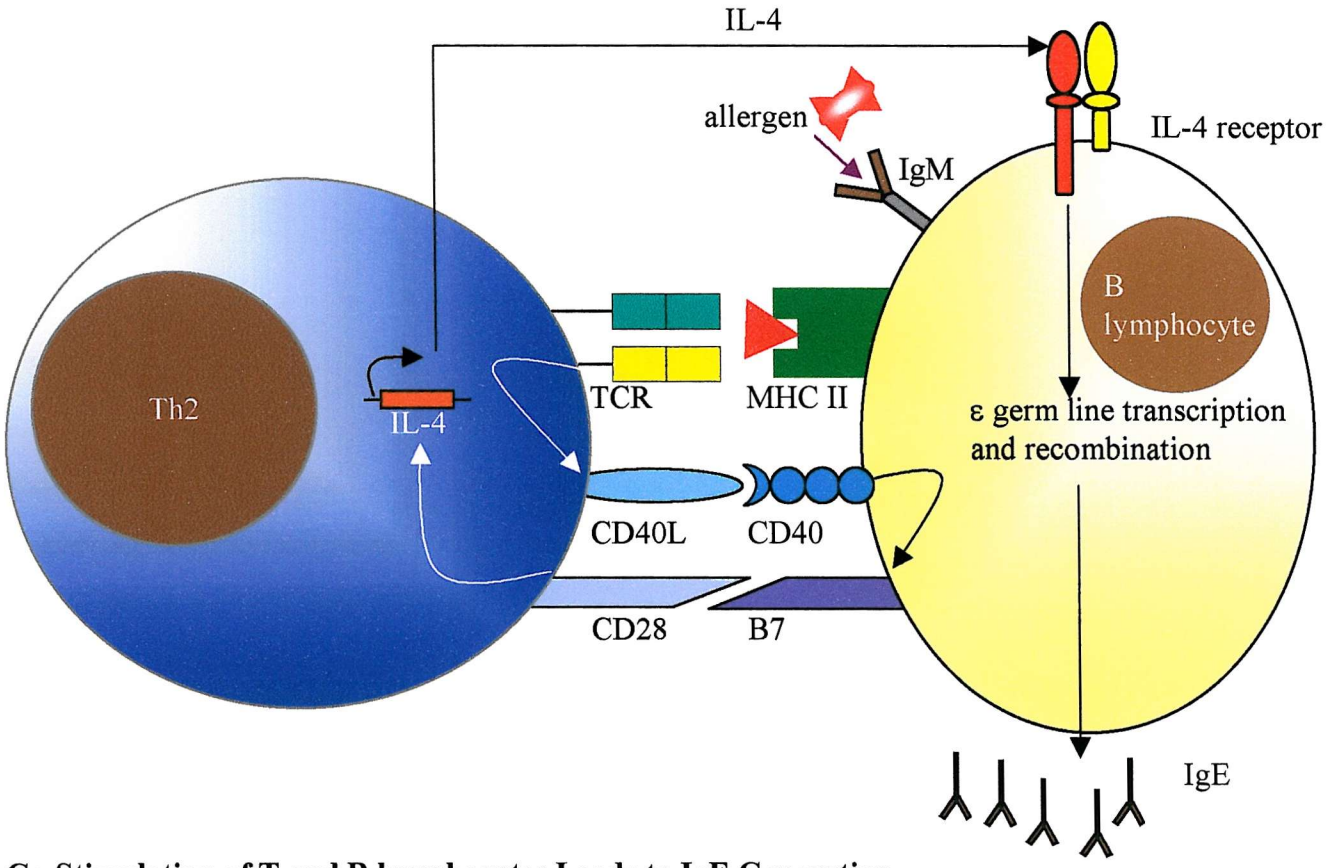


Figure 1.2 Co-Stimulation of T and B lymphocytes Leads to IgE Generation.
Every B cell begins expressing IgM, which can bind allergen at the cell surface. The allergen is then processed and presented at the cell surface in the cleft of MHC class II molecules. Ligation of the MHC class II molecule with the an antigen specific T cell receptor stimulates expression of CD40 ligand on the Th2 cell surface, which interacts with CD40 on B cells. This leads to high rate secretion of IL-4 which binds to its receptor on the surface of B cells. IL-4 acts on the promoter of the ε germline gene to initiate transcription which leads to isotype switching of the antibody heavy chain from IgM to IgE. Figure adapted from Djukanovic & Holgate 1999, [5].

binds to its receptor on the surface of the B cell [5]. Engagement of the IL-4 receptor results in transcription of the ε germ line gene which causes the cells to switch from producing IgM antibodies to IgE [18]. It has also been shown that IL-13, can also induce germline ε gene mRNA synthesis and IgE switching and release from B lymphocytes, independently of IL-4 [19]. Once produced, IgE binds to its high affinity receptors on mast cells until subsequent exposure to allergen.

1.2.4 *Mast Cells in the Acute Phase of Asthma*

Mast cells in the airway submucosa have long been considered the primary effector cells of the early asthmatic response. Their levels are significantly higher in both atopic and non atopic asthmatics compared to atopic and normal controls [20] and they are positioned at the interface between the external and internal environments, which enable them to respond rapidly to stimuli with mediator and cytokine secretion. Mediators and cytokines are stored within granules in the cytoplasm so that they can be rapidly released upon cellular activation. Mast cells express high numbers of high affinity IgE receptors (FcεRI) and allergen-specific cross-linkage of IgE bound to these receptors is the dominant signal for mast cell activation [21]. The numbers of FcεRI bearing cells is significantly increased in both atopic and non-atopic asthmatic subjects, compared to control subjects [22]. Antigen cross-linking causes receptor dimerisation and activates a signalling cascade, which results in a process called degranulation, where the preformed mediators such as histamine, proteases and an array of cytokines are released. Following activation, a number of mediators such as leukotrienes and prostanoids are rapidly synthesised from membrane phospholipids [23]. The range of cytokines and mediators released from mast cells and their cellular targets are summarised in figure 1.3. Release of a huge array of cytokines and mediators cause many local effects in the airways for example histamine, leukotriene C4 (LTC4) and prostaglandin D2 (PGD2) can all cause smooth muscle contraction [24]. Both LTC4 and PGD2 are both also vasodilators with a resultant increase in vascular permeability, which may contribute to the oedema observed in asthmatic airways. As will be discussed further below, cytokines such as IL-4 and IL-13, as well as causing Th2 cell differentiation, can induce mucus secretion from goblet cells in the epithelium [25]. Also in addition to these local acute effects, several of these cytokines and mediators are important for recruitment of eosinophils into the airways from the pulmonary circulation, which causes the late asthmatic response and the development of chronic airways inflammation.

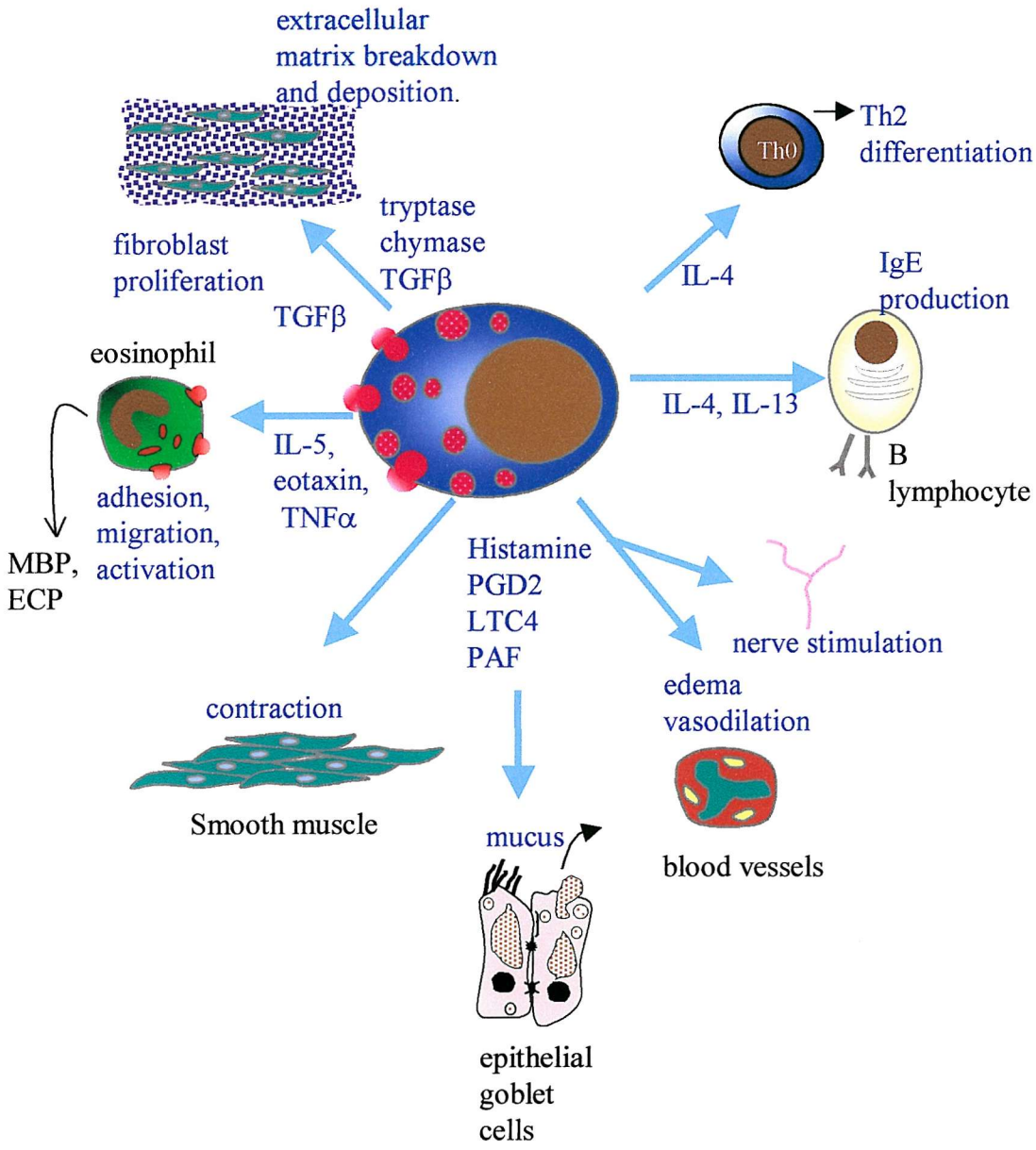


Figure 1.3 Mast Cell Products.
The highly diverse role of the products of mast cell degranulation. PAF, platelet activating factor; TNFα, tumour necrosis factor α.

1.2.5 *Eosinophils as Effectors of the Late Asthmatic Response*

One of the most prominent features of asthmatic airways is an accumulation of eosinophils where their levels been shown to correlate with disease severity [26]. This increase in eosinophils is apparent in both atopic and non-atopic asthmatics [7]. Mediators released from mast cells provide the necessary signals, for endothelial cells of the post capillary venules and leucocytes to promote transmigration of eosinophils across the vessel wall into the tissues [27]. This highly complex process is controlled by the timed expression of adhesion molecules on both the endothelial cells and eosinophils. Initial tethering of leukocytes to the endothelial wall involves upregulation of the adhesion molecule E-Selectin on the endothelial cells, which in turn, interacts with the sialyl-Lewis^x moiety on eosinophils. This interaction cannot anchor the cells against the blood flow and instead they roll along the endothelium continually making and breaking contact. This binding does however allow stronger interactions, which occur as a result of the induction of vascular cell adhesion molecule 1 (VCAM-1) on the endothelial cell [28]. TNFα and IL-4 act in synergy to induce VCAM expression on pulmonary microvascular endothelial cells [29], both of which are released from mast cells. Once expressed, VCAM interacts with the integrin, very late antigen 4 (VLA-4), which is constitutively expressed on the surface of eosinophils. Tight binding between these molecules arrests the rolling and allows the eosinophil to squeeze between the endothelial cells that form the wall of the vessel to migrate through the extracellular matrix along a chemoattractant gradient [16].

The mechanisms involved in eosinophil recruitment, priming, activation and survival are not well understood. However, cytokines which serve as chemoattractants, as well as activators, for eosinophils include GM-CSF [30], eotaxin [31] and IL-5 [32]. Similar to mast cells, eosinophils have granules within their cytoplasm. These store cytotoxic proteins such as major basic protein (MBP) and eosinophil cationic protein (ECP) which have the capacity to kill cells by making pores in their membranes, leading to osmotic lysis [33]. These proteins have been suggested to be a major cause of bronchial epithelial cell damage, thus rendering it more fragile [34]. Eosinophils also have the capacity to release lipid mediators such as LTC4, which, as described above, can cause airflow obstruction.

Whilst the eosinophil is thought to be the primary cytotoxic cell in inflamed airways, at the severe end of the disease spectrum neutrophils may also contribute to damage [35]. Accumulation of neutrophils within the airway mucosa can lead to the generation of

tissue damaging free radicals such as hydrogen peroxide and or superoxide$^{\cdot}$. These oxidants released from neutrophils may also modulate mucus production within the airways through activation of pathways that regulate cell growth and differentiation [36].

1.3 AIRWAY STRUCTURAL CELLS AND REMODELLING

As described in section 1.1.2 and depicted in figure 1.4, profound structural changes occur in the airways of subjects with asthma, such that the airways of these subjects are described as being remodelled. The features observed in asthmatic airways are epithelial cell damage, thickening of the *lamina reticularis* as a result of deposition of extracellular matrix (ECM) proteins, smooth muscle and vascular hyperplasia and oedema [37]. In the airway wall of asthmatic subjects, mucous glands may be present where they are normally absent and hypertrophy of these glands is likely to be the major cause of the excessive mucus production observed in fatal asthma. Goblet cell hypertrophy and hyperplasia are also features of the asthmatic airways, and may also result in increased secretion of mucus. This increased mucus secretion from these glands, along with the inflammatory exudates, increases the surface tension of the airways favouring airway closure [38]. This mucus also blocks the airways and its viscosity may reduce the efficiency of mucociliary clearance, all enhancing airway occlusion [39].

Patients with asthma develop airways hyperreactivity, where their airways respond to lower concentrations of inhaled spasminogens such as methacholine or histamine. They also exhibit airways hyperresponsiveness where the airways can narrow to a greater extent. In asthmatic subjects this is due partly to the increase in smooth muscle mass [38], which may be due to cell hyperplasia, cellular hypertrophy or reduced apoptosis [40]. Many growth factors and cytokines, some of which are expressed at increased levels in asthma, can act as mitogens for smooth muscle cells including epidermal growth factor (EGF) and endothelin-1 (ET-1) [41]. As with other cell types in the airways, smooth muscle cells upon proliferation undergo a phenotypic change to a synthetic phenotype which can result in the deposition of collagen [42]. This has the potential to contribute to the airway wall thickening observed in asthma. It is also possible that asthmatic smooth muscle cells have an intrinsic abnormality, which increases their proliferative potential [43].

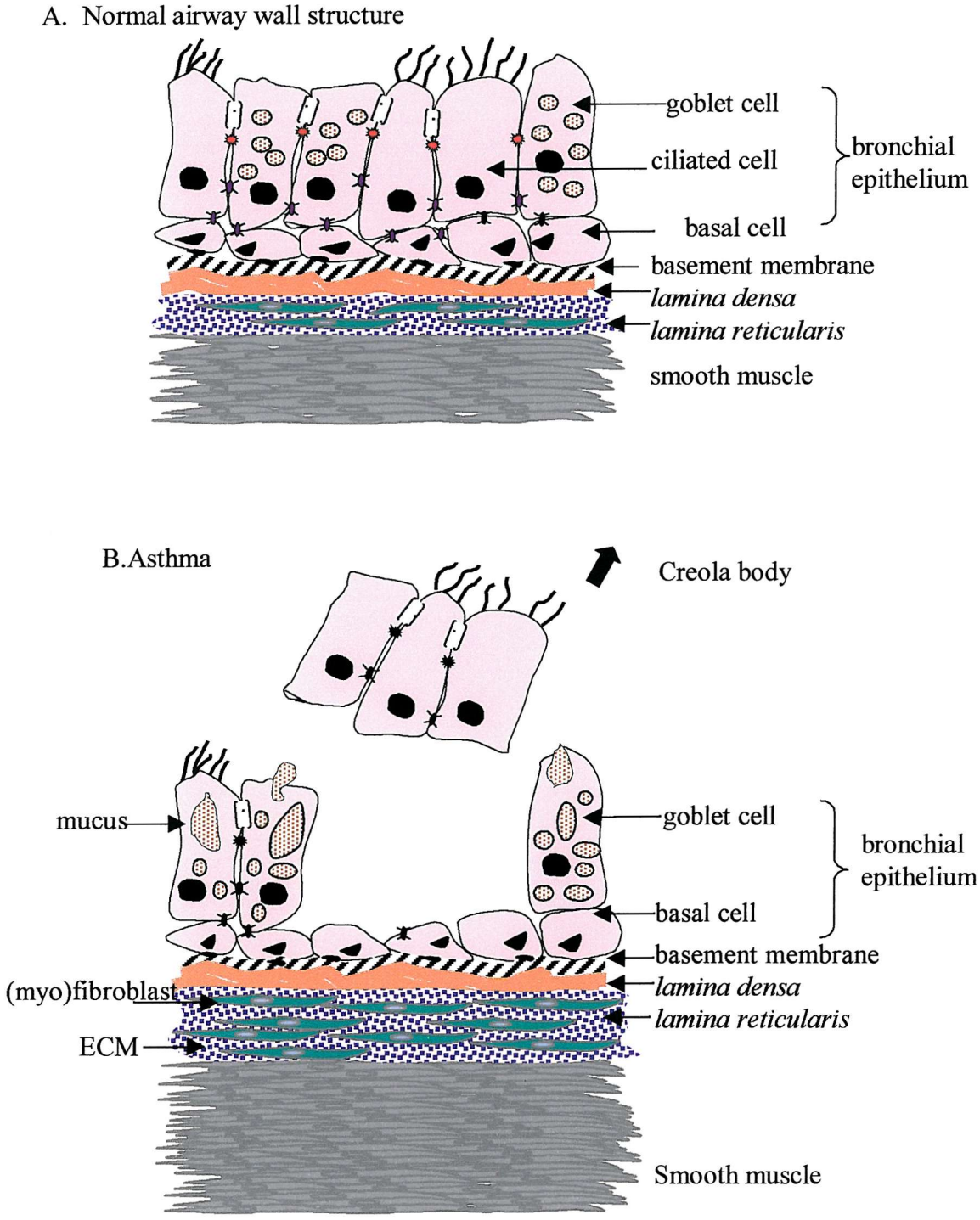


Figure 1.4 Airway Wall Structure.
This figure illustrates the components of the airway epithelium and the components of the underlying mesenchyme.
A. Normal airway wall structure. The integrity of the epithelium is maintained through cell junctions. tight junctions[ ], desmosomes, intermediate junctions, and hemidesmosomes.
B. In asthma the barrier function of the epithelium is impaired as a result of epithelial shedding. The *lamina reticularis* is thicker and the smooth muscle layer is hypertrophic, resulting in reduced airway caliber upon constriction.

In the airways of asthmatic subjects there are changes in the bronchial microcirculation, with oedema being a feature in *post mortem* studies of asthma deaths. In the airways, the blood vessels are adjacent to the epithelial basement membrane, and during the chronic inflammatory process they dilate and become permeable to allow the transmigration of inflammatory cells into tissue, which results in the exudation of plasma and subsequently oedema. Bronchial biopsies from patients with mild asthma are more vascular than those of normal control subjects, there are more vessels and asthmatic vessels are larger than those of control subjects [44]. It has been observed that newly generated vessels are hyperpermeable which would further increase the oedema due to the chronic inflammation [45]. There are many proinflammatory mediators and growth factors that can induce angiogenesis and vasodilation including vascular endothelial growth factor (VEGF) [46]. Also leukotrienes and prostanoids can induce vasodilation and microvascular leakage. However despite many factors, which induce microvascular leakage being present in asthma, it has proved difficult to study oedema in bronchial biopsies [46].

The consequences of all the features described above is increased airway wall thickness, which reduces the extent of bronchoconstriction required to cause airway closure [47]. For a long time, remodelling of the airways was considered to be a consequence of the damage evoked by the inflammatory cells in the airways. However it is now becoming apparent that that the airway structural cells are not passive, and can themselves release many cytokines and growth factors many of which could contribute to inflammation and airway wall remodelling.

1.3.1 *The Bronchial Epithelium*

Whilst asthma has classically been considered a disease of allergic origin, allergy and asthma are not always co-expressed [48], as demonstrated by individuals suffering from intrinsic asthma or atopic non-asthmatic subjects. Evidence from lung transplant studies has shown that non-asthmatic recipients of lungs from asthmatic subjects, develop asthma, whilst asthmatic subjects who receive lungs from non-asthmatic subjects lost their asthma [49]. This suggests that asthma is a local disease, and that an individuals structural cells have the potential to confer susceptibility to the disease. Recently, there has been much interest in the role of the bronchial epithelium in disease pathogenesis by acting as a central co-ordinator in airways inflammation and remodelling.

1.3.1.1 Structure of the Bronchial Epithelium

The bronchial epithelium has a polarised structure and forms a continuous layer throughout the bronchial tree. It consists of various cell types forming two layers as shown in figure 1.4. The lower layer of basal cells is in contact with the basement membrane, whilst the upper layer of, ciliated columnar cells, intermediate cells, neuroendocrine cells and mucus secreting goblet cells come into contact with the lumen of the airway [50]. These cells vary in distribution throughout the tree. In the main bronchi, ciliated columnar cells predominate, where, with goblet cells and basal cells, they form a stratified epithelium [51]. Here the sweeping action of the cilia combines with secretions from both goblet cells and mucoserous glands in the submucosa to form the so-called mucociliary escalator. This helps to expel particles from the airways and helps prevent damage to the more delicate lower airways. In the smaller peripheral branches of the airways, the epithelium becomes less complex, cilia are not present and goblet cells become less numerous. Here the epithelium becomes columnar and surfactant secreting Clara cells and neuroendocrine cells become more numerous [50;51]. The structural integrity of the epithelium is maintained through a network of adhesion molecules and junctional complexes, which link adjacent cells and adhere them to the underlying basement membrane, as shown in figure 1.4.

1.3.1.2 The Bronchial Epithelium as a Physiological Barrier

One of the primary functions of the bronchial epithelium is to act as a barrier to defend the airways against the entry of noxious substances such as gas, allergens and microorganisms. This defence is mediated in part via the integrity of the epithelium that contributes to the physical barrier. Mucus released from bronchial epithelial cells provides a protective layer over the epithelial cells by binding noxious agents and microorganisms, including viruses. The co-ordinated beating of cilia subsequently clears the trapped particles and this is aided by surfactant, which changes the surface charge properties, making the particles less sticky. Bronchial epithelial cells also secrete protective mediators to provide protection against injurious agents for example the antibacterial substance lactoferrin, anti-proteases, anti-oxidants and IgA. Bronchial epithelial cells also express the anti-oxidant enzymes, super oxide dimutase, catalase and glutathione peroxidase which can convert H_2O_2 into H_2O [52].

1.3.1.3 The Bronchial Epithelium in Asthma

1.3.1.3.1 Epithelial Damage

As depicted in figure 1.5, the bronchial epithelium undergoes extensive damage in asthma [53] and it has long been recognised that asthmatic subjects display a substantial degree of epithelial shedding [4]. The major structural site of this damage is between the columnar and basal cells and between adjacent columnar cells while the basal cells usually remain attached to the basement membrane [54]. Epithelial shedding leads to occurrence of large clusters of cells known as creola bodies in BAL fluid and it has been demonstrated that there is a significant inverse correlation between the number of epithelial cells in BAL fluid of asthmatic subjects and AHR [55]. Whether the degree of damage to the epithelium reflects either increased fragility or increased damage as a consequence of inflammation is unknown, but both factors are likely to play an important role. As described in section 1.2, inflammatory cells release a number of cytotoxic mediators which have the potential to damage the epithelium, indeed both activated T lymphocytes and eosinophils have been identified at increased levels in the epithelium of asthmatic subjects [56 26]. However, bronchial epithelial cells obtained from brushing asthmatic subjects have been reported to be less viable than those from normal control subjects [57] and thus it is possible to speculate that they also more susceptible to insults from the environment. In support of this hypothesis, is the finding that primary bronchial epithelial cells obtained from asthmatic subjects are more susceptible oxidant –induced injury, as determined by the number of cells entering apoptosis following treatment with hydrogen peroxidase, compared to those obtained from normal subjects [58]. Damage to the epithelium results in a reduction of the barrier function of the bronchial epithelium, allowing entry of potentially harmful environmental agents in the respiratory tissue. Also, loss of columnar cells reduces the amount of the protective mediators, described in section 1.3.1.2, which can be synthesised, as well as reducing the function of the mucociliary escalator.

Following epithelial damage and shedding, repair mechanisms are activated which result in migration of the surrounding columnar cells over the damaged area to cover it. This is followed by proliferation and differentiation of the cells into columnar cells and eventually restoration of the epithelium [59]. The mechanisms involved in epithelial repair have not yet been fully elucidated but during this process the epithelium displays a repair phenotype with increased expression of specific markers such as CD44 [60] and the epidermal growth factor receptor (EGFR) [61]. *In vitro* studies have demonstrated that rapid activation of the EGFR also occurs following damage. This in turn activates

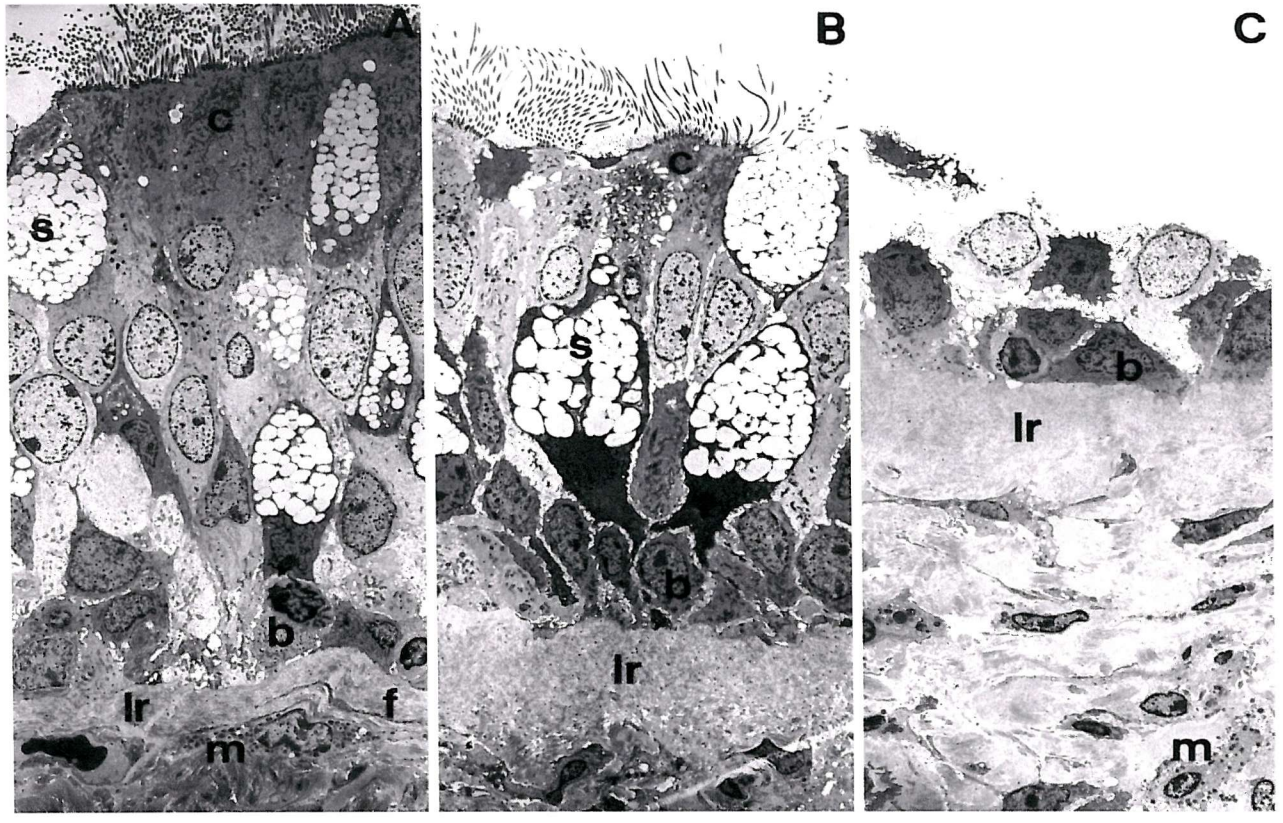


Figure 1.5 Ultrastructure of the Airway Wall.
Ultrastructure of normal (A) and asthmatic (B, C) bronchial mucosa. A and B, intact stratified bronchial epithelium composed of basal (*b*), secretory (goblet) (*s*), and ciliated (*c*) cells. Note the hypertrophic appearance of goblet cells and increased thickness of the *lamina reticularis* (*lr*) in asthmatic (B) bronchial mucosa. Mast cells (*m*) and fibroblasts (*f*) can also be seen. C, Disrupted appearance of bronchial epithelium frequently observed in asthma. (Original magnification x1600). Reproduced with kind permission from Holgate *et al* 2000.[98].

intracellular signalling pathways which produce proliferation signals and processes which are involved in cellular movement, both of which are necessary for repair [62]. Furthermore studies have revealed that whilst the addition of epidermal growth factor to wounded monolayers can accelerate repair, blockade of the EGFR inhibits both basal and EGF induced repair [61]. The role of the EGFR in these *in vitro* studies is interesting as it has also been shown that expression of this receptor *in vivo,* is increased in asthmatic subjects relative to healthy controls [61]. However the epithelium in asthma is not hyper-proliferative, perhaps as a result of increased expression of the cell cycle inhibitor $P21^{waf}$ in asthmatic subjects, [63]. Persistent up regulation of EGFR in the asthmatic epithelium suggests that these cells are in a constant repair phenotype. Furthermore, transcription factors such as NFκB are also upregulated in the asthmatic bronchial epithelium all indicating that the cells are stressed [64].

The repair process is also likely to involve a number of growth factors and indeed *in vitro* studies have shown that epithelial damage results in increased release of a number of growth factors. A study by Zhang *et al* showed that both chemical and mechanical damage to the epithelium resulted in increased release of basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), insulin like growth factor (IGF), ET-1 and transforming growth factor beta 2 ($TGF\beta_2$) [65]. All of these growth factors have the potential to contribute to the thickening of the *lamina reticularis* observed in asthma. Basic FGF, PDGF, and IGF are known to be potent mitogens for connective tissue cells including fibroblasts [65] [66]. Endothelin 1 can also stimulate fibroblast proliferation and acts as a chemoattractant for these cells [67]. Further studies by Morishima *et al*, have shown that induction of myofibroblast differentiation and proliferation by epithelial wounding is dependent on TGFβ [68]. The pro-fibrogenic effects of TGFβ are well documented [69] and are discussed in depth below. From studies such as these, it is possible to appreciate how mediators released from the epithelium may contribute to the pathological changes observed in asthmatic airways.

1.3.1.3.2 Pro-Inflammatory Potential of the Bronchial Epithelium

The bronchial epithelium has long been regarded as a passive barrier that is subjected to insults from the environment and inflammatory cells. However the bronchial epithelium itself is a significant source of many bioactive molecules, which have the potential to contribute to chronic airways inflammation and airway wall remodelling. Bronchial epithelial cells are an important source of cytokines, including IL-5, IL-8, IL-6, regulated

on activation normal T cell expressed and secreted (RANTES), GM-CSF, and eotaxin [70]. Amongst the roles of these proteins, is to act as attractants for a number of inflammatory cells including eosinophils and neutrophils. IL-8, GM-CSF, IL-6 and eotaxin are expressed at greater levels in asthmatic bronchial epithelial cells than in normals and it is possible that activation of bronchial epithelial cells serves to propagate inflammation [52]. Other chemoattractants released from bronchial epithelial cells that are increased in asthma include the arachidonic acid metabolites 15-hydroxyeicosatetranoic acid (15-HETE) and LTB_4, both of which are potent attractants for eosinophils, neutrophils monocytes and can induce mucus production. Expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) is increased on asthmatic epithelial cells thus aiding transmigration and localisation of leukocytes at the site of inflammation [70]. All of these mediators have the ability to sustain and propagate inflammation in the airways and may contribute to the remodelling effects observed. Therefore, it is possible to appreciate how the epithelium can play a central role to maintain the inflammatory response, as well as releasing mediators that can affect the underlying mesenchyme.

1.3.2 *Fibroblasts and Subepithelial Fibrosis in Asthma*

As stated in section 1.3, thickening of the *lamina reticularis* layer of the airway wall is apparent in asthmatic subjects (figure 1.5). This is due increased proliferation of connective tissue cells and increased synthesis of ECM proteins. Fibrosis within the *lamina reticularis* is present even in mild forms of the disease [71] and has been suggested to contribute to the overall airway narrowing, disease severity [72] and AHR [73].

The cells responsible for the fibrosis are reported to be myofibroblasts, whose numbers are in increased in asthma in proportion to the thickness of the *lamina reticularis* [74]. Thus, proliferation of myofibroblasts is one component of the fibrotic response. The hypothesis that the myofibroblast might be of importance in the subepithelial fibrosis observed in asthma was based on the findings that in the rare condition of collagenous colitis, which is characterised by a dense band of subepithelial fibrosis in the colonic mucosa similar to that observed in asthma, activated myofibroblasts were present [75]. Myofibroblasts have features of both fibroblasts and smooth muscle cells, and studies of colonic myofibroblasts have shown that these cells express desmin, myosin and filamin, all components of the contractile machinery found in smooth muscle cells [76]. Colonic myofibroblasts also contain numerous polyribosomes in their cytoplasm, suggestive of a synthetic phenotype [76]. Similarly ultrastructural analysis of bronchial myofibroblasts has

shown characteristic intracellular bundles of filaments, abundant rough endoplasmic reticulum and irregularly shaped nuclei [71].

In addition to the increased population of myofibroblasts, there also appears to be increased levels ECM proteins in the *lamina reticularis* of asthmatic subjects. ECM proteins are macromolecules which form a complex network that fills the extracellular space of the airway wall [45]. Increased deposition of the fibrous proteins, fibronectin, interstitial collagens of type I, III and V, but not type IV and VII have been reported in asthmatic subjects [77]. In addition to being present at increased levels, the density of the collagen bands is much greater in asthmatic subjects. Similarly, increased levels of tenascin have been observed in asthmatic subjects compared to controls [78].

The ECM is a dynamic structure, and equilibrium between synthesis and controlled degradation of ECM components is required for the maintenance of its homeostasis. Deposition of ECM proteins is an integral component of tissue repair and this requires degradation upon resolution of the damaged area. The finding that ECM proteins are persistently present at increased levels even in mild asthma [79] suggests that there is increased synthesis of these proteins, and that the airway wall is in a persistent state of repair in these individuals. One of the major elements controlling ECM build up are proteases, which degrade it and their inhibitors, which reduce this degradation. Whilst many proteases can degrade ECM molecules, the family of Zn^{++} matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), have been suggested as being the normal physiologically relevant mediators of ECM degradation [80]. The MMP family includes, collagenases, gelatinases, stromelysins, and membrane type MMPs. These can degrade many proteins including collagen and fibronectin, which are both present at abnormally high levels in the airway wall of asthmatic subjects. Thus it has been hypothesised that an imbalance between the regulation of MMPs and TIMPs may make an important contribution to subepithelial fibrosis. Of the MMPs, MMP-9 has been found to be the prominent MMP in BAL of asthmatic subjects following antigen challenge [81]. Furthermore, the levels of both MMP-9 and TIMP-1 were found to be increased in bronchial biopsies from asthmatic subjects compared to controls. However the levels of MMP-9 were significantly greater than that of TIMP-1 in the biopsies analysed suggesting that in asthmatic subjects suggesting that there is an imbalance between the MMPs and the TIMPs [82]. In other studies of this kind, the ratio of TIMP-1: MMP-9 is increased in unstable asthma [80], suggesting that in asthma, there is inappropriate activation of repair

mechanisms. Therefore it seems plausible that dysregulation of the MMPs and the TIMPs could factor in the increased levels of ECM molecules which are deposited in asthma.

1.3.2.1 TGFβ as an Inducer of Subepithelial Fibrosis in Asthma

As described in section 1.3.1.3.1, damage to the epithelium results in the release of several pro-fibrogenic growth factors, which can stimulate myofibroblast differentiation and ECM secretion. Of the growth factors released, TGFβ has received much attention regarding its pro-fibrogenic effects, which are well documented in diseases such as, liver fibrosis and renal interstitial fibrosis. However, despite its potentially pathological effects, TGFβ plays a fundamental role in wound healing as demonstrated by the finding that dermal wound healing in rabbits was significantly increased when collagen scaffolds impregnated with TGFβ were applied to the wounds [83]. Thus, TGFβ has both physiological and pathological functions and as a result its bioactivity is highly regulated at all levels.

TGFβ exists as three isoforms, designated $TGF\beta_{1, 2}$ and $_3$. $TGF\beta_1$ is thought to be the main isoform secreted from mesenchymal cells such as fibroblasts and smooth muscle, whilst $TGF\beta_2$ is the major isoform secreted from epithelial cells [65]. As depicted in figure 1.6, TGFβ is synthesised as a large precursor molecule that is proteolytically cleaved by endoproteases such as furin, in the Golgi apparatus to produce mature TGFβ and an amino terminal remnant termed latency-associated peptide (LAP). TGFβ and LAP remain associated by non-covalent interactions, and LAP prevents TGFβ from binding to its receptors. These latent TGFβ complexes are significantly more stable than bioactive TGFβ. Within the Golgi, LAP covalently interacts with latent TGFβ binding proteins (LTBPs). Four types of LTBPs have been cloned and alternative splicing is common. In the lung it is LTPB-2 that is expressed at the greatest levels [84]. The existence of four different LTBPs with only partially overlapping expression patterns suggests important functions in different tissues. The LTBPs are thought to be necessary for the secretion and stability of TGFβ [85]. They also act to direct TGFβ to ECM where the large latent TGFβ molecules can be stored through interactions of the LTBP with ECM proteins such as decorin and biglycan. Interactions with biglycan and decorin reduce the activity of TGFβ, possibly by protecting it from the proteolysis necessary for its activation [85]. Other proteins such as α2 macroglobulin also act to reduce binding of TGFβ to its receptors [86].

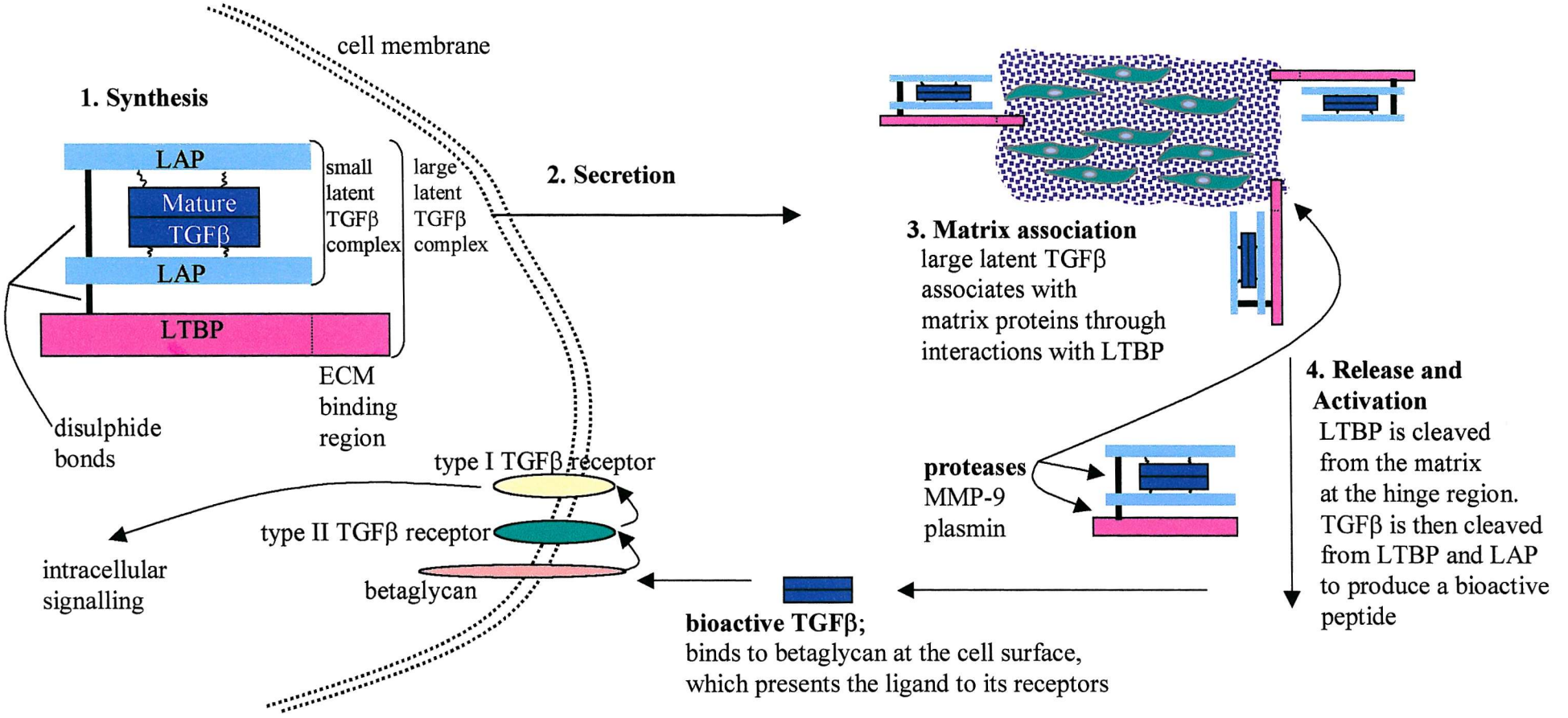


Figure 1.6 Schematic Representation of TGFβ Release and Activation.
TGFβ is released from the cell as a part of a large latent molecule, composed of mature TGFβ, latency associated peptide (LAP) and latent binding protein (LTBP). The large complex binds to extracellular proteins such as decorin, through interactions with a specific region of the LTBP. This sequesters TGFβ to the matrix. Release of bioactive TGFβ involves cleavage from the extracellular matrix and cleavage of LTBP and LAP. TGFβ subsequently binds to betaglycan at the cell surface, which in turn presents it to functional receptors resulting in signal transduction.

The production of bioactive TGFβ involves release from the ECM by cleavage of the LTBP at protease sensitive sites. The cleavage of LAP from TGFβ also probably occurs at the same time. Proteases which can cleave TGFβ to give rise to bioactive molecules include plasmin, mast cell chymase, leukocyte elastase, thrombospondin and more recently MMP-9. Physicochemical methods of activation include an acidic cellular microenvironment as would be the case during wounding and reactive oxygen species which can be released from several inflammatory cells including eosinophils and neutrophils [84]. The effect of alternative splicing of LTBPs on the storage of TGFβ in the ECM and its activation is unknown, but it has been reported that sites involved in these two processes are deleted or mutated in some variants [87]. Once activated, TGFβ binds to betaglycan (formerly the type III TGFβ receptor), which acts to present the ligand to the constitutively active type II TGFβ receptor kinase. In turn, binding of TGFβ to the type II receptor results in phosphorylation of the type I TGFβ receptor kinase, which then activates intracellular signalling molecules. The central signalling molecules in the TGFβ signalling pathway are the SMAD proteins, which reside in the cytoplasm. Phosphorylation of SMAD2 or SMAD3 by the type I receptor kinase results in heterodimerisation with a common subunit SMAD4. These activated complexes then translocate to the nucleus where they act as transcription factors [88 89 90].

TGFβ is present at greater levels in BAL fluid recovered from asthmatic subjects both at baseline and following antigen challenge [91]. It has also been shown that TGFβ is expressed at greater levels in both epithelial and mucosal cells of bronchial biopsies obtained from asthmatic subjects compared to normal controls [92]. Furthermore, a positive correlation was found between the number of epithelial or submucosal cells expressing TGFβ and basement membrane thickness and fibroblast number [92]. *In vitro* TGFβ is a potent inducer of fibroblast proliferation and myofibroblast differentiation, as determined by ultrastructural analysis of cells. These changes are also accompanied by an increase in collagen I gene expression [93]. Increased expression of α-smooth muscle actin, a marker of myofibroblast differentiation, has also been reported in rats treated subcutaneously with TGFβ [94]. Treatment of myofibroblasts with $TGF\beta_2$ also results in the release of VEGF and ET-1 [93], which can cause increases in smooth muscle and microvasculature. Another mechanism by which TGFβ may contribute to increased myofibroblast number is through inhibition of IL-1β-induced apoptosis [95]. In addition to inducing deposition of ECM, in certain cell types TGFβ has been implicated in the inhibition of MMPs which breakdown ECM and upregulation of the TIMPs [96 97].

1.3.3 *Epithelial Mesenchymal Trophic Unit*

It is thus apparent that mediators released from the epithelium following damage can act on the underlying mesenchymal cells, the fibroblasts, myofibroblast, smooth muscle cells and blood vessels, causing cellular proliferation or differentiation, which could potentially result in airway remodelling. Communication between the epithelium and the mesenchymal cells suggests that the two cell types work as a trophic unit and may involve mechanisms similar to those that drive physiological tissue remodelling during foetal branching morphogenesis [98]. The extensive deposition of collagens and other matrix proteins in the *lamina reticularis* in asthma has led to the suggestion that the processes involved in lung development are reactivated in asthma but that the set point for communication between the compartments, leading to a subsequent increase in mesenchymal volume [98].

It is also likely that interactions between the inflammatory cells and the airways structural cells also contribute to disease pathology. Mediators released from inflammatory cells have the potential to act on the airway epithelium to stimulate further mediator release, which can contribute to both airways inflammation, through recruitment of inflammatory cells and possibly remodelling, through the release of pro-fibrogenic growth factors. Thus, the pathogenesis of asthma is highly complex and to begin to understand it, the interactions between all the different components must considered. This concept is shown in figure 1.7.

1.4 Th2 CYTOKINES

As detailed throughout section 1.2, Th2 cytokines play a critical role in orchestrating the asthmatic inflammatory response and contribute to the overall pathogenesis of asthma. The Th2 cytokines include IL-4, IL-5, IL-9, IL-13 and GM-CSF and their genes are found on chromosome 5q [99] a region has been linked to increased serum IgE and AHR [100 101]. Whilst all Th2 cytokines probably have a role in disease pathology, there is substantial evidence *in vivo* to indicate a crucial role for IL-4 and IL-13. Studies in humans have shown that treatment with a soluble form of the IL-4 receptor produces a significant improvement in forced expiratory volume (FEV_1) and reduced β_2 agonist use [102]. However, whilst eosinophilia is a prominent feature of the airways of asthmatic subjects and the role of IL-5 in this process is well recognised, treatment with anti-IL-5 blocking

antibody whilst reducing the numbers of these cells in the airways, had no effect on AHR [103]. This study brings into question the importance of eosinophils in the development of asthma symptoms. Genetic studies also provide strong evidence for dysfunction of IL-4 and IL-13 in relation to asthma and atopy. Polymorphisms within the genes encoding IL-4 and IL-13 have been mapped, for example a 590 C/T mutation has been linked to elevated IgE levels and enhanced IL-4 activity *in vitro*. Similarly polymorphisms within the gene encoding IL-13 have been identified which influence mRNA transcription and receptor binding [104].

1.4.1 *IL-4 and IL-13*

IL-4 protein is composed of 129 amino acids with a molecular weight of 15-19kDa. It was first identified in 1982, based on its ability to enhance IgM induced DNA synthesis in resting murine B lymphocytes [105]. However, like most other cytokines it has subsequently been shown to be pleiotropic in its actions. For example it also causes induction of MHC class II molecules and CD23 [106]. IL-13 was first described in 1993 [107] and like IL-4 it was also found to stimulate B cell proliferation, MHC class II expression and CD23 expression [108]. It has a molecular mass of 12kD and despite having a largely similar effector profile as IL-4, it shares only 25 % homology but is structurally quite similar [99]. The reason for their overlapping functions is due to shared use of a receptor system and signalling pathway by these two cytokines. However, the most apparent difference between IL-4 and IL-13 is the inability of IL-13 to induce Th2 cell differentiation and this is due to lack of IL-13 receptor expression by T lymphocytes [109] [110]. Both IL-4 and IL-13 are now known to exert their actions on a number of immune effector cell types, in addition to B cells and in the case of IL-4, T cells, including natural killer cells, mononuclear phagocytes and granulocytes [99].

1.4.2 *IL-4 and IL-13 in Asthma*

Since it was discovered that IL-4 is essential for the generation of the Th2 clones, and both IL-4 and IL-13 are involved in the generation of IgE, these two cytokines have received much attention regarding their role in the pathogenesis of asthma. Bronchial biopsies of atopic and non-atopic asthmatic subjects show an increased number of cells containing IL-4 mRNA and protein [111], with the major site of IL-4 expression been $CD4^+$T lymphocytes, eosinophils and mast cells [112]. Indeed, these three cell types all infiltrate the airway mucosa in both atopic and non-atopic asthmatic subjects [20].

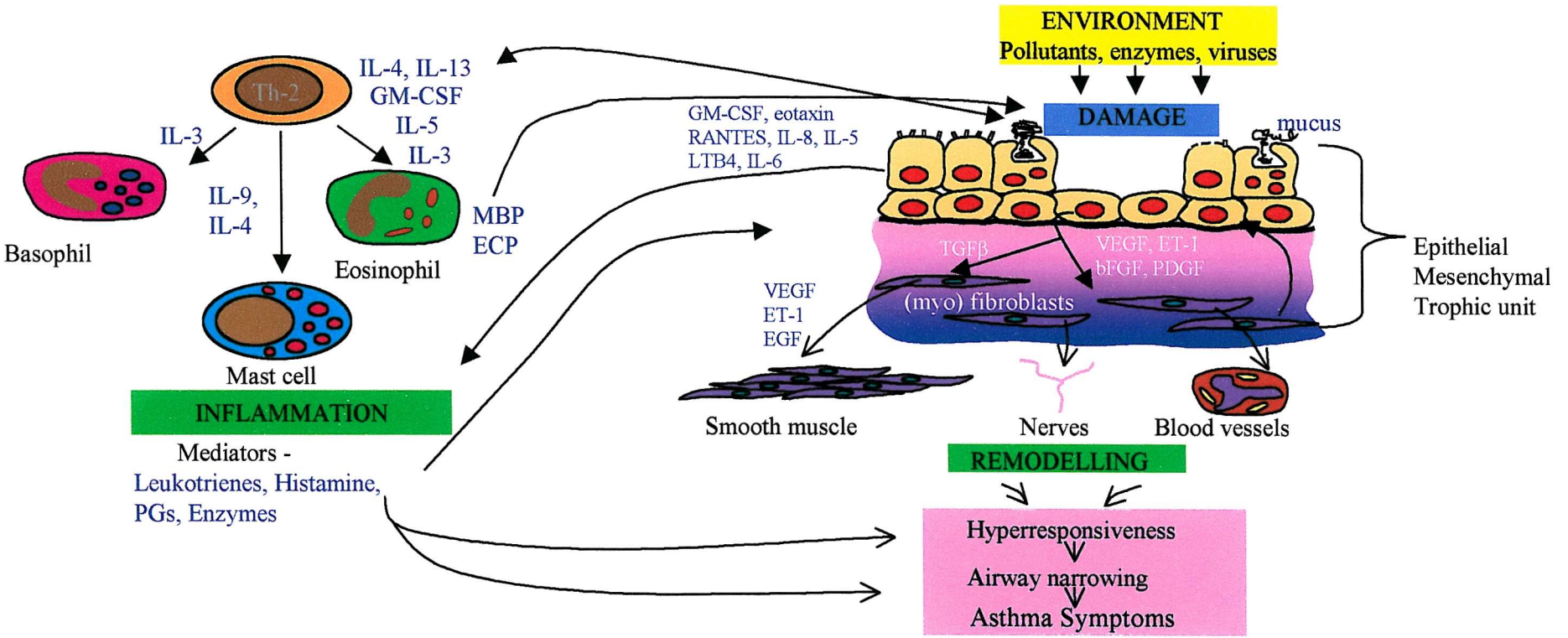


Figure 1.7 Schematic Representation of the Interactions Between Airway Structural Cells and Inflammatory Cells In Asthma. This figure illustrates how damage to the epithelium can activate the underlying mesenchymal cells which in turn can release growth factors that propagate and amplify the remodelling response. Inflammatory cells and mediators also impinge on this system giving rise to asthma. Reproduced with kind permission from Holgate et al.,2000. [98].

Similarly, increased levels of IL-13 mRNA has been observed in the bronchial mucosa of atopic and non-atopic asthmatics compared to healthy controls [113]. Furthermore greater concentrations of both IL-4 and IL-13 are recovered in BAL fluid collected from asthmatic subjects [12] following antigen challenge. Therefore, in asthmatic subjects there are many sources of IL-4 and IL-13, which could potentially contribute to pathological changes.

Further evidence for the importance of IL-4 and IL-13 in asthma, comes from animal models of allergic disease. Mice expressing a lung specific IL-4 transgene display epithelial cell hypertrophy with an increase in mucin positive cells [114] increased numbers of inflammatory cells in their bronchoalveolar fluid and increased base line airway resistance [115]. IL-13 transgenic mice also display these characteristics, but in addition, show increased production of the eosinophil chemoattractant eotaxin and subepithelial fibrosis [116], compared to a modest deposition of collagen in the airways of IL-4 transgenic mice [114]. Similarly administration of recombinant IL-13 to mice induces AHR and eosinophil influx [117]. However the involvement of eosinophils in the development of AHR has recently come under question, based on the finding that targeted deletion of IL-13 in mice abolished AHR, despite the presence of eosinophilic inflammation [118]. This is in keeping with the finding that whilst blockade of IL-5 in humans reduces the number of eosinophils in the airways, it does not affect AHR [103]. The phenotype of the transgenic mice contrasts with the features observed in IL-4 knockout mice which are unable to generate Th2 cytokines or IgE [119]. Similarly, blockade with a neutralising protein, results in attenuation of allergen induced airway hyperresponsiveness eosinophil influx and goblet cell metaplasia in sensitised mice [120].

1.5 IL-4 AND IL-13 SIGNALLING

The biological effects of IL-4 and IL-13 are brought about thorough complex intracellular signalling pathways involving several molecules. The initial stage involves activation of IL-4 and IL-13 receptors upon ligand binding, which initiate a series of phosphorylation events involving associated tyrosine kinases, termed Janus kinases (JAKs). This process ultimately leads to activation of the signal transducer and activator of transcription (STAT6) and insulin receptor substrate 1 (IRS-1) and IRS-2, which are thought to control gene expression and proliferation respectively [121] [122] (figure 1.8). However, as will be

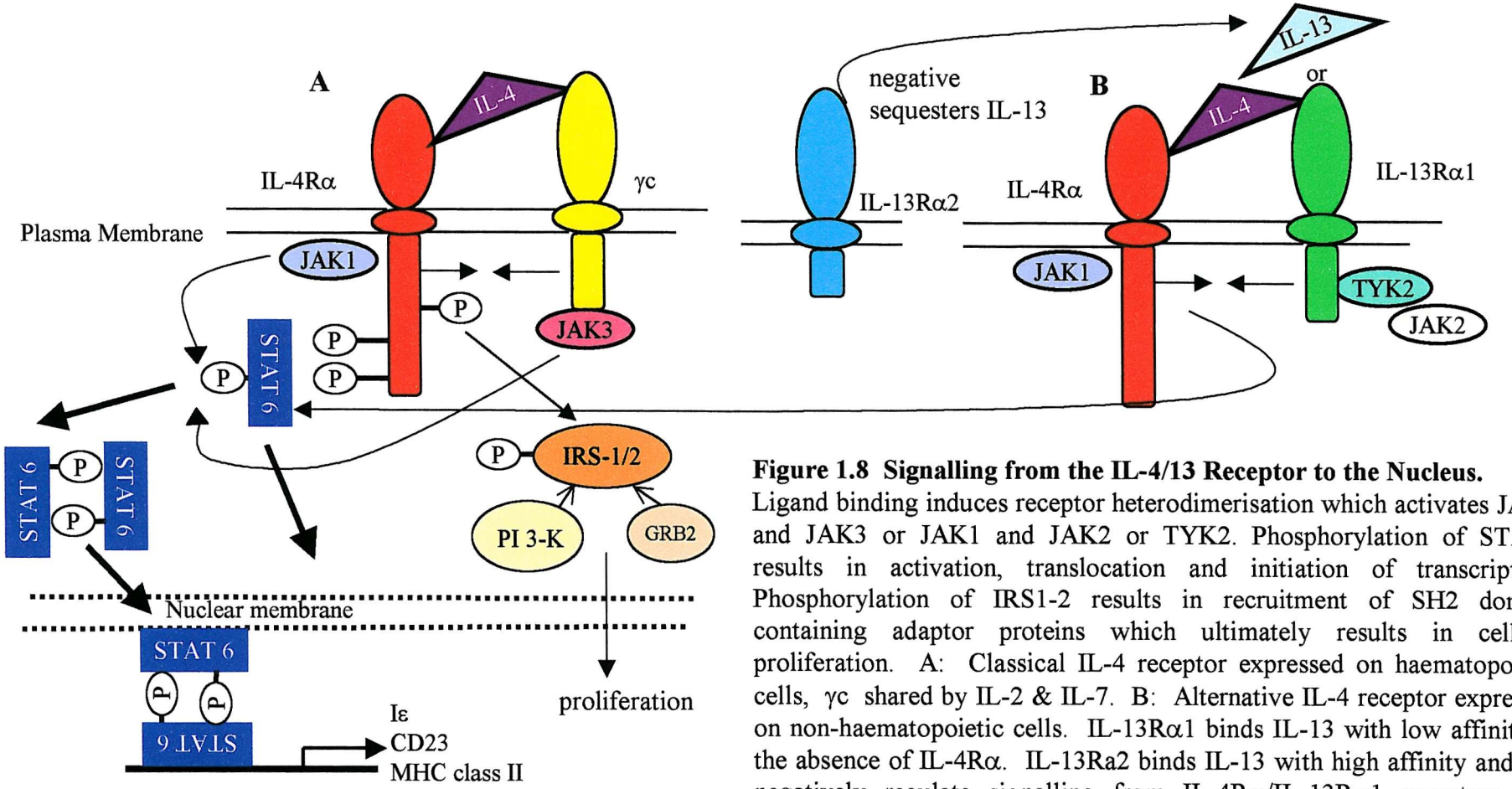


Figure 1.8 Signalling from the IL-4/13 Receptor to the Nucleus. Ligand binding induces receptor heterodimerisation which activates JAK1 and JAK3 or JAK1 and JAK2 or TYK2. Phosphorylation of STAT6 results in activation, translocation and initiation of transcription. Phosphorylation of IRS1-2 results in recruitment of SH2 domain containing adaptor proteins which ultimately results in cellular proliferation. A: Classical IL-4 receptor expressed on haematopoietic cells, γc shared by IL-2 & IL-7. B: Alternative IL-4 receptor expressed on non-haematopoietic cells. IL-13Rα1 binds IL-13 with low affinity in the absence of IL-4Rα. IL-13Ra2 binds IL-13 with high affinity and can negatively regulate signalling from IL-4Rα/IL-13Rα1 receptors. A receptor composed of IL-13Rα1/IL-4Rα/γ2 has also been proposed (not shown).

described in detail below, STAT6 is considered to be the major intracellular signalling pathway activated selectively by IL-4 and IL-13.

1.5.1 *IL-4 and IL-13 Receptors*

The effects of IL-4 and IL-13 involve signalling through their receptors, which are expressed at the cell surface. The IL-4 receptor (IL-4R) is a complex composed of at least two distinct subunits, one of which is the 140kDa IL-4 high affinity binding subunit termed IL-4Rα [123]. This chain is composed of 800 amino acids with 207 forming the extracellular domain, 24 forming the transmembrane domain and 569 forming the intracellular domain [121]. In haematopoietic cells IL-4Rα associates with the γ chain of the IL-2 receptor now termed the common γ chain (γ_c) [124] forming the classical form of the receptor. Expression of γ_c increases the binding affinity of IL-4 by 2-3 fold [121]. In non-haematopoietic cells γ_c is generally not expressed and the IL-4Rα chain associates with the IL-13Rα1 chain to comprise the alternative IL-4 receptor [125 126].

To date two IL-13 receptor chains have been cloned, IL-13Rα1 and IL-13Rα2. The 65-70kDa IL-13Rα1 protein, has a low affinity for IL-13 when expressed alone in a fibroblast cell line but when co-expressed with IL-4Rα the affinity of binding of both IL-4 and IL-13 was increased [127]. This binding was blocked by adding an excess of either IL-4 or IL-13 indicating the specificity of the ligands [128]. The latter receptor binds both IL-4 and IL-13 with high affinity, and this is dependent on expression of IL-4Rα [127]. In contrast the IL-13Rα2 chain has a high affinity for IL-13 and was first identified in a renal carcinoma cell line for its ability to bind IL-13 with high affinity [129]. This binding was diminished by IL-13 but only partly by IL-4 [128] suggesting that the IL-13α2 chain is not a component of the IL-4 receptor. It has recently been proposed that the IL-13α2 chain acts as a decoy receptor, based on the finding that upon binding to IL-13 it is internalised at a high rate and does not activate the down stream signalling molecule STAT6 [130]. Whether the IL-13α2 chain is expressed as a homodimer, or in association with other receptor chains in unknown, but studies have shown that when IL-13Rα2 was co transfected with IL-4Rα1 and IL-13Rα1, STAT6 activity was decreased [130]. Other forms of IL-13 and IL-4 receptors have been described in transfection experiments as functional, for example a combination of IL-13Rα1, IL-4Rα and γ_c. Expression of all three chains, forms a receptor which can bind both IL-4 and IL-13 with high affinity in

some cell types [131]. However it has been reported that in some cell types the expression of γc with IL-4Rα and IL-13Rα1 results in a dramatic decrease in IL-13 binding [132].

1.5.2 *Ligand Binding Induces Activation of the Constitutively Associated Tyrosine Kinases*

IL-4 and IL-13 receptors lack consensus sequence motifs for tyrosine kinase activity [133] and thus signal transduction is initiated by tyrosine kinases which physically associate with the cytoplasmic domains of the receptor. In this receptor system, as with many other cytokines, it is members of the Janus family of tyrosine kinases, the JAKs, which carry out this function.

The JAK family of tyrosine kinases comprises JAK1, JAK2, JAK3 and TYK2. Different JAKs are activated by IL-4 and IL-13 which is due to their differential association with the different receptor chains [134]. Treatment of haematopoietic cells with IL-4 or IL-13 results in activation of JAK1, however, only IL-4 and IL-2, and not IL-13 induces JAK3 activation [135]. These results indicate that JAK3 associates with the γ_c chain [136], where as JAK1 [137] is more widely distributed and is associated with a receptor component common to both IL-4 and IL-13, the IL-4Rα chain [128]. Similarly in non-haematopoietic cells JAK1, JAK2 and TYK2 are rapidly activated when treated with IL-4 or IL-13 [138]. Thus JAK1 associates with the IL-4Rα chain, JAK3 with the γ_c chain and JAK2 or TYK2 with the IL-13Rα1 chain. However as with the receptor chains, it is likely that the JAK involved in signalling varies depending on the cell type. Activation of the JAKs is necessary for phosphorylation of the downstream signalling molecules STAT6 and IRS-1. However, as described below, there is evidence to suggest that STAT6, especially with regard to asthma, is the major signalling pathway activated by IL-4 and IL-13.

1.5.3 *Signal Transducers and Activators of Transcription*

STATs are a family of latent cytoplasmic transcription factors which are activated mainly by cytokine receptors, but are also utilised by non-cytokine receptor families, for example the epidermal growth factor receptors [139]. To date, seven STAT proteins have been identified, STAT1,2,3,4,5a,5b, and 6. Not all of these molecules are coded for by the same gene but the STAT proteins share several conserved structural domains. The SH2 domain is conserved between the family members and is essential for recruitment of STAT molecules to the receptor, interaction with JAKs and STAT dimerisation and

associated ability to bind to DNA. The DNA binding domain, within STAT molecules is a highly conserved area unlike those observed in other DNA binding proteins. Similarly the N-terminus is highly conserved and critical to STAT function. Gene expression is believed to be regulated in some way by the carboxy terminus of the STAT proteins, which is highly divergent amongst the family members and could explain how they all bind very similar DNA sequences but affect gene expression uniquely [140]. The activation profile of the STATs does vary; STAT1, 5a and 5b been activated by a number of ligands whilst STAT2, STAT4 and STAT6 are selectively activated by IFN-α, IL-12 and IL-4/IL-13 respectively. Despite this the STATs participate in a number of diverse biological responses.

1.5.3.1 STAT6

STAT6 is the major down stream signalling molecule activated by IL-4 and IL-13, and this accounts for their overlapping effector profile. STAT6 was initially cloned in 1994 [141] based on the identification of an IL-4 induced DNA binding factor which was necessary for transcription of the CD23 and IgG heavy chain germline epsilon genes [142] [143] [144]. Subsequent characterisation revealed STAT6 to be composed of approximately 850 amino acids with a highly conserved tyrosine residue at position 641 and a molecular weight of 105 kDa [145]. STAT6 resides in the cytoplasm and upon activation of the IL-4/IL-13 receptor, it is phosphorylated on tyrosine at position 641 [146]. Studies using a cell line deficient in the IL-4/IL-13 receptor associated tyrosine kinase, Jak1, have revealed that STAT6 phosphorylation is dependent on this tyrosine kinase [147]. Following dimerisation and translocation to the nucleus, STAT6, binds to specific recognition sequences termed gamma interferon activation site (GAS) elements [148], like other members of this family. The sequence recognised by STAT6 is TTN_6AA a sequence found in the promoter of a number of IL-4 responsive genes including the genes for MHC class II molecules, CD23 and the constant region of the IgE heavy chain.

Three naturally occurring variants of STAT6 exist, STAT6a (referred to as STAT6), STAT6b and STAT6c [149]. STAT6b has an NH_2 terminal truncation of at least 110 amino acids, which reduces its molecular weight from 100kDa to 95Kda. Studies show that this variant is not phosphorylated to the same extent and demonstrates reduced transcriptional activity. STAT6c exhibits an SH_2 domain deletion and is not phosphorylated in response to IL-4 or IL-13. This isoform is unable to form dimers, cannot regulate transcription and can reduce the activity of STAT6 by an unknown mechanism. Of the isoforms STAT6 is

believed to be the most abundant in human tissues with the dominant negative being present in greater amounts in the lung compared to other human tissues[149]. In addition, a novel STAT6 isoform has been identified in mast cells, which binds to the STAT6 consensus sequence but migrates at a faster, mobility due to its reduced molecular weight of 65kDa. This variant is not detected by antibodies raised against the carboxy terminus suggesting that this is where the truncation is [150]. How initiation of transcription by this isoform differs from others has yet to be determined, but the carboxy terminus of STAT molecules has been shown to influence transcriptional activity.

1.5.3.2 Role of STAT6 in IL-4 and IL-13 Mediated Biological Functions and Asthma.

STAT6 has been shown to map to mouse chromosome 10, bands q11-1 to q22 [139], and thus to dissect the role of STAT6 in IL-4 signalling, it has been possible to generate mice deficient in this molecule by gene targeting. The first of these studies revealed that STAT6 deficient (STAT $6^{-/-}$) mice are unable to increase expression of CD23 or MHC class II molecules in response to IL-4 unlike the wild type. Proliferation of splenic B lymphocytes isolated from the $STAT6^{-/-}$ mice and incubated with IL-4 was also impaired and T lymphocytes were unable to differentiate into the Th2 phenotype [151]. These studies indicate the essential role of STAT6 in IL-4 mediated functions, including those such as B cell proliferation, which were previously thought to be mediated primarily by IRS-1.

Transcription factors are believed to play an important role in amplifying the inflammatory process in disease, due their ability to switch on inflammatory genes leading to increased synthesis of inflammatory proteins [152]. More recent studies on STAT6 knockout mice have revealed that STAT6 is likely to play a major role in mediating the pro-inflammatory responses of IL-4 and IL-13, and thus makes a significant contribution to disease pathology. STAT $6^{-/-}$ mice demonstrate strikingly contrasting characteristics to the IL-4 and IL-13 transgenic mice. These mice do not develop AHR and have reduced levels of the Th2 cytokines IL-4 and IL-5 in BAL upon challenge with the bronchoconstrictor acetylcholine [153]. $STAT6^{-/-}$ mice show reduced levels of serum IgE and, in contrast to wild type sensitised mice, no increase is observed upon treatment with antigen. Furthermore, STAT $6^{-/-}$ mice are protected from an increase in mucus containing epithelial cells in response to antigen [153]. Measurement of cells in BAL fluid collected from these mice showed reduced numbers of eosinophils in one study [153] with eosinophilia being completely abolished in another [154]. This is likely to be due to the reduced levels of the Th2 cytokines necessary for recruitment of eosinophils. In contrast,

STAT6$^{-/-}$ mice did not show reduced levels of lymphocytes or macrophages and the level of expression of the adhesion molecule VCAM was not affected. Overall, lung histology of wild type sensitised mice showed patchy inflammatory infiltrates around the small bronchi, bronchioles and blood vessels. This inflammatory response was abolished in STAT6$^{-/-}$ mice [154]. From these studies alone it is possible to postulate the pivotal role of STAT6 in mediating many of the inflammatory processes in the lungs observed in asthma. In addition to these animal models, mice lacking B cell lymphoma gene-6 (BCL-6), a transcription factor which binds to DNA sequences very similar to those recognised by STAT6, have increased levels of STAT6 activity also display Th2 like inflammation with increased numbers of Th2 cytokines in their BAL [155]. Further evidence for the role of STAT6 in the pathogenesis of asthma is that the STAT6 gene is assigned to chromosome 12q, at a site of genetic linkage to asthma [156] and polymorphism within the STAT6 gene on chromosome has been associated with atopy in a Japanese population [157].

1.5.4 *Activation IL-4 and IL-13 Receptors*

When IL-4 and IL-13 bind to their receptors they induce receptor dimerisation. This activates the JAKs by bringing them into close proximity such that they activate each other by cross phosphorylation of tyrosine residues. Phosphorylation of the tyrosine residue within the catalytic domain allows access to the substrate, i.e. tyrosine residues on the cytoplasmic domain of the IL-4Rα chain [158 159]. The phosphotyrosine residues on the cytoplasmic domain of the receptor serve as docking sites for the latent cytoplasmic transcription factor STAT6 [151], which binds to the receptor via its SH2 domain [160], and IRS-1 [161] (figure 1.8). It has however, been demonstrated that STAT6 and IRS-1 are dependent on phosphorylation of different residues within IL-4Rα for their activation suggesting differential control of gene expression and proliferation respectively. There are reports that phosphorylation of a tyrosine at position 497 is essential for IRS-1 activation [161], whilst STAT6 activation is dependent on tyrosine residues within the 557-657 region of the receptor [162]. However the region to which STAT6 and IRS are recruited is likely to vary depending on the cell type, as there are reports that STAT6 activation is dependent on amino acids between position 353-393 of the IL-4Rα chain [163].

Recruitment of STAT6 and IRS-1 to the receptor brings them into contact with the JAKS, and, like the receptor, they too become phosphorylated on tyrosine. Phosphorylation of STAT6 on a specific tyrosine residue at position 641 is believed to provide a mechanism by which two STAT6 molecules can form homodimers through reciprocal SH2 domain

phospho-tyrosine interactions [164]. These dimers then translocate to the nucleus where they bind to specific recognition sequences within the promoter region of genes [139 140].

Activation of IRS-1 is essential for proliferation of haematopoietic cells [165] and causes activation of down stream signalling molecules such as of Grb-2 and the p85 subunit of phosphatidyl inositol 3-kinase (PI_3 kinase) [166 122], which are involved in mitotic responses. Activation of STAT6 is not impaired by the absence of IRS-1, nor is its binding to GAS responsive elements on target genes [167]. In some cell types, activation of STAT6 is not sufficient to induce cellular proliferation [167] but in STAT6 null animals IL-4 induced B cell proliferation is impaired [151]. The latter suggests that there may be complex interactions between the two signalling pathways, which vary depending on the cell type and species studied. More recently it has been demonstrated that, in human lung fibroblasts, IL-4 and IL-13 activate extracellular signal-related kinase (Erk) and c-Jun NH_2-terminal kinase (JNK), which are components of the mitogens activated protein (MAP) kinase cascades [168]. The MAP kinase pathway has been associated with asthma [169], and thus there is the potential for cross talk between the pathways.

The mechanisms which control IL-4 and IL-13 signalling are not well understood, and like many other components of the signalling cascade are likely to differ depending on the cell type. Transfection of NIH 3T3 cells with the protein tyrosine phosphatase, SH2-containing tyrosine phosphatase –1 (SHP1) has been reported to substantially reduce STAT6 phosphorylation in response to IL-4 [170]. Similarly the tyrosine phosphatase SHP2 and the inositol phosphatase, SH2-containing inositol 5'-phosphatase (SHIP), also associate with immunoreceptor tyrosine based inhibitory motifs (ITIM) located within IL-4Rα [171]. However the role of SHIP in regulating IL-4 signal transduction remains unclear. Transfection of cells with SHIP enhances cell proliferation, but it has no effect on the activity of IRS-1 or STAT6 [172]. Therefore it has been hypothesised that SHIP positively regulates signalling through interactions with components of the phosphatidyl inositol pathway, unlike in other cytokine receptor systems. Another molecule which can regulate IL-4/IL-13 mediated biological responses is the transcriptional regulator BCL-6. It has been shown that cells transfected with this proto-oncogene product have reduced CD23 expression and its inducibility by IL-4 is abolished. [155].

Cytokine signalling can also be regulated by a family of inducible proteins, termed the suppressors of cytokine signalling (SOCS). These proteins are rapidly induced by a number of cytokines and are thought to be part of a negative feedback loop [173]. SOCS

were initially identified for their ability to inhibit the tyrosine kinase activity of JAK1, 2 and 3 [174]. Subsequently, cloning experiments revealed three members of this family, SOCS-1, 2 and 3, where SOCS-1 was shown to inhibit IL-6 receptor phosphorylation and STAT activation [175]. In the IL-4 mediated biological responses, SOCS-1 has been shown to inhibit JAK1 phosphorylation, the formation of STAT6-DNA binding complexes and inhibition of CD23 promoter activity in response to IL-4 [176]. In some studies transfection of cells with SOCS3 has also been shown to inhibit receptor associated JAK1 activity and cause a 50 % reduction in STAT6-inducible luciferase reporter construct activity, compared to complete ablation in SOCS-1 transfected cells [177]. These studies suggest that it is SOCS-1 and 3 which regulate IL-4 signalling, and both of these proteins have been identified in primary bronchial epithelial cells, where they are rapidly induced by IL-4 and IL-13 [178].

1.6 IL-4, IL-13 and Bronchial Epithelial Cells

Bronchial epithelial cells can respond to both IL-4 and IL-13 in a number of ways, indicating expression of functional receptors on their membrane. Indeed, expression of IL-4Rα and γc mRNA and protein has been demonstrated in both primary bronchial epithelial cell cultures and bronchial biopsies from both asthmatic and normal subjects [179]. Similarly both IL-13Rα1 and IL-13Rα2 protein have also been identified in epithelial cells within bronchial biopsies [180 181]. However the composition of IL-4/IL-13 receptors on bronchial epithelial cells is not well understood at this time and there are conflicting reports regarding the differences in IL-4Rα expression in normal and asthmatic subjects [179 182]. However polymorphisms within both IL-4 and IL-13 receptor chains have been associated with asthma and allergic disease. For example, an Arg551Val mutation in the IL-4Rα chain is linked with severe eczema and hyper IgE syndrome. *In vitro* analysis of this mutation has shown it to increase expression of CD23 and reduce dephosphorylation and thus inactivation of the receptor [104]. Similarly mutations within the genes of down stream signalling molecules are now being linked with the asthmatic phenotype in some populations.

The bronchial epithelium has the potential to play a pivotal role in asthma. A number of studies have demonstrated that the sphere of influence of IL-4 and IL-13 can be extended beyond their effects on immune and inflammatory cells, with the finding that they can

also act on bronchial epithelial cells. Unlike many other cell types, airway epithelial cells express an inducible form of nitric oxide synthase (iNOS), the enzyme responsible for nitric oxide (NO) production, and this is believed to be partly under the control of IL-4 [183]. This multifunctional mediator influences a number of processes in the lung including airway tone and mucus secretion [183]. However, NO may also have detrimental effects on the airways as it can react with superoxide anions to yield peroxynitrite anions, these in turn can nitrate –SH groups, tyrosine residues and lipids. The consequences of these reactions include binding to and inactivation of iron/sulphur centres of enzymes, including those involved in the mitochondrial electron transport chain, the citric acid cycle and DNA synthesis.

Goblet cell hyperplasia and mucus hypersecretion are characteristic features of the asthmatic airways. This mucus may contribute significantly to the morbity and mortality of the disease by plugging the airways. As described above, both IL-4 and IL-13 transgenic mice show goblet cell hyperplasia with an increase in mucin positive cells, thus, implicating a role for these cytokines in mucin synthesis or release. *In vitro* experiments using a bronchial epithelial cell line have demonstrated that treatment with IL-4 resulted in an increase in MUC2 gene expression and the production of mucus, [25]. In contrast IL-4 transgenic mice, despite having goblet cell hyperplasia and producing increased amounts of mucus, showed no difference in MUC2 expression from normal littermates. In these mice it was MUC5AC expression that was increased, highlighting a potential difference between animal models and humans [184]. More recently, it has been shown that administration of IL-13 to rats causes increased release of mucin from epithelial cells. From this study it was proposed that the actions of IL-13 were indirect and due to IL-13 induced release of a chemoattractant, the rat homologue of IL-8. This in turn results in recruitment of neutrophils to the airways which release oxygen free radicals that activate the EGFR, resulting in mucus production [185]. Therefore it is possible that IL-4 and IL-13 stimulate increases in mucin production by both direct and indirect mechanisms, but the evidence from STAT6 knockout mice suggests that this transcription factor is important in regulating mucus production.

As was hypothesised in the study in rats described above, IL-4 and IL-13 significantly increase the release of IL-8, a potent chemoattractant and activator of neutrophils, from both human bronchial epithelial cell lines and primary bronchial epithelial cells [186] [187]. IL-4 and IL-13 also directly act on primary human bronchial epithelial cell cultures *in vitro* to stimulate the release of GM-CSF, a cytokine involved in the proliferation,

differentiation, migration, activation and survival of eosinophils [188]. The C-C chemokine eotaxin is a potent eosinophil chemoattractant and its expression is enhanced upon allergen challenge in animal models [31]. *In vitro* studies on bronchial epithelial cell lines has revealed that both IL-4 and IL-13 enhance eotaxin mRNA expression and protein release, which could have important implications in eosinophil recruitment in the airways [189]. Whilst IL-4 and IL-13 increase eotaxin levels alone, when combined with TNFα a much greater increase is observed. This effect is thought to be due to interactions between STAT6 and NFκB of the two transcription factors activated by IL-4 and TNFα respectively, during transcription of the eotaxin gene [190]. More recently it has been proved that IL-4 and IL-13 induced eotaxin transcription in epithelial cells is dependent on STAT6. In these experiments, transfection of cells with a dominant negative STAT6 isoform, significantly the response was significantly reduced [191]. Thus, it is becoming apparent that epithelial expression of STAT6 is important in mediating many the effects of IL-4 and IL-13 on these cells, which as described throughout this chapter may be important in co-ordinating airways inflammation.

1.7 HYPOTHESIS

In summary, IL-4 and IL-13 are central components of airways inflammation, through their actions on both airway structural and inflammatory cells. Both cytokines are expressed at increased levels in the airways of asthmatic subjects, giving rise to the potential for amplified responses to these cytokines in disease. Polymorphisms throughout the IL-4 and IL-13 signalling pathway are associated with asthma or atopy, providing further evidence for the role of these cytokines in this disease. The strikingly contrasting characteristics observed in STAT6 knockout mice and IL-4 and IL-13 transgenic mice implies that STAT6 mediates many of those responses that contribute to the asthmatic phenotype. Recent experiments *in vitro,* have demonstrated that STAT6 activation is essential for mediating inflammatory responses to IL-4 and IL-13. The finding that IL-13 transgenic mice display subepithelial fibrosis also suggests that in addition to their inflammatory effects, IL-4 and IL-13 can contribute to airway wall remodelling. Furthermore, both IL-4 and IL-13 act on bronchial cells, a cell type known to release a number of mediators relevant to subepithelial fibrosis. These observations and the finding that the majority of IL-4 and IL-13 mediated responses rely on STAT6 led to the hypothesis that *IL-4 and IL-13 have the potential to act in a STAT6 dependent*

manner on the airway structural cells to bring about responses which induce subepithelial fibrosis. In this way, dysregulation of the STAT6 signalling pathway has the ability to impinge on the airways structural cells as well as the inflammatory cells, thus contributing to both components of disease pathology.

1.8 AIMS

Whilst STAT6 has been identified in primary bronchial epithelial cells, its levels of expression have not been examined in asthmatic airways. Thus the initial aim this project was to *characterise STAT6 expression in the bronchial epithelium and relate it to disease severity by staining archival bronchial biopsies with STAT6 antibodies. In addition the expression of STAT6 variants, which have the potential to influence down stream gene activation, have also been assessed.*

Fibroblast proliferation and extracellular matrix deposition cause subepithelial fibrosis and the pro-fibrogenic growth factor TGFβ is thought to play a prominent role in this process. Most of the effects so far described mediated by IL-4 and IL-13 relate to the classical inflammation observed in asthma, but it has recently been shown that IL-4 can stimulate TGF-β_1 release from eosinophils [192]. Regulation of epithelial TGFβ synthesis by IL-4 or IL-13 in the airway cells would provide a link between both the inflammation and the airways remodelling described above. Thus *the effect of IL-4 and IL-13 on TGFβ2 mRNA production and release from bronchial epithelial cells has been examined. Furthermore, involvement of STAT6 and TGFβ processing enzymes in this response has also been assessed.*

CHAPTER 2

2 MATERIALS AND METHODS

2.1 MATERIALS and EQUIPMENT

All materials were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1.1 *Cell Culture Reagents*

Minimal Essential Medium (MEM) (Eagle) 25nM HEPES with Earle's salts, RPMI 1640, Hanks Balanced Salt Solution (HBSS) without $Ca^{2+}$and Mg^{2+}, trypsin EDTA (10 x liquid), heat inactivated foetal bovine serum (FBS) and penicillin/streptomycin solution (5000 units penicillin & 500μg streptomycin/ml), were all purchased from Gibco BRL, Life Technologies Ltd, Paisley, UK. Trypan blue was obtained from Flow Laboratories, UK. Ultra Culture serum free medium was obtained from Bio Whittaker, UK. Nunclon tissue culture plastics were all purchased from Gibco BRL, Life Technologies, Paisley, UK.

2.1.2 *General Reagents for Immunohistochemistry*

Microscope slides were purchased from Surgipath, Europe Ltd. Diaminobenzene (DAB) and Aminoethylcarbozole (AEC) liquid systems were purchased from Menarini Diagnostics, Finchampstead, Berks, UK. Crystal mount was purchased from Biomedia Biogenesis, Poole, UK. Swine serum and streptavidin biotin-peroxidase (Strept ABC-HRP) were purchased from DAKO, Glostrup, Denmark. Supercut microtome was purchased from Leica, Milton Keynes, UK.

2.1.3 *Reagents for SDS-PAGE and Western Blotting*

Acrylamide/bis-acrylamide gel mix was purchased from National Diagnostics, Hull, UK. Full range rainbow molecular weight markers, Hybond–C; nitrocellulose membrane, Hybond-P; hydrophobic polyvinylidene difluoride (PVDF) membrane and ECL Plus were all purchased from Amersham Life Sciences, Buckinghamshire, UK. Biotinylated SDS-PAGE standards (broad range), Mini Protean Systems and Transblot apparatus were purchased from Bio-Rad Laboratories Ltd, Hertfordshire, UK.

2.1.4 *Immunological Reagents*

Anti-human STAT6 polyclonal (S-20) rabbit antibodies and mouse monoclonal (C-9) antibodies were both purchased from Santa Cruz Biotechnologies. Anti-human JAK1 and biotinylated anti-phosphotyrosine (PY20) monoclonal mouse antibodies, and PY20-agarose conjugates were purchased from Transduction Laboratories (distributed by Becton Dickinson, Cowley, Oxford, UK). Phospho-STAT6 (Tyr641) rabbit polyclonal antibodies were purchased from New England Biolabs Ltd, Hertfordshire, UK. Rabbit anti-mouse, swine anti-rabbit horseradish peroxidase conjugated antibodies and biotinylated rabbit anti-mouse and swine anti-rabbit antibodies were purchased from DAKO Ltd, Glostrup, Denmark. The concentrations of these antibodies used for immunohistochemistry and immunostaining of Western blots are depicted in Table 1.

2.1.5 *Molecular Biology Reagents*

All reagents used for these techniques were molecular biology grade.

STAT6 expression vectors were a kind gift from Tularik Inc, San Francisco, California, USA. TRIzol® total RNA isolation reagent, super-script first strand synthesis system for RT-PCR, glycogen, 50bp ladder and Tris/Boric acid/EDTA (TBE) buffer were purchased from Gibco BRL, Life Technologies Ltd, Paisley, UK. Effectine transfection reagent, RNeasy mini kit and RNase free DNase set for RNA clean up were purchased from Qiagen Ltd, Crawley, UK. All PCR primers were purchased from MWG Biotech Ltd, Milton Keynes, UK. TaqMan™ probes, TaqMan™ universal master mix, TGFβ pre-developed assay reagent, endogenous 18S ribosomal RNA control and micro amp optical 96 well reaction plate and caps were all purchased from Perkin-Elmer Biosystems, Warrington, UK. MOPS and agarose were purchased from Promega, Southampton, UK. Bio Rad wide mini sub cell GT was purchased from Bio-Rad Laboratories Ltd, Hertfordshire, UK. RNase Away was purchased from Molecular BioProducts Inc. California, USA. Capillary cell for Gene Quant spectrophotometer and spare capillaries were purchased from Amersham Life Sciences, Buckinghamshire, UK.

2.1.6 *Miscellaneous Reagents*

Human recombinant IL-13 and IL-4 were purchased from PeproTech Ltd, London, UK. Both cytokines were reconstituted to a concentration of 10μg/ml, in filter sterilised DulA containing 0.1% BSA. Reconstituted cytokines were stored in aliquots at –80°C. Cytokines were made up to the working concentrations in serum free medium prior to the

Table 1. Antibody Dilutions for Immunohistochemistry and Western Blotting.

Antibodies for Immunohistochemistry			
Primary Antibody			Secondary Antibody
Antigen	**Species / Isotype**	**Dilution**	
STAT6 (S-20): carboxy terminus of human protein (Santa Cruz)	rabbit polyclonal	1:30 from 200μg/ml stock	biotinylated swine anti-rabbit at 1:300. From 0.57g/l stock. (inc. 1:5 swine sera)
JAK1: kinase 1 domain (aa 551-766) of human protein (Transduction Labs)	mouse monoclonal IgG2b	1:60 from 250μg/ml stock	biotinylated rabbit anti-mouse at 1:300. From 1g/l stock.
Antibodies for Western Blotting			
Primary Antibody			Secondary Antibody
Antigen	**Species / Isotype**	**Dilution**	
STAT6 (C-9):aa 280-480 of mouse protein (Santa Cruz)	mouse monoclonal IgG2b	1:1000 from 200μg/ml stock	rabbit anti-mouse HRP 1:1000 on nitrocellulose or 1:4000 on PVDF. From 1.3g/l stock.
PY-20 (Transduction Labs)	biotinylated mouse monoclonal IgG2b	1:1600	streptavidin-biotinylated rabbit anti mouse at 1:1000 on nitrocellulose. From 1.8mg/ml stock.
human STAT6 phosphorylated at tyrosine 641 (New England Biolabs)	rabbit polyclonal	1:1000 from 50μg/ml stock	swine anti-rabbit HRP at 1:4000 on PVDF. From 1.3g/l stock.
β actin – N terminus	mouse monoclonal IgG1	1:4000 from 2.5mg/ml stock	rabbit anti-mouse HRP 1:4000 on PVDF. From 1.3g/l stock.

experiment. Micro BCA protein assay reagent Kits were purchased from Pierce and Warringer (UK) Ltd, Chester, UK. TGFβ_2 E_{MAX} Immunoassay Kit was purchased from Promega, Southampton, UK. MMP inhibitor II (N-hydroxy-1,3-di-(4-methooxybenzenesulfonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide) was purchased from Calbiochem (UK) Ltd. The matrix metalloproteinase inhibitor (MMPI), specific for MMP-9, 1, 3 and 7, was reconstituted in DMSO to a concentration of 1mM. This was stored at –20°C and working solutions were made up in serum free medium prior to the experiment.

2.2 METHODS

2.2.1 *Immunohistochemistry*

2.2.1.1 Tissue processing and staining

To analyse STAT6 and JAK1 expression, anonymous archival bronchial biopsies (originally obtained following ethical approval) were obtained from eleven normal subjects, fourteen mild asthmatics and nine severe asthmatics. Subjects were all nonsmokers and had not experienced recent symptoms of upper respiratory tract infection. The clinical and physiological details of the subjects in the three groups, together with all medication taken at the time of the study, are summarized in table 2. Definition of disease severity was based on treatment and was according to the GINA guidelines, table 3. The biopsies were embedded into glycol-methacrylate (GMA) using the method described by Britten *et al.*, 1993 [193]. 2μm sections were cut using a Leica supercut and were floated out onto 0.002% (v/v) ammonia water for 1 minute, before being picked up onto poly-L-lysine coated slides. The sections were left to dry for at least one hour, after which time they were either stained or wrapped in aluminium foil and stored at –20°C, for use within one week.

For increased sensitivity, a four stage staining method was utilised for immunohistochemistry. This relies on the specificity of an antibody - antigen interaction, in that a primary antibody against an antigen of the cellular component under study was applied to the tissue section. A secondary antibody raised against the primary antibody and labelled with the vitamin biotin was then applied. The third stage involved addition

Table 2. Summary of the Clinical and Physiological Characteristics of Subjects for Immunohistochemistry Study.
S, Salbutamol; ICS, inhaled corticosteroids; OCS, oral corticosteroids.

		Age (yr)	Sex	Atopy	FEV_1 (% predicted)	Treatment
Healthy controls						
1		46	F	N	110	None
2		23	M	N	94	None
3		21	M	N	89	None
4		21	M	N	89	None
5		18	M	N	108	None
6		22	F	Y	116	None
7		23	M	Y	98	None
8		21	F	N	96	None
9		21	F	N	111	None
10		22	M	N	108	None
11		24	F	N	86	None
Mean		23.81			102.09	
SD		7.5			9.89	

Mild Asthmatics						
1		30	F	Y	75	S
2		25	M	Y	91	S
3		54	M	Y	75	S
4		27	M	Y	96	S
5		21	F	Y	99	S
6		23	M	Y	93	S
7		19	M	Y	95	S
8		29	M	Y	101	S
9		21	M	Y	71	S
10		20	F	Y	95	S
11		18	M	Y	114	S
12		36	F	Y	88	S
13		25	M	Y	96	S
14		47	F	Y	106	S
Mean		28.21			93.90	
SD		10.71			12.45	

Severe Asthmatics						ICS ug/day	OCS mg/day
1		44	F	Y	67	4000	7.5
2		50	F	Y	95	3000	5
3		53	F	Y	55	3200	2.5
4		25	F	Y	82	4000	35
5		13	F	Y	68	2000	100
6		15	F	Y	53	2000	60
7		24	F	Y	77	4000	50
8		22	M	Y	87	2400	5
9		17	F	Y	91	2000	60
Mean		29.22			75		
SD		15.52			15.20		

Table 3. Summary of Classification of Disease Severity.

Asthma Classification
PEF: peak expiratory flow

Mild Asthma

- PEF > 80% predicted
- PEF variability <20%
- PEF normal after bronchodilator
- Intermittent brief symptoms <1-2 per week
- Nocturnal asthma symptoms less than twice per month
- Asymptomatic between episodes

Mild and Moderate Persistent Asthma

- PEF 60-80% predicted
- PEF variability 20-30%
- PEF normal after bronchodilator
- Patients with exacerbations .1-2 per week
- Nocturnal asthma symptoms more than twice per month
- Daily requirements for inhaled β2 agonists

Severe Asthma

- PEF<60% predicted
- PEF variability >30%
- PEF constantly below normal despite optimal treatment
- This form of disease is associated with frequent exacerbations, continuous symptoms and frequent nocturnal symptoms. Physical activities are limited due to respiratory status

of streptavidin biotin peroxidase complex. Streptavidin binds to biotin with high affinity such that the complex becomes attached to the secondary antibody. The role of the peroxidase is to convert a colourless chromagen into a coloured end product, which is easily detectable under a light microscope.

Initially, to reduce non-specific staining, endogenous peroxidases were inhibited by applying a solution of 0.1% sodium azide and 0.3% hydrogen peroxide in reverse osmosis water for 30 minutes. Following three five-minute washes with TBS, the slides were drained and blocking medium (80% v/v DMEM, 20%v/v FCS, 0.01% w/v FCS, stored at $-20^{\circ}C$) applied for thirty minutes to inhibit non-specific protein interactions. If a polyclonal primary antibody was being used, a 1:20 dilution of normal swine serum was included in the blocking medium.

After blocking, the slides were then drained and the primary antibodies applied at the appropriate concentration in Tris buffered saline (TBS) (1.36M NaCl, 0.005M Tris, 0.0038% HCl, in dH_2O pH to 7.6) as shown in Table 1. For incubation with monoclonal primary antibodies, coverslips were placed over the sections which were then left in a moist atmosphere at room temperature overnight. The incubation time for polyclonal antibodies was reduced to one hour with all other conditions remaining constant.

After binding of the primary antibody the slides were washed, as described above, and biotinylated secondary antibodies, made up to the appropriate concentration in TBS (table 1), applied to the sections. After two hours, the slides were washed and streptavidin biotin-peroxidase (Strept-ABC-HRP) was applied in accordance with the manufacturers directions. Sections were routinely incubated for two hours, except when sections had been treated with a polyclonal primary antibody. In this case the incubation times for the biotinylated secondary antibody and the strept-ABC-HRP were reduced to one hour, and a 1:5 dilution of normal swine serum was included in the former step.

Following washing, the chromagen DAB was applied for fifteen minutes, after which time the sections were washed in running tap water for five minutes. The sections were then counterstained with Mayer's haematoxylin for one and a half minutes and blued in running tap water for five minutes. After draining, crystal mount was applied and the slides were baked at $80^{\circ}C$ for approximately fifteen minutes. Once cool, the slides were mounted in *p*-xylene-*bis*-pyridium bromide (DPX).

All immunostaining experiments included control slides unexposed to primary antibody and a matched isotype control was carried out for each antibody, which revealed no positive staining. As a further control, the STAT6 antibody was incubated at 4°C with a 14 fold molar excess of the immunising peptide overnight, prior to application to several tissue sections from each clinical group. Biopsy sections stained with preadsorbed antibody revealed a small amount of staining along the brush border, an area subsequently excluded from analysis. Within the epithelium a small amount of staining was also apparent, however this was less than 1% of the entire intact epithelium.

2.2.1.2 Image Analysis

Following staining, airway epithelial expression of immunoreactive STAT6 in GMA sections was quantified by computer-assisted image analysis (Colourvision 1.7.6 Improvision, Coventry, UK). For each biospsy specimen, the entire intact epithelium in two nonserial sections was systematically assessed based on red, blue, green (RBG) colour balance. At the beginning of each session, the image analysis system was standardized using the same secton of bronchial mucosa stained for STAT6 to ensure reproducibility of analysis. The digitised image of the standard section was used to interactively sample an example of the positive staining and the system was then allowed to select all the pixles in the RBG colour balance (i.e positive staining) within the image. The area of epithelium was then delineated interactively and the percentage of positive staining within the epithelium was determined; the colour balance and percentage staining was recorded for future sessions. At the beginning of each subsequent session, the image analyser was calibrated using this section and was adjusted to within $\pm$ 5% of the original pixle reading. Once the system had been set up using the 'standard slide', the test sections were analysed using the same parameters. For the measurement of STAT6, slides were coded such that one was unaware of the clinical group from which the biopsy specimen was derived.

2.2.2 *Cell Culture Techniques*

2.2.2.1 Culture of Continuous Cell Lines

Two cell lines, 16HBE 14o-, an SV-40 Large T antigen transformed human bronchial epithelial cell line [194], and NC1-H292, a mucoepidermoid bronchial epithelial cell line [195], were routinely cultured and used for experiments. All culture techniques were carried out in Microflow Class II Biological safety cabinets and cells were maintained in Heraeus incubators at 37°C, 5%(v/v) CO_2 in a humidified atmosphere.

H292 cells were cultured in RPMI 1640, containing 10% (v/v) heat inactivated FBS, 50 IU/ml penicillin, 50µg/ml streptomycin and 2mM L-glutamine. 16-HBE 14o-cells were cultured in MEM 25mM HEPES with Earle's salts containing the same additions as the RPMI 1640 medium. All medium and other reagents were pre-warmed to 37°C before being added to the cell monolayer.

2.2.2.1.1 Trypsinisation of Confluent Cell Monolayers

Confluent cells were routinely passaged by detachment with trypsin and plating down in new flasks. 1x trypsin-EDTA (made up in Ca^{2+}, Mg^{2+} free HBSS) was defrosted and warmed to 37°C prior to use. The culture medium was removed from the cell monolayer, which was then washed twice in 10ml HBSS and left in this medium for five minutes to prevent inhibition of trypsin by traces of serum. 1-2mls of 1x trypsin-EDTA, sufficient to cover the monolayer was then added and the excess removed. The cells were incubated at 37°C for ten to fifteen minutes during which time the majority round up, as determined microscopically. The flask was then tapped sharply to detach the cells and 10mls of the appropriate culture medium added to halt the action of trypsin. To avoid selecting for a sub population, at least 95% of the cells were detached and occasionally the above procedure had to be carried out twice for this to occur.

A single cell suspension was obtained by aspiration of the cell solution through a 25-gauge needle attached to a syringe and, to remove traces of trypsin, the cell suspension was centrifuged at 660xg for five minutes before being resuspended in 10ml of the appropriate medium. For routine passage 12ml of culture medium was inoculated with approximately 0.5ml or 0.25ml of 16HBE, or H292 cell suspensions respectively.

2.2.2.1.2 Viable Cell Counting

A 500µl or 1ml aliquot of the single cell suspension was removed and centrifuged at 600xg for 5 minutes before being resuspended into the same volume of HBSS to prevent inhibition of dye uptake by traces of serum. 20µl of this suspension was then diluted into 30µl of HBSS and 50µl of 0.4%(w/v) Trypan Blue. The number of viable cells in suspension was then counted using an improved Neubauer haemocytometer.

2.2.2.1.3 Long Term Cryogenic Storage

Cells were routinely prepared for long term cryogenic storage at –170°C in liquid nitrogen vapour. Single cell suspension was centrifuged at 600xg for five minutes. Following removal of culture medium the pellet was resuspended in 1ml of chilled freezing medium (culture medium containing 10%(v/v) dimethylsulphoxide (DMSO) as a cryoprotectant). The suspension was then placed in cryotubes and stored at -70°C overnight before being placed into liquid nitrogen vapour.

2.2.2.2 Collection and Culture of Primary Bronchial Epithelial Cells

Following ethical approval, primary bronchial epithelial cells were collected by qualified personnel within the Division of Respiratory Cell and Molecular Biology. The cells used for experiments described within this thesis were kindly cultured and provided by Dr. S. Puddicombe.

2.2.2.2.1 Fibreoptic Bronchoscopy

Epithelial brushings were obtained by bronchoscopy using a fibreoptic bronchoscope (Olympus FB-20D, Tokyo, Japan) in accordance with standard published guidelines. Bronchial epithelial cells were obtained using a standard sterile single-sheathed nylon cytology brush (Olympus BC 9C-26101; Tokyo, Japan). On average five to six consecutive brushings were sampled from the bronchial mucosa of the second and third generation bronchi, and for large scale culture a maximum of 12-14 brushings were performed. Cells were harvested into 5ml sterile phosphate-buffered saline (PBS) after each brushing. At the completion of the procedure, 5ml RPMI with 10% FBS was added and the sample centrifuged at 150xg for 5min to harvest the cells. Epithelial cell purity was assessed by performing differential cell counts of the harvested cell suspension.

2.2.2.2.2 Primary Bronchial Epithelial Cell Cultures

Primary cultures were established by seeding freshly brushed bronchial epithelial cells into culture dishes containing 3ml of serum-free hormonally-supplemented Bronchial Epithelium Growth Medium (BEGM; Clonetics, San Diego, CA). When confluent, the cells were passaged (p1) using trypsin and were allowed to further expand until used for experimentation at passage 2 or 3; control experiments confirmed that there was no significant difference between the responses of the cells at p2 or p3. Viability was assessed by exclusion of trypan blue dye and the epithelial nature of cells assessed by immunocytochemistry using a pan-cytokeratin (CK) antibody and antibodies specific for CK13 and CK18.

2.2.3 *SDS-Polyacrylamide Gel Electrophoresis and Western Blotting*

2.2.3.1 Background

This technique depends on the separation of proteins in solution based on their movement through a porous gel that serves as a molecular sieve. The proteins move through this gel to varying degrees, with those that are small compared with pores in the gel moving easily through it, so that they separate according to their molecular weight. Prior to loading, the protein samples are dissolved in a solution containing sodium dodecyl sulphate (SDS), an anionic detergent that disrupts the non-covalent interactions in the native proteins, making them rod shaped. SDS also gives them a net negative charge and thus aids migration the gel towards the anode, at the bottom. This technique was utilised to detect STAT6 expression in the chosen cell lines and to analyses changes in phosphorylation in response to IL-4 and IL-13.

2.2.3.2 Preparation of Whole Cell Lysates for SDS-PAGE

Cells were counted as described above and plated down at a density of approximately $1x10^5$/ml and cultured for five days until they were about 80% confluent. At this stage they were transferred in to serum free medium for twenty four hours before being treated and harvested for SDS-PAGE. Following stimulation with IL-4 or IL-13 (made up to the concentrations stated in the results chapters, in serum free medium) the cells were washed twice in PBS (one oxoid DulA buffer tablet dissolved in 100ml of H_2O to give a solution comprising 136.89mM NaCl, 2.68mM KCl, 7.89mM Na_2HPO_4, 1.47mM KH_2PO_4, pH

7.3) containing protease and phosphatase inhibitors (1mM Na_3VO_4, 1mM NaF, 1mM PMSF 1.54μM aprotinin, 21μM leupeptin,) and lysed into denaturing lysis buffer (10mM Tris-HCl (pH 7.4), 1% w/v SDS made up in dH_2O, pH7.4) containing 5mM EGTA and 5mM EDTA in addition to the other phosphatase and protease inhibitors stated above, and placed on ice.

Following sonication for fifteen seconds and heating for five minutes at 95°C, the lysates were centrifuged for ten minutes (16000xg at 4°C) for clarification, and a 20μl aliquot taken for protein determination. The remainder of each was stored at –80°C until required. Prior to electrophoresis samples were diluted to 0.8μg/μl in dH_2O and 5x sample buffer (0.3125M Tris-Cl pH6.8, 50% (v/v) glycerol, 25%(v/v) 2-mercaptoethanol, 10% (w/v) SDS and 0.01% (v/v) bromophenol blue made up in dH_2O) was also added to give a final concentration of 1x sample buffer.

2.2.3.3 Immunoprecipitation of Tyrosine Phosphorylated Proteins

To investigate the levels of tyrosine phosphorylation in samples, tyrosine phosphorylated proteins were specifically immunoprecipitated from whole cell lysates. Whole cell lysates were prepared as described in the previous section and approximately 1000μg of lysate was diluted in 2x Triton buffer (20mM Tris pH 7.4, 2% (v/v) Triton X-100, 1%(v/v) NP40, 300mM NaCl, pH 7.4) containing protease and phosphatase inhibitors (10mM EGTA, 10mM EDTA, 2mM Na_3VO_4, 100mM NaF, 2mM PMSF, 42μM leupeptin, 3.08μM aprotinin) at a ratio of 1:1. Phosphotyrosine proteins were captured by addition of 50μl of PY-20-agarose conjugate and mixing on for ninety minutes at 4°C on a rotary shaker. The samples were then centrifuged at 600xg for fifteen minutes at 4°C and the supernatant carefully removed and discarded. Following three washes in 1x Triton buffer (600xg for five minutes at 4°C), the pellet was resuspended in 80μl of 2x sample buffer and denatured at 95°C for five minutes. The samples were then stored at –80°C until required for polyacrylamide gel electrophoresis.

2.2.3.4 Protein Determination

The concentration of protein within whole cell lysate samples was determined using a Micro BCA Protein Assay Reagent kit, in accordance with the manufacturers instructions (Pierce and Warringer (UK) Ltd, Chester). A standard curve, ranging from 40 - 0.625μg/ml BSA was generated by carrying out serial doubling dilutions in diluent solution (1/25 dilution of denaturing lysis buffer in dH_2O). Samples were diluted 1:25

and then applied to the plate, followed by Micro BCA working solution. The plates were then incubated at 60°C for one hour, before the absorbance at 562nm was read on a plate reader. The concentration of protein within the sample was determined by reading off the standard curve, a typical example of which is shown in figure 2.1.

2.2.3.5 Polyacrylamide Gel Preparation

A 7.5% separation gel solution was prepared using 22.5ml 30% (w/v) acrylamide / 0.8% bis-acrylamide, 22.5ml 1.5M Tris-HCl pH 8.8, 37.1ml H_2O and 0.45ml 20% SDS. For each pair of gels with 1mm spacers 10ml of the gel mix was used with 30µl of ammonium persulphate (APS) and 5µl of N,N,N',N'-tetramethylenediamine (TEMED), which were added to initiate polymerisation. Once poured into the cast, the gel was overlaid with water saturated propanol, to exclude oxygen, and left to set for approximately thirty minutes.

After this time, the polymerised gel was rinsed with distilled water, dried with filter paper and overlaid with stacking solution. Stock stacking gel solution was prepared with 12.5ml acrylamide / bis-acrylamide 25ml of 0.5M Tris-HCl pH 6.8, 62ml H_2O and 0.5ml of 20% SDS. For each pair of gels, 5ml of this mix was used with 16.7µl APS and 3.8µl TEMED. The stacking gel was then poured over the separation gel, to fill the cassette. To form channels for the samples, 10 or 15 well combs were inserted into the stacking gel, ensuring no air bubbles were trapped. The gel was then left to polymerise for approximately twenty minutes. Once the gel had set, the combs were removed and the apparatus assembled in the gel tank, which contained chilled running buffer (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS, pH 8.3).

The samples were heated at 95°C for three minutes, and then 15µl or 25µl loaded into the small and large wells respectively, using a Hamilton syringe. 10µl of rainbow marker and a biotinylated marker (only when a biotinylated antibody was used) were added to two wells, so that the molecular weight of the separated proteins could subsequently be determined. Electrophoresis proceeded at a constant 200V for about forty to forty-five minutes until the dye front reached the bottom of the separation gel.

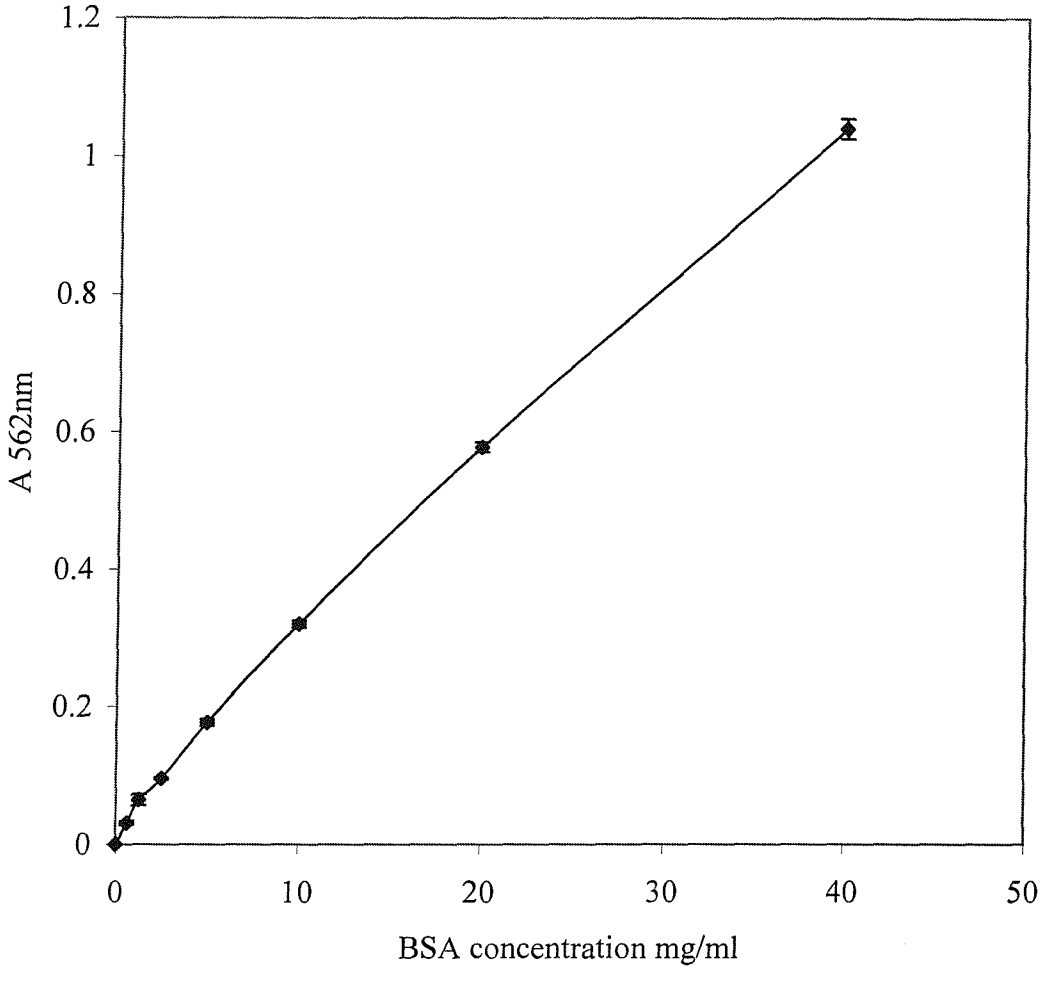


Figure 2.1 Typical Micro BCA Standard Curve for Protein Determination.

This curve was generated from the mean values of three individual experiments ± SE as described in chapter 2, and was linear between 1-20 mg/ml BSA.

2.2.4 *Western Blotting*

This involves transferring the separated proteins from the polyacrylamide gel onto Highbond nitrocellulose or PVDF membranes so that they can be probed with appropriate antibodies for identification of protein.

The gel was placed carefully into chilled transfer buffer (25mM Tris, 192mM glycine and 20% (v/v) methanol made up in dH_2O, pH 8.4) for fifteen minutes to remove salts and to preshrink the gel. Nitrocellulose pre-cut to the size of the gels, filter paper and the transfer cassettes containing scotchbrite pads were also treated in the same way. When PVDF membranes were used, the membrane was hydrated in methanol for thirty seconds and washed in dH_2O for five minutes prior to being placed in transfer buffer. The gel and membrane were then placed into the transfer cassette on top of a pad of scotchbrite and two layers of Whatman 3MM filter paper. Air bubbles were removed by gently rolling a test tube over the layers and two more layers of filter paper and another scotchbrite pad were added to the cassette to ensure a tight fit. The cassette was inserted into the Transblot tank, containing transfer buffer with the NC sheet nearest the anode so that the proteins would migrate onto it. A constant voltage of 50V was then applied overnight at 4°C or 90V for two hours. Once the transfer was complete the membrane was stored dry at 4°C until required.

2.2.4.1 Immunostaining of Western Blot

The NC membranes were rehydrated in 10ml of PBS/Tween (1 X PBS {5X stock prepared; 727mM NaCl 45mM Na_2HPO_4, 5.7mM $NaH_2PO_4 \bullet H_2O$ made up in H_2O}, 0.5% v/v Tween 20, pH 7.4) containing protease and phosphatases inhibitors (1mM EDTA, 1mM EGTA, 1mM NaF, 1mM Na_3VO_4, 70μM PMSF) with shaking for ten minutes. The PVDF membrane was rehydrated in methanol for thirty seconds and washed in dH_2O for five minutes prior to being placed in PBS/Tween buffer for ten minutes.

To reduce non-specific antibody binding, the membranes were incubated for thirty minutes in 10ml of blocking buffer (1X PBS, prepared from 5X stock, 0.5% v/v Tween 20, 5% w/v dried low fat skimmed milk plus protease and phosphatases inhibitors at the same concentrations as in the PBS/Tween buffer above). To remove any residual buffer the blots were washed in PBS/Tween buffer for five minutes. Initially blots were incubated with a range of antibody concentrations to determine those which gave optimal

staining. Primary antibodies directed against STAT6, phosphotyrosine, phospho-STAT6 or β-actin were made up to the appropriate concentrations in antibody buffer (150mM NaCl, 50mM Tris, 0.25% w/v BSA, 0.5% v/v Tween 20 plus protease and phosphatases inhibitors as above, pH 7.4), as shown in table 1. Each antibody solution was added to an individual polythene envelope, to which a NC blot was added prior to sealing and incubating at room temperature for one hour.

Following three five-minute washes to remove any unbound antibody, the secondary antibodies were diluted, as indicated in table 1, and incubated with the blots for a further hour. For detection of phosphotyrosine, a streptavidin biotinylated horseradish peroxidase conjugated antibody was used, based on the principle that streptavidin binds biotin with high affinity. The final wash sequence consisted of 1x fifteen minutes and 4x five minutes in PBS / Tween. For detection, enhanced chemiluminescence plus (ECL plus) was used. This reaction relies on the oxidation of luminol by horseradish peroxidase and hydrogen peroxide under alkaline conditions. The product emits light and this was detected using Hyperfilm ECL. In later experiments protein bands were visualised using the GeneSnap image capture programme on a Syngene Genome image analysis system (Synoptics Ltd, UK). The intensity of bands detected on the western blots was determined using the Gene Tools programme from Syngene (Synoptics Ltd, UK).

2.2.5 *Enzyme Linked Immunosorbent Assay (ELISA)*

2.2.5.1 Preparation of Conditioned Medium

H292 cells were seeded onto a 24 well plate at a density of $5x10^4$ or $2.5x10^4$ cells per well and left to adhere for 24h before changing to serum free medium for twenty-four hours. Following this treatment cells were stimulated with IL-4 or IL-13, as indicated in the results sections. At the end of the stimulation period the conditioned medium was harvested and clarified by centrifugation (16,000xg for 10min at 4°C). The cells were washed twice in PBS containing Ca^{2+} & Mg^{2+} and formyl saline (4% formaldehyde in 0.9% saline solution) applied for 30 minutes as a fixative. These plates were stored wrapped in cling film at room temperature until cell number was determined by uptake of methylene blue.

2.2.5.2 TGFβ$_2$ ELISA

Conditioned cell culture medium was assayed for both total and active TGFβ$_2$ using a five-stage antibody sandwich format TGFβ$_2$ E_{max} ImmunoAssay System, in accordance with the manufacturers instructions. This kit is designed for the specific recognition of biologically active TGFβ$_2$ and thus, to detect the amount of total TGFβ$_2$ within the samples collected, the TGFβ$_2$ had to artificially activated using an acid treatment procedure. Therefore, prior to being assayed an aliquot of each sample was acidified, by the addition of 0.1M HCl for fifteen minutes followed by 0.1M NaOH, which activated total cellular TGFβ$_2$. The assay was performed in Costar 96 well plates, which were coated with a monoclonal antibody directed against TGFβ$_2$ and incubated at 4°C for sixteen to twenty hours. The plate was then blocked for thirty five minutes at 37°C to inhibit non-specific protein interactions. 100µl of sample was then added to the plate in duplicate and left at room temperature for ninety minutes with shaking. After this time the plate was washed in TBST wash buffer (20mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, made up in dH_2O and pH to 7.6) and anti-TGFβ$_2$ polyclonal antibodies applied for two hours. The plate was then washed and the HRP conjugate applied for two hours, before washing again. The TMB and peroxidase solution was then added and the colour left to develop at room temperature for approximately fifteen minutes. The reaction was stopped with 1M phosphoric acid, and the absorbance at 450nm was read using a plate reader. The inter assay variation on a given sample was less than 10% and a typical standard curve is shown in figure 2.2.

2.2.6 *Methylene Blue Assay*

This assay, which utilises a protocol first described by Oliver et *al.* (1989)[196], was used to estimate the number of adherent cells present on the twenty four well plates from which the supernatants assayed for TGFβ$_2$ were taken. This was necessary to determine any effects that IL-4 and IL-13 may have on epithelial cell proliferation, which could account for increases in mediator release in their presence.

As described in section 1.2.5.1, following the removal of conditioned from the 24 well plates the cells were fixed in formyl saline and stored until required for the methylene blue assay. One ml of 1% methylene blue dye in 0.01M borate buffer (pH 8.5) was added to each well of the twenty-four well plates, which were then left at room temperature for

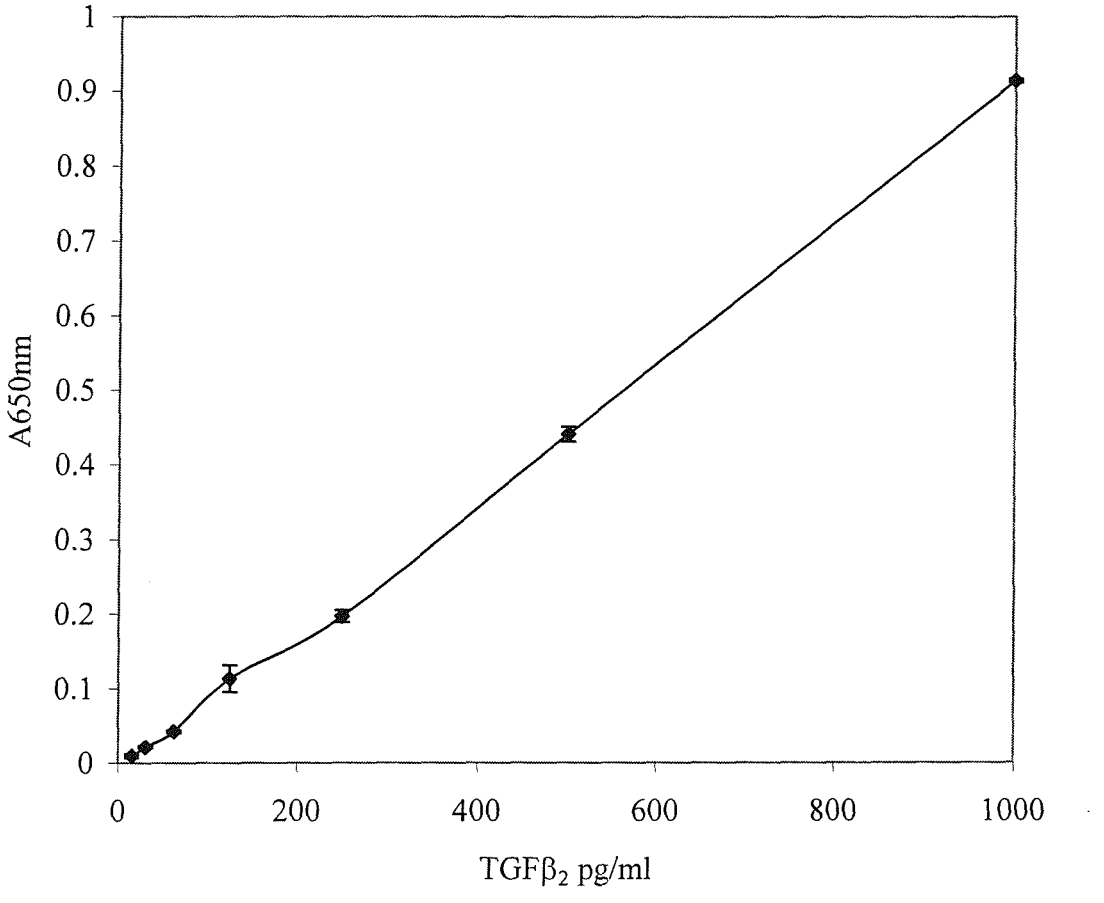


Figure 2.2 Typical TGFβ_2 Emax Immunoassay Standard Curve. This curve was generated using the mean ±SE of three independent experiments as described in chapter two and is linear between 32-1000pg/ml.

thirty minutes. Unbound dye was removed by immersion into water until all the water ran clear and the wells blotted dry. To elute the bound dye 500µl of 1:1 v/v 0.1M HCl/ethanol was then added to each well and the plate incubated for thirty minutes at room temperature. Following this treatment, 100µl/well of HCl/ethanol solution containing eluted methylene blue was transferred to 96 well plates and the absorbance at 650nm read using a plate reader. To allow this absorbance value to be related to cell number a standard curve was generated using a known number of cells, this is shown in figure 2.3.

2.2.6.1 Analysis of ELISA and Methylene Blue Assay Results

To correct for changes in cell density between the wells each, each value obtained from the ELISA was adjusted to 1 absorbance unit, as determined by the methylene blue assay. From a standard curve of H292 cell growth this was determined to represent $4x10^5$ cells, allowing the data to be presented as pg $TGF\beta_2/4x10^5$ cells. All results are expressed as mean ± standard error (SE) unless otherwise stated.

2.2.7 RNA Preparation and Reverse Transcription

For all work involving RNA, special precautions were taken to reduce the risk of degradation and contamination. This involved wiping the area with RNase away, the use of ultra pure RNase free water, RNase and DNase free plastics, sterile glassware, ultra pure reagents designated for molecular biology and pipettes designated for RNA work with barrier pipette tips. During work involving RNA, gloves were also changed regularly.

2.2.7.1 Preparation of Total RNA from Bronchial Epithelial Cells

NCI-H292 bronchial epithelial cells were seeded at a density of $7.25x10^4$/ml in a $57cm^2$ petri-dish and left to grow for forty-eight hours. After this time, the medium was changed to ultra culture serum free medium and the cells were left for a further twenty-four hours. Cells were treated with 1.5nM IL-4 for 6, 12, 24, 48 and 72 hours. In experiments where the matrix metalloproteinase inhibitor (MMPI) was used, this was applied to the cells thirty minutes prior to the addition of IL-4. At the end of each incubation period the supernatant was collected and stored as described above and total RNA extracted from the cell monolayer. 1ml of TRIzol® reagent was added to the monolayer, which was then

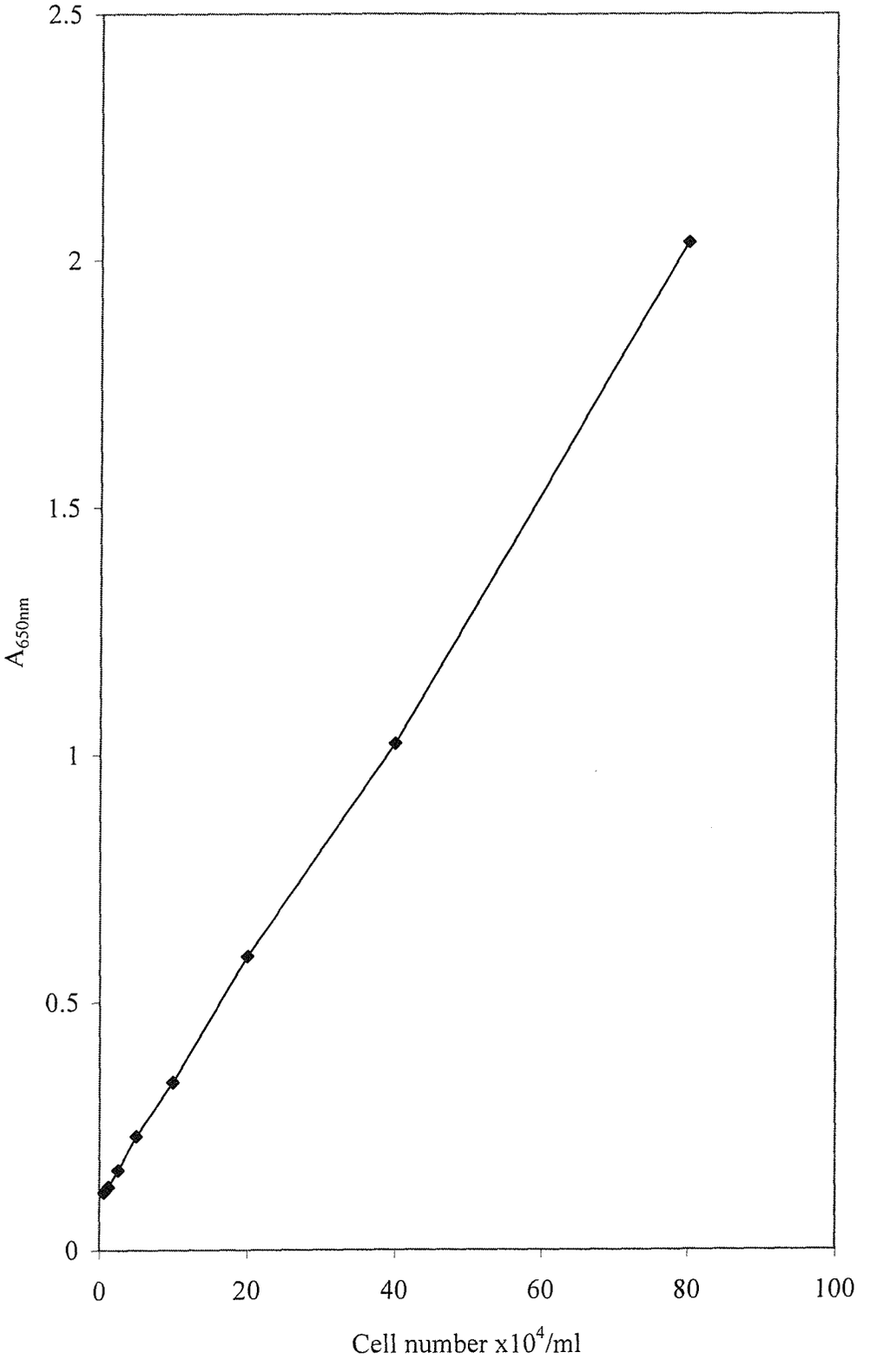


Figure 2.3 H292 Standard Curve.
This chart shows the absorbance at 650nm, as determined by methylene blue assay for a known number of H292 cells ($n = 2$).

disrupted using a sterile pipette tip and left for five minutes at room temperature before being transferred to a sterile microfuge tube. 0.2ml of chloroform was then added to the lysate, which was shaken and left to settle for two to three minutes before being centrifuged at 12,000xg for fifteen minutes at 4°C. Following centrifugation this mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase containing the RNA. The upper aqueous phase was transferred to a fresh tube leaving behind a layer of this phase to prevent disturbance of the DNA interface and DNA contamination carry-over. The RNA was then precipitated by mixing with 0.5ml of chilled isopropranol and left at room temperature for at least twenty minutes, before centrifuging at 12,000xg for ten minutes at 4°C. The supernatant was then removed carefully to avoid disturbing the gel-like RNA pellet. The pellet was washed in 1ml of chilled 75% ethanol, vortexed and centrifuged at 7,500xg for five minutes at 4°C. Following removal of the supernatant, the pellet was left to air-dry and resuspended in up to 40µl of ultra pure H_2O. Using this method, the RNA yield was approximately 100µg.

2.2.7.2 RNA Preparation from Primary Bronchial Epithelial Cells

Cells were previously grown in a 24 well plate by Dr. James Lordan, and were provided in 200µl of TRIzol® reagent. To maximise the RNA yield from the small number of cells glycogen was used as a carrier for the RNA. 0.2µg/µl of glycogen was added to the lysate before adding 40µl of chloroform and leaving on ice for fifteen minutes. The preparation was then centrifuged and separated as described above. An equal volume of ice cold isopropanol was then added and the RNA was left to precipitate over night at –20°C. The pellet was washed twice in ethanol as described above and resuspended in 10µl of H_2O.

2.2.7.3 RNA Quantitation

The RNA yield was assessed using a Pharmacia GeneQuant spectrophotometer with a capillary cell. RNA was diluted 1:10 in RNase free water and the absorbance measured at 260nm to establish the concentration of nucleic acids and at 280nm to establish the concentration of protein contaminants within the sample. To assess the quality of the RNA, samples were also run on a 1% agarose formaldehyde gel (section 1.2.10.2) to observe the 28S and 18S ribosomal RNA. A typical gel is shown in figure 2.4.

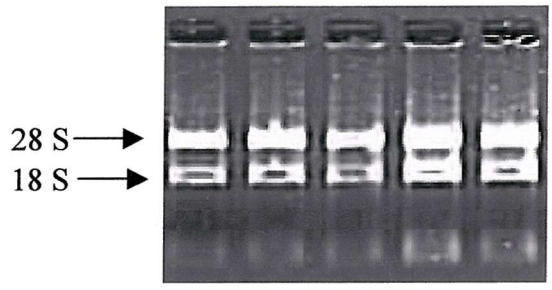


Figure 2.4 RNA Gel Electrophoresis.
Typical pattern of RNA separation in five samples, following electrophoresis on a 1% agarose formaldehyde gel. The smear down each lane represents total human RNA. The two bright bands represent 28S and 18S ribosomal at approximately 5kb and 1.9kb respectively.

2.2.7.4 DNase Treatment

To purify RNA and reduce genomic DNA contamination, the samples were cleaned using the RNeasy mini kit for RNA cleanup according to the manufacturers instructions. This method involved selective binding of RNA to a membrane within a mini spin column, and digestion of DNA by DNase 1, contaminants were efficiently washed away and high quality RNA was eluted into ultra pure water.

For experiments with H292 cells, 50ug of RNA was DNase treated for use in future experiments. 50ug of RNA was made up in 100µl of RNase free water and cleaned according to the manufacturers instructions. Lysis buffer and ethanol were added to the sample to provide conditions which promote selective binding of the RNA to the RNeasy column. The sample was then treated with 27Kunitz units of DNase 1 for fifteen minutes, to remove DNA contamination. Following a series of washes, the RNA was eluted into 50µl of RNase free water and the quantity of RNA recovered assessed using a Pharmacia GeneQuant as described above. Variable and low amounts of RNA were isolated from primary cell cultures so maximum amounts, usually less than 50µg, were cleaned using the above protocol and eluted into 20µl of RNase free H_2O. Recovery ranged from as much as 25% to 75%, depending on the purity of the original sample.

2.2.7.5 cDNA Synthesis (Reverse Transcription)

For experiments with bronchial epithelial cell lines 2µg of RNA, or approximately 1µg of RNA from primary bronchial epithelial cells, was reverse transcribed for use in polymerase chain reaction (PCR). Reverse transcription (RT) was carried out using a superscript first-strand synthesis system for RT-PCR according to the manufacturers instructions.

2 or 1 µg of RNA, 25ng of random hexamers per µg of RNA and 1µl of 10mM dNTP were made up to 10ul in DEPC H_2O, incubated at 65°C for five minutes and left on ice for at least one minute. Reaction mixture containing 2µl 10X RT buffer, 4µl 25mM $MgCl_2$, 2µl 0.1M DTT and 1µl of the RNase inhibitor RNaseOUT® was added to each sample, which were then mixed gently and left at 25°C for two minutes. 50 units (1µl) of the reverse transcriptase, superscript, or ultra pure H_2O for –RT controls, was then added to the samples. The samples were then incubated at 25°C for ten minutes and 42°C for fifty minutes, before the reaction was terminated by incubation at 75°C for fifteen minutes.

The first strand cDNA obtained from this reaction was stored at –80°C until required for PCR.

2.2.8 *Polymerase Chain Reaction*

All gene sequences were retrieved from the National Centre for Biotechnology Information, using the Entrez world wide web server, (http://www3.ncbi.nlm.nih.gov/Entrez).

2.2.8.1 Real Time PCR For the Identification of STAT6 Variants and TGFβ.

This method of PCR exploits the 5' nuclease activity of AmpliTaq Gold DNA polymerase to cleave the reporter dye (FAM) at the 5' end of the probe during the extension phase. Cleavage of the probe separates the reporter dye from the 3' quencher dye (TAMRA), resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the accumulation of fluorescence of the reporter dye, figure 2.5. The 7700-sequence detection system has a built in thermal cycler and a laser, directed via fibre optic cables, to each of the 96 sample wells and this allows the fluorescence to be detected. This has the advantage of allowing PCR products to be detected during the exponential phase when none of the reaction components are rate limiting [197].

2.2.8.1.1 TaqMan™ Primer and Probe Composition

TaqMan™ primers and probes were designed for the coding sequence of genes of interest using the primer express version 1.0 programme on a Macintosh computer. A BLAST search was carried out on all the primers and probes used in this thesis to ensure their specificity to the gene of interest.

2.2.8.1.1.1 Investigation of STAT6 Isoform mRNA Expression

Analysis of the STAT6 isoforms was achieved using a forward primer common to all of the variants and reverse primers which specifically targeted transcripts containing (a) the SH2 domain, which is absent in STAT6c, (b) the sequence spanning the splice site formed by the deletion of 84 base pairs in the SH2 domain to form the dominant negative variant, STAT6c and (c) a sequence common to both STAT6 and STAT6c molecules [149].

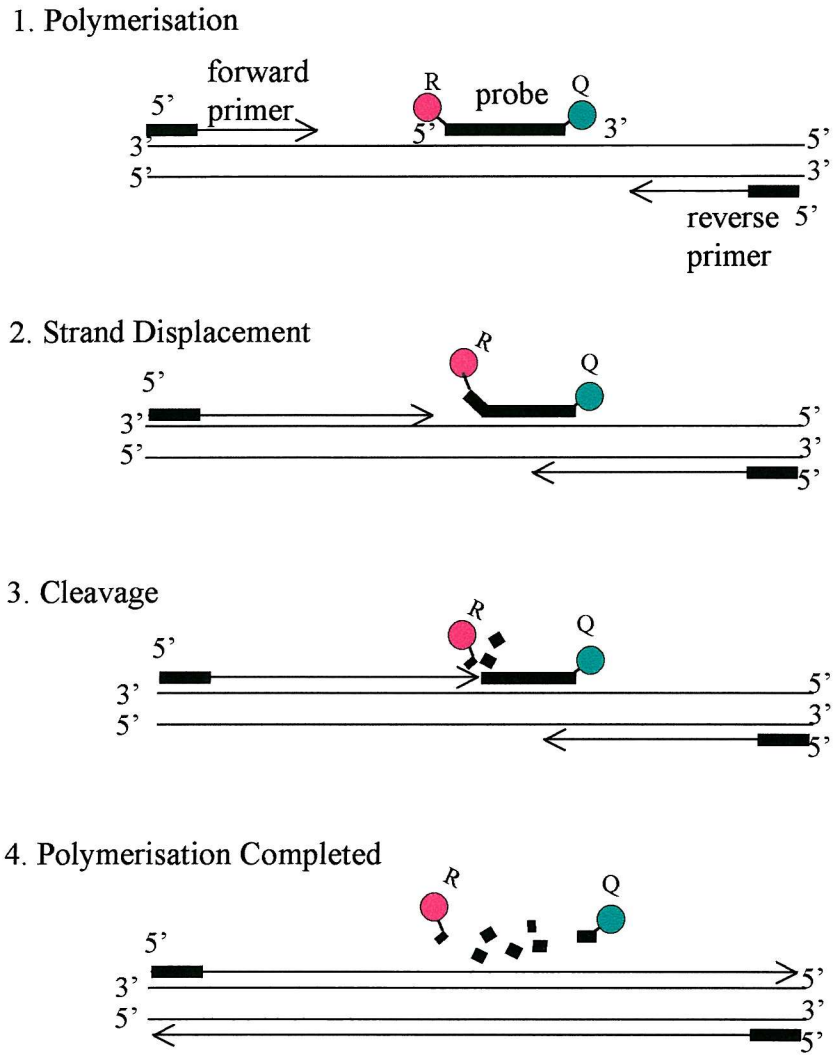


Figure 2.5 Schematic Representation of One Real Time PCR Cycle.
1. The forward and reverse primers and TaqMan™ probe hybridise to the cDNA and polymerisation is initiated by Taq polymerase. The Taqman™ probe is labelled with a fluorescent reporter dye such as FAM at the 5' end and a quencher dye such as TAMRA at the 3' end. The close proximity of quencher to the reporter, quenches the fluorescence emitted by the reporter dye .
2. As polymerisation continues the Taqman™ probe is displaced by the extending DNA strand.
3. The 5'-3' endonucelase activity of Taq Polymerase cleaves the 5' end of the probe and the reporter dye is released. The separation of the reporter dye from the quencher dye results in an increase in the level of fluorescence emitted from the reporter. This increase is measured and is a direct consequence of amplification of the target sequence and occurs in each cycle of the PCR reaction.
4. Polymerisation continues until a complete complimentary strand has been synthesised.
Figure adapted from Livak et al 1995. [197].

A single 5'FAM, 3'TAMRA labelled TaqMan™ probe was used to detect all isoforms, figure 2.6. STAT6 primer and probe sequences are shown in the table below.

	Forward primer (5'-3')	**Reverse primer (3'-5')**	**TaqMan probe**	**Product size**
SH2 domain STAT6	ttt tgg cag tgg ttt gat ggt	gtt tgc tga tga agc caa tga tc	acc tca cca aac gct gtc tcc gga	114bp
STAT6 common	ttt tgg cag tgg ttt gat ggt	cca atc tct gag tcg cgg tc	acc tca cca aac gct gtc tcc gga	190bp
STAT6c	ttt tgg cag tgg ttt gat ggt	tga cat ggg caa tgg tga tg	acc tca cca aac gct gtc tcc gga	83bp
STAT6 2	gtg gct tca cct ttt ggc a	tga gcg aat gga cag gtc ttt	acc tca cca aac gct gtc tcc gga	235bp

STAT6 primers were used at a concentration of 50nM for TaqMan™ reactions, with the aliquot composition for 100 TaqMan™ reactions being 13µl of forward primer, 13ul isoform specific reverse primer (10µM stocks) and 0.5µl of TaqMan™ probe (5 pmol/µl stock).

The expression of the various transcripts was investigated in primary bronchial epithelial cells derived from four normal control subject and seven asthmatic subjects, the clinical details of which are summarised in table 4 (page 64). As described in section 2.2.1, subjects were classified according to GINA guidelines.

2.2.8.1.1.2 Investigation of TGFβ mRNA Expression

To investigate TGFβ expression a pre-developed assay kit containing primers and probe, directed against pan TGFβ was purchased. For each set of samples endogenous 18S ribosomal RNA was used as a housekeeping gene control, so that other genes could be normalised against this to assess any changes in expression. All primers and probes were stored at –20°C until required for use. For PCR in which the product was visualised on an

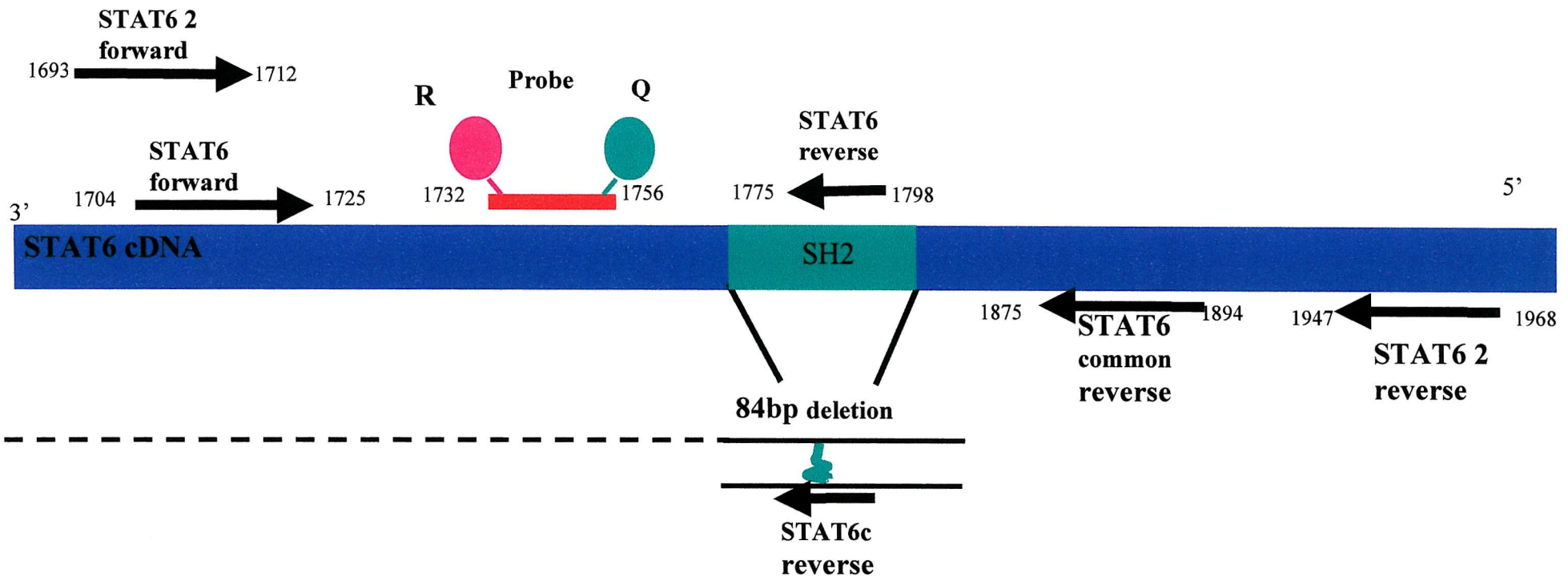


Figure 2.6 STAT6 Isoform Primer Design.
For analysis of STAT6 isoform expression a single forward primer and 5'FAM, 3' TAMRA labelled Taqman™ probe (R; reporter, Q; quencher common to all STAT6 transcripts was designed using the Perkin Elmer Primer Express program. Detection of the individual isoforms was achieved by designing reverse primers specific to the insoforms. STAT6 reverse primer hybridizes in the SH2 domain (STAT6 reverse) thus does not detect the dominant negative type. To detect this a primer was designed over the join created by deletion of 86 base pairs within the SH2 domain (STAT6c reverse). A reverse primer common to all full length STAT6 transcripts was also designed (STAT6 common). A second pair of primers were designed to detect full length STAT6 (STAT6 2)were also designed.

Table 4. Summary of the Clinical and Physiological Characteristics of Subjects for the Investigation of STAT6 Isoform Expression.

S, Salbutamol; ICS, inhaled corticosteroids; mod/sev, moderate to severe asthma

		Age (yr)	Sex	Atopy	FEV_1 (% predicted)	Treatment
Healthy controls						
N1		20	M	N	104	none
N2		25	M	N	99	none
N3		21	M	N	103	none
N4		20	M	N	110	none
Mean		21			104	
SD		2.38			4.54	

Asthmatics						
	Severity					
A1	mild	27	M	Y	86	S
A2	mod/sev	55	M	Y	68	400μg ICS
A3	mod/sev	25	M	Y	69	1000μg ICS
A4	mod/sev	28	M	N	86	1000μg ICS
A5	mod/sev	39	M	Y	78	800μg ICS
A6	mild	22	M	Y	90	S
A7	mild	21	F	Y	94	S
Mean		31			81.57	
SD		12			10.16	

agarose gel, the primers were used at the same concentration but the probes were omitted from the reaction.

2.2.8.1.2 Preparation of Real-Time RT-PCR Reactions

For TaqMan ™ experiments, a reaction mix was prepared using the primer/probe aliquots, or pre-developed assay kit, along with TaqMan universal master mix and ultra pure dH_2O. The amounts of each component for one reaction are as follows:

18s Reaction Mix (one reaction): 0.375µl pre-developed control reagent, 12.5µl TaqMan master mix and 7.125µl H_2O.

TGFβ Reaction Mix (one reaction): 1.25µl pre-developed assay reagent, 12.5µl TaqMan master mix, 6.25µl H_2O.

STAT6 Isoform Reaction Mixes (one reaction): 0.76µl primer-probe aliquot, 12.5µl TaqMan master mix 6.74µl H_2O.

Following preparation of the reaction mix 20µl was added to each well of a micro amp optical 96 well reaction plate. For use in PCR reactions, cDNA samples were diluted to a concentration of 5ng/µl in ultra pure H_2O. Following the addition of reaction mix, 5µl of each sample was added to micro amp optical reaction plate, to give a final reaction volume of 25µl. For each TaqMan™ PCR experiment, a cDNA standard curve typically ranging from 100ng to 0.1ng was also included on each plate. The source of cDNA for the standard curve was pooled H292 or primary bronchial epithelial cell cDNA from several experiments. Each sample, including each concentration for the standard curve, was assayed in duplicate and the mean used.

Following addition of reaction mix and sample to the plate, the lids were tightly sealed and the plate spun gently to collect the liquid. PCR was carried out using a Perkin-Elmer ABI PRISM 7700 sequence detection system, where samples are initially heated to 50°C for two minutes and 95°C for ten minutes to activate the Taq polymerase within the universal master mix. This is followed by 40 cycles of: 95°C for fifteen seconds to allow denaturation of the RNA:cDNA complex and 60°C for one minute to allow annealing of the primers and extension.

For visualisation of the STAT6 PCR products (to check product size), 5ng/µl of primary bronchial epithelial cell cDNA was amplified on an Eppendorf mastercycler using the

conditions described above. For these PCR reactions the primers were used at the same concentration but the probes were omitted from the reaction.

2.2.8.1.3 Analysis of Results

At the end of the reaction, the 7700-sequence detector sets a baseline based on the initial stages of the PCR reaction when there is little fluorescence and a fixed fluorescence threshold can be set just above this baseline. The cycle number at which fluorescence from a sample crosses this threshold is defined as the Ct value, as shown in figure 2.7. To obtain a standard curve, the Ct value obtained for different starting cDNA concentrations is plotted against the log dilution factor of the sample. The mean of the standard curves from five experiments for 18S ribosomal RNA, TGFβ, STAT6 common and STAT6c, SH2 domain STAT6 and STAT6 2 are shown in figure 2.8, 2.9 and 2.10. This in turn allowed the relative amount of product in the unknown samples to be read from the relevant standard curve, using their Ct values. These values were then normalised for the corresponding 18S values, which takes into account differences in the starting amount of cDNA within samples that could be due to variation in the efficiency of the reverse transcripts among the different RT reactions. A typical example of the calculation used to obtain the normalised relative amount of a target gene is shown in appendix 1.

2.2.8.2 PCR to Identify IL-4Rα, IL-13Rα1 and IL-13Rα2 Transcripts in Bronchial Epithelial Cells

Primers designed for the coding region of IL-4Rα, IL-13Rα1 and IL-13Rα2 were kindly provided by Dr. A. Konstantindis (Human Genetics, Southampton General Hospital). The primer sequences were as follows.

Fragment	**Forward primer (5'-3')**	**Reverse Primer (3'-5')**	**Product length**
IL-4Rα	ctg acc tgg agc aac ccg tat	ccg ctt ctc cca ctg tga ccc	450
IL-13 Rα1	tca tgg tcc ctg gtg ttc	cgg tgc gcg act caa cat aaa	555
IL-13Rα2	gga gca tac ctt tgg gac ct	ttg gcc atg act gga aac tg	427

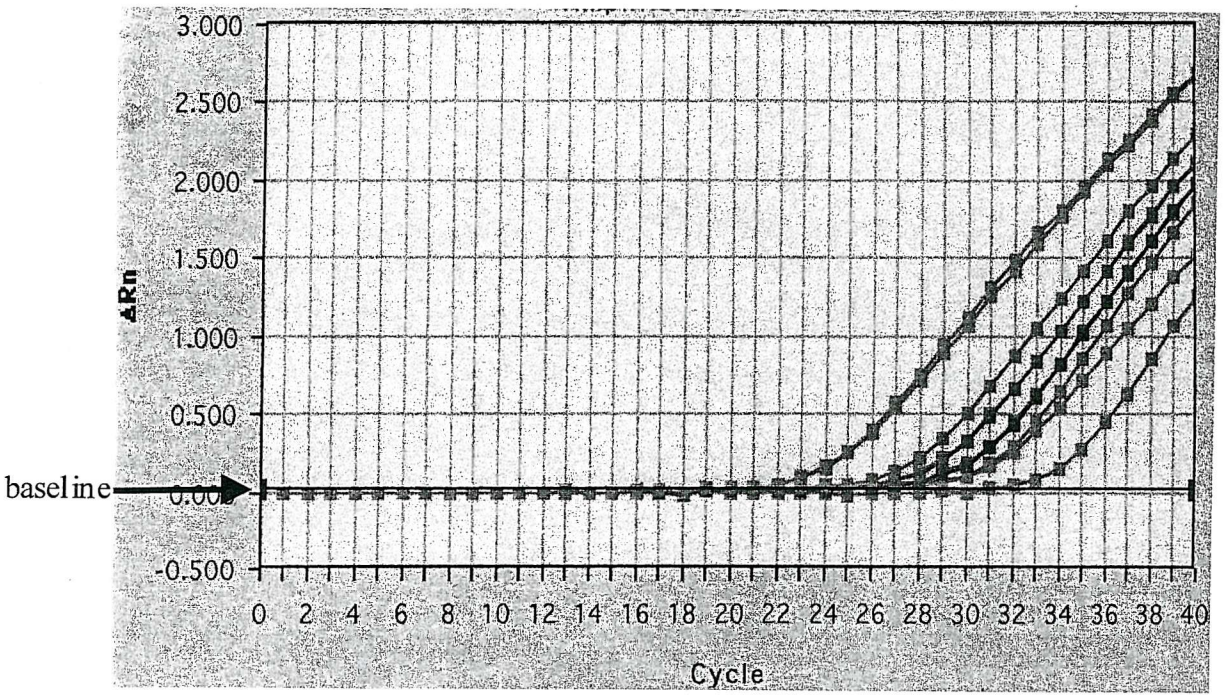


Figure 2.7 Typical TaqMan™ PCR Standard Curve.
This figure shows the typical output from the ABI Prism 7700 Sequence Detector used for TaqMan™ PCR. ΔRn is the fluorescence emitted by each individual reaction following cleavage of the probe by Taq polymerase which increases with each PCR cycle. A baseline is set along the first 8 cycles and the cycle number at which ΔRn crosses this threshold is the Ct value. This figure was generated using different starting concentrations of cDNA thus giving increasing Ct values.

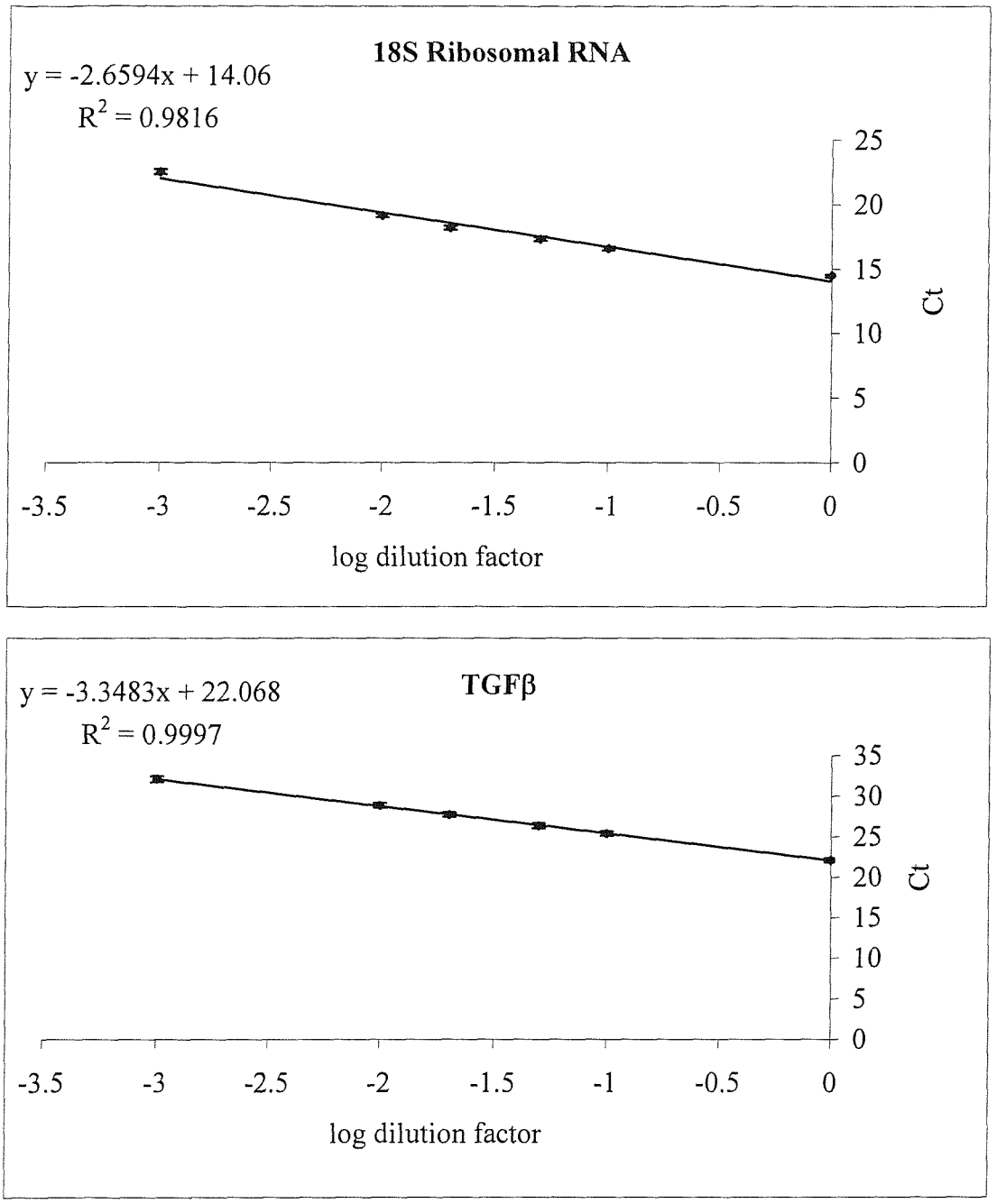
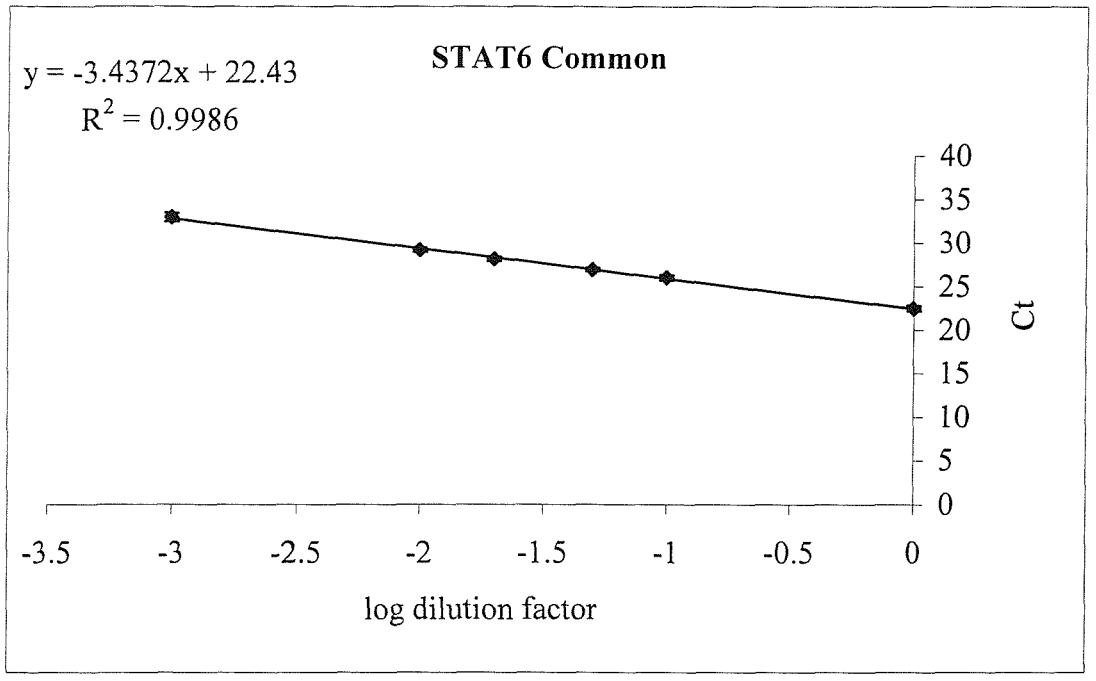


Figure 2.8 18s Ribosmal RNA and TGFβ TaqMan Standard Curves.
Pooled cDNA was serially diluted to 20, 2, 1, 0.4, 0.2 and 0.02 ng/μl and assayed in duplicate to provide a standard curve to measure relative amounts of the samples of interest. For analysis the log of the dilution factor was used to generate the standard curve. The curves above show the mean±SE of 5 standard curves generated independently for 18S ribosomal RNA and TGFβ.



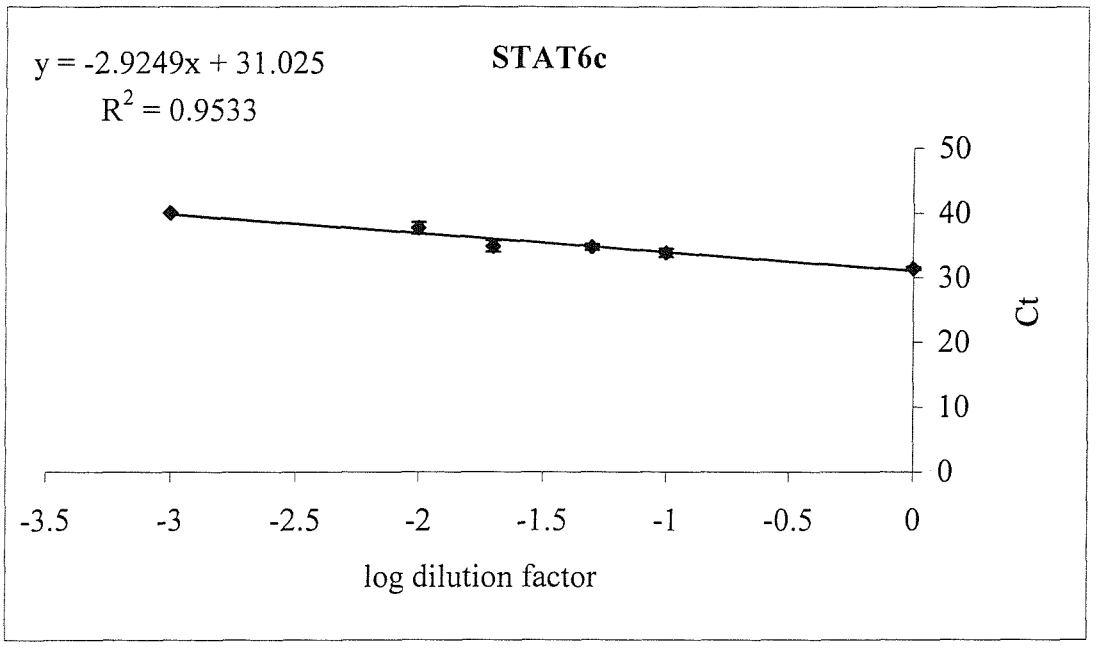
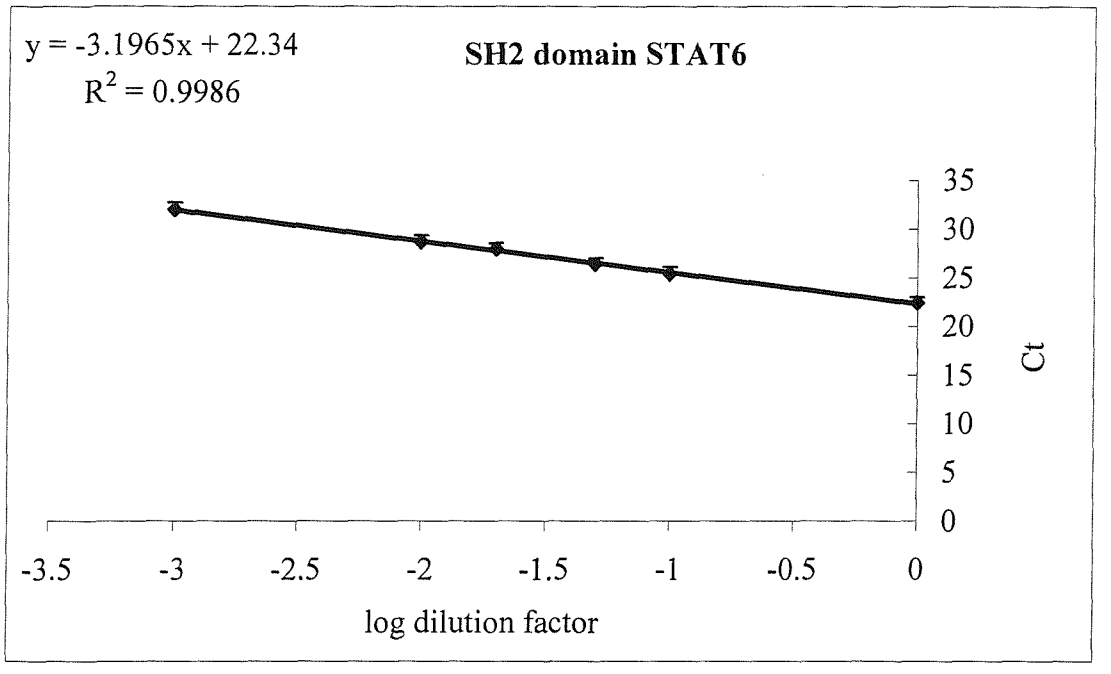


Figure 2.9 STAT6 Common and STAT6c TaqMan Standard Curves.
Pooled cDNA was serially diluted to 20, 2, 1, 0.4, 0.2 and 0.02 ng/ml and assayed in duplicate to provide a standard curve to measure relative amounts of the samples of interest. For analysis the log of the dilution factor was used to generate the standard curve. The curves above show the mean±SE of 5 standard curves generated independently for STAT6 common and STAT6c.



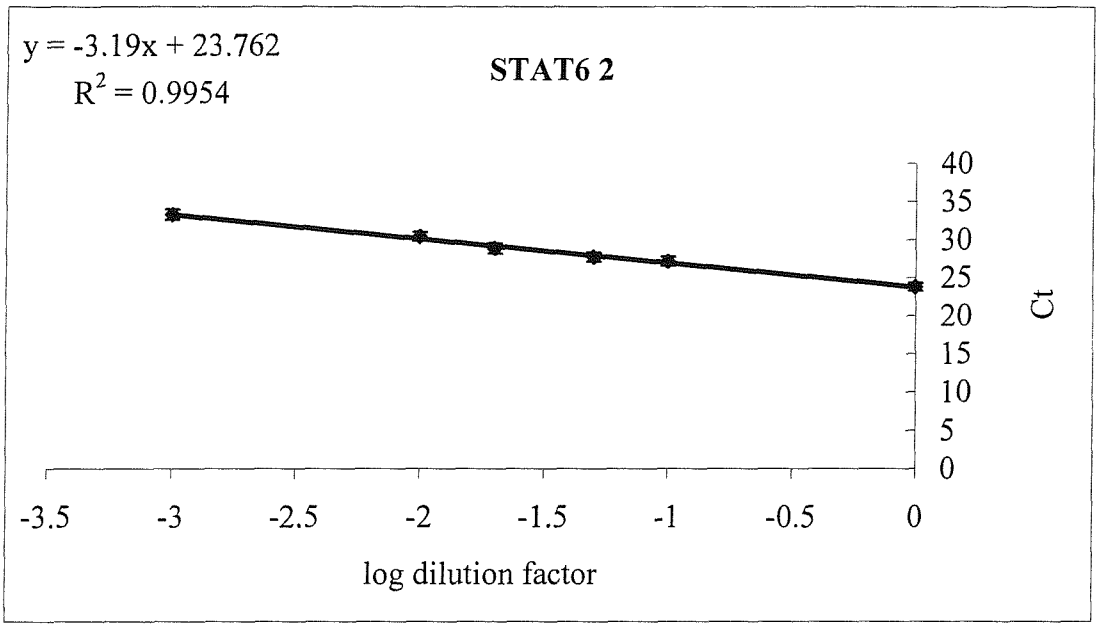


Figure 2.10 SH2 Domain STAT6 and STAT6 2 TaqMan Standard Curves. Pooled cDNA was serially diluted to 20, 2, 1, 0.4, 0.2 and 0.02 ng/ml and assayed in duplicate to provide a standard curve to measure relative amounts of the samples of interest. For analysis the log of the dilution factor was used to generate the standard curve. The curves above show the mean $\pm$ SE of 4 standard curves, generated independently for SH2 domain STAT6.

For each PCR reaction, 0.2μg of cDNA (2ul of a 0.1μg/μl preparation) or negative RT sample was added to a PCR master mix containing the following reagents per reaction, shown in the table below. A no template control was also included for each primer set.

Reagent	Final Concentration	Stock Concentration	Volume
Water			17.25μl
Forward primer	0.2μM	10μM	0.5μl
Reverse primer	0.2μM	10μM	0.5μl
PCR buffer II	1 X	10X	2.5μl
$MgCl_2$	1.5mM	25mM	1.5μl
dNTP	200μM	10mM	0.5μl
Taq Polymerase	0.025U/μl	2.5U/μl	0.25μl
Final Reaction Volume = 25μl			

The PCR amplification was performed in DNA engine tetrad under the following conditions.

Primer	Denature	Anneal	Extend	Cycles
IL-13Rα1	94°C, 30"	58°C, 30"	72°C, 40"	35
IL-13Rα2	94°C, 30"	64°C, 30"	72°C, 30"	35
IL-4Rα	94°C, 30"	58°C, 30"	72°C, 40"	35

For all conditions, PCR reactions had an initial denaturation step of two minutes and a final extension for five minutes. The PCR product was subsequently visualised using agarose gel electrophoresis, as described below.

2.2.9 *Agarose Gel Electrophoresis*

2.2.9.1 DNA Gel Electrophoresis

STAT6 and IL-4/13R PCR products were separated on a 3% (3g agarose, 100ml TBE buffer) or 2% (2g agarose, 100ml TBE buffer) slab agarose gel respectively. The agarose and buffer were heated in a microwave until the agarose dissolved (approximately four minutes on medium power). The solution was then poured into the gel cast, the combs inserted and left to cool for approximately thirty minutes until the gel was set. After this time, the appropriate PCR product was mixed with loading buffer (30% glycerol, 0.25% xylene cyanol FF, 0.25% bromophenol blue made up in distilled H_2O, stored at 4°C) in a 2:1 ratio, and loaded into the gel, 2µl of 50 base pair DNA ladder in 6µl of sample buffer was also added. The electrophoresis tank was then filled with TBE buffer and electrophoresis was left to proceed at 80-100V for thirty to sixty minutes depending on the size of the expected product. The gel was then removed from the holder and placed in either a 0.0004% solution of vistra green or ethidium bromide (4µl in 40ml H_2O) for thirty minutes, washed under running tap water and visualised under UV light on a Genegenius for agarose gels, Syngene (Synoptics Ltd, UK).

2.2.9.2 RNA Gel Electrophoresis

For this procedure all equipment was made RNase free by baking glassware at 250°C for four hours and wiping down all surfaces and the Bio Rad wide mini sub cell GT with RNase away. 1% agarose formaldehyde gel was prepared by heating 0.4g agarose, 4ml 10x MOPS buffer, 34ml H_2O in a microwave to melt the agarose (forty-five seconds on medium heat). This solution was left to cool before 2.162ml of formaldehyde was added. This solution was then poured into the gel holder and left to set for thirty to sixty minutes. Once the gel was set, it was inserted into the electrophoresis tank and covered with 1X MOPS. Approximately 1µl RNA was added to 9µl of sample loading buffer (50% deionised formamide, 1X MOPS, 2.15M 37% formaldehyde, 5% glycerol, tiny bit of bromophenol blue to give blue colour, 0.1mg/ml ethidium bromide, made up in H_2O) and loaded into the gel. Electrophoresis was left to proceed at 90V for forty minutes after which time the RNA was visualised under UV light on a Genegenius for agarose gels, Syngene (Synoptics Ltd, UK).

2.2.10 *Transfection of NCI-H292 Cells with STAT6 Expression Vectors*

Plasmids containing wild type STAT6 or STAT6 tyrosine 641/ tryptophan mutation, which acts as a dominant negative, were a kind gift from Tularik Inc. San Francisco. Both have been fully characterised in Mikita *et al.*, 1996 [146]. The plasmids were cloned and purified in Southampton by Dr.R.Powell. Unfortunately the empty pcDNA3 plasmid (used for STAT6 constructs) was not provided and is no longer commercially available thus a plasmid of similar size, pRc/CMV was used as a control vector (figure 2.11).

H292 cells were seeded in twenty-four or six well plates at a density of $2.5x10^4$ or $7.5x10^4$/ ml respectively and cultured as described in section 2.2.2.1 for twenty-four hours, after which time the cells were approximately 40% confluent. The culture medium of the cells was then changed and the cells were transfected using Effectene transfection reagent according to the manufacturers instructions. Using this system, the DNA is initially condensed by addition of enhancer in a ratio of 1ug of DNA to 8ul of enhancer, in the supplied DNA condensation buffer. Following a five minute incubation at room temperature, the effectene reagent was added at a DNA:Effectene ratio of 1:10. This mix was then incubated for ten minutes at room temperature, after which time the effectene-DNA complexes were mixed with culture medium and added drop-wise directly to the cells. The concentrations and volumes of reagents (per well) used for transfection of cells in 24 and 6 well plates are shown below.

Culture Format	**DNA µg**	**Enhancer µl**	**Final volume of DNA in Buffer EC (µl)**	**Effectine Reagent µl**	**Volume of medium added to cells (µl)**	**Volume of medium added to complexes (µg)**
24-well plate	0.4	3.2	60	4	350	350
6-well plate	0.8	6.4	100	8	1600	600

After six hours the medium containing the transfection reagents was replaced with serum free medium for eighteen hours prior to stimulation. Cells in the 24 well plates were

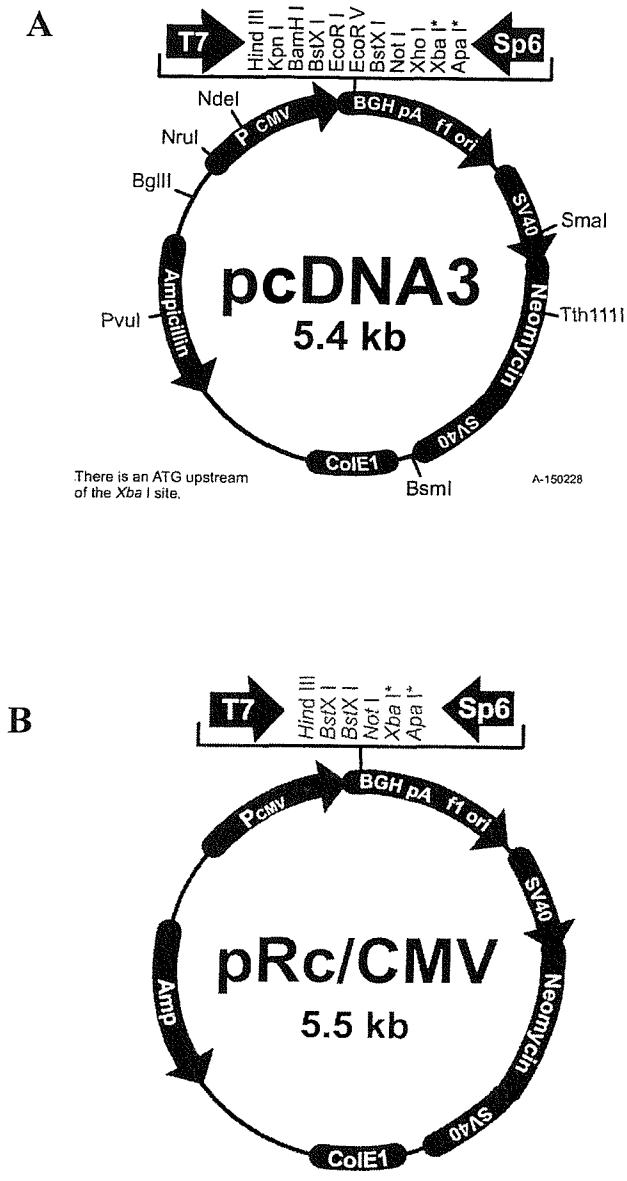


Figure 2.11 Plasmids used for Transfection of H292 Cells.
STAT6 wild type a the tyrosine 641 dominant negative mutant constructs were inserted into pcDNA3 (A) using the EcoRI and NotI sites in the polylinker region. (B) pRc/CMV was used as an empty plasmid for control experiments due to its structural similarities to pcDNA3.

incubated with 1.5nM IL-4 or fresh serum free medium (control) for forty-eight hours. After this time the supernatant was collected for ELISA and the cells fixed in formyl saline as described above. The cells in the 6 well plates were also treated with 1.5nM IL-4 or fresh serum free medium (control) in duplicate and left for either one hour or forty-eight hours for collection of whole cell lysates or RNA respectively. Collection of whole cell lysates from these experiments involved direct lysis into sample buffer containing DTT in place of mercaptoethanol (5X stock; 0.77g DTT, 1g SDS, 4ml Tris-HCl pH 6.8, 5ml glycerol, 1ml dH_2O). Following lysis cells were sonnicated and centrifuged at 16000xg. When the cells were lysed in this way, a protein assay was not performed and a western blot with a β-actin antibody was carried out to ensure equivalent protein loading.

2.2.11 *Statistical Analysis of Results*

Statistical analyses of the results contained within this thesis were carried out using SPSS (version 10) software. The expression of STAT6 protein in bronchial biopsy specimens and STAT6 mRNA expression in primary bronchial epithelial cells was analysed using the non-parmatric Mann Whitney test. A repeated measures ANOVA test, to take into account multiple testing, and a paired students T test, was used to analyse the effect of increasing concentrations of IL-4 and IL-13 on $TGF\beta_2$ release. A paired students T test was also used to analyse the effect of IL-4 on TGFβ and STAT6 mRNA expression and $TGF\beta_2$ release from transfected cells at discrete time points. A P value of less than 0.05 was generally considered significant.

CHAPTER 3

3 STAT6 EXPRESSION IN BRONCHIAL EPITHELIAL CELLS

3.1 Introduction

As described in chapter one, the bronchial epithelium in asthma displays an activated phenotype with increased expression of many cytokines, growth factors and transcription factors. These proteins can induce further expression of other pro-inflammatory cytokines and growth factors, which have the potential to contribute to the development of chronic airways inflammation and remodelling.

The transcription factor STAT6 mediates many of the effects of IL-4 and IL-13, two pivotal cytokines associated with allergic disease, and evidence from STAT6 knockout mice indicates its vital role in the manifestation of allergic inflammation [154] [153]. The presence of the IL-4Rα [179] and IL-13Rα1 and IL-13Rα2 [180] chains on bronchial epithelial cells has been confirmed, but expression of the down stream signalling molecules STAT6 and one of its upstream activators, the tyrosine kinase JAK1, has not been investigated.

In addition to full length STAT6 several naturally occurring variants have been described in human tissues, including a dominant negative splice variant which is capable of inhibiting full length STAT6 activation [149]. Thus, the aims of this chapter were to analyse the expression of STAT6 and JAK1 in the human bronchial epithelium *in vivo* and to investigate whether levels of expression relate to disease severity. The expression of dominant negative STAT6 in human bronchial epithelial cells was also investigated. Several *in vitro* studies have utilised human bronchial epithelial cell lines to assess the involvement of STAT6 in IL-4 and IL-13 mediated responses. The presence of STAT6 in a carcinoma derived bronchial epithelial cell line was investigated to establish a suitable model in which to carry out future experiments.

3.2 Results

3.2.1 *STAT6 Expression in Airway Tissue*

Archival glycol methacrylate (GMA) embedded bronchial biopsies (originally obtained from anonymous subjects following ethical approval) were obtained from 11 control subjects without asthma (5 female; age, 22 (18-46)); nine subjects with severe asthma (8 female; age, 24 (13-50)) and fourteen subjects with mild asthma (9 female; age, 25 (18-54)); the clinical characteristics of these volunteers are detailed in table 2, chapter two.

Immunohistochemical analysis revealed the columnar cells of the bronchial epithelium to be the major site of STAT6 expression in the airways of all subjects, (Figure 3.1 panel A-C) with both the ciliated cells and mucus secreting goblet cells showing positive immunoreactivity. STAT6 was expressed largely in the cytoplasm, but in the few sections, which had been cut vertically through the epithelium, a nuclear ring pattern of staining was apparent in both normal and asthmatic subjects suggesting a degree of transcription factor activity irrespective of disease status (Figure 3.1 panel E). In contrast, the cells in the basal layer of the epithelium were only weakly positive.

The specificity of STAT6 immunoreactivity was confirmed by pre-adsorbing the antibody with an excess of its immunising peptide. This abolished all staining apart from a small amount along the brush border, which was probably due to non-specific association of the antibody to mucus in the brush border (Figure 3.1 panel D). As a result, the brush border was carefully excluded from the area selected for quantitation. Computerised image analysis revealed that the level of STAT6 expression in the bronchial epithelium in mild asthma did not differ significantly when compared to normal controls (% epithelial staining, median (range): (3.4(0-16.0) *versus* (4.7(0-20.0)). In severe asthma, epithelial STAT6 expression (13.7 (4.8-25.7)) was significantly increased when compared to with mild asthma (P=0.001) and normal controls (P=0.037) (Figure 3.2).

Positive immunoreactivity for JAK1 was much weaker and more diffuse than that observed for STAT6. As with STAT6, the principal site of JAK1 expression was the cytoplasm of columnar epithelial cells with much lower levels in the basal layer. In some sections staining around the plasma membrane was evident which is where one would expect this surface receptor associated kinase to be located (Figure 3.1 panel F). The low intensity of staining for JAK1 in the bronchial epithelium made it difficult to quantify

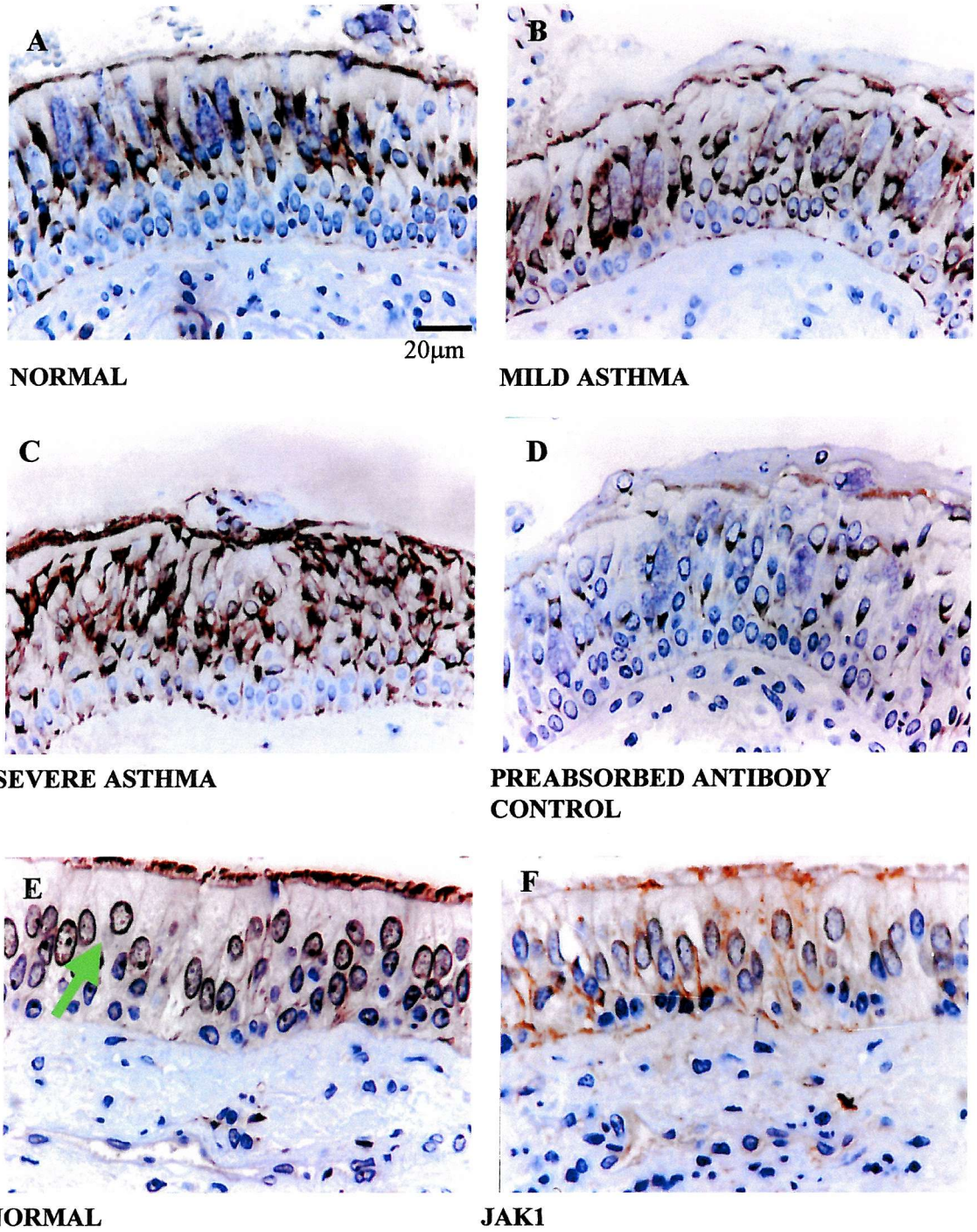


Figure 3.1. Immunohistochemical Analysis of STAT6 and JAK1 Expression in Bronchial Epithelial Cells.

Bronchial biopsies from normal and asthmatic subjects were stained for the presence of STAT6 as described in Materials and Methods. The figure shows representative staining of biopsies from Normal (A), mild (B,D) or severe asthmatic (C) subjects using anti-STAT6 (A-C) or preadsorbed antibody control (D). Plate E shows a normal biopsy displaying nuclear staining for STAT6 which was observed to be similar in a subset of both normal and asthmatic biopsies. Plate (F) shows a typical section stained for JAK 1 with weak staining localised to the cell membranes. Scale bar=20μM.

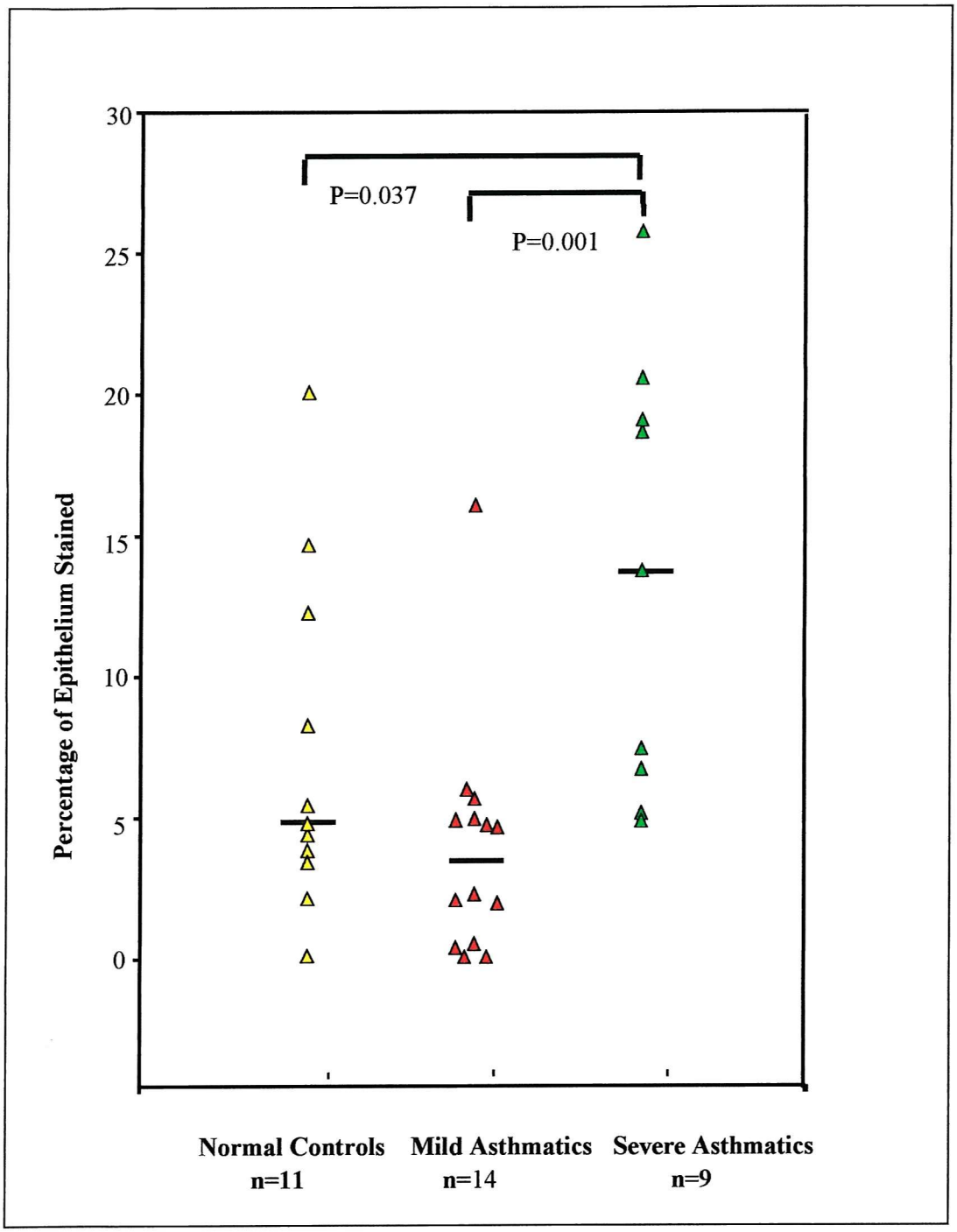


Figure 3.2 Quantitative Analysis of Epithelial STAT6 Staining in Bronchial Biopsies.

Immunostaining for STAT6 was quantified in the entire intact epithelium in each bronchial biopsy using computerised image analysis. The figure shows % epithelial staining for each subject and the bar shows the median value.

Data were analysed using Mann Whitney U test.

accurately using the colour vision image analysis system currently available, therefore analysis was not undertaken.

3.2.2 *STAT6 and IL-4/IL-13 Receptor Expression in Bronchial Epithelial Cell Lines*

Following the identification of STAT6 in human airway tissue, experiments were carried out to investigate the expression of IL-4Rα, IL-13Rα1, IL-13Rα2 and STAT6 expression in the H292 bronchial epithelial cell line. Studies from other groups have shown that these cells respond to IL-4 [25]. However, it was considered necessary investigate what the receptor profile of the cells was before examining IL-4 and IL-13 mediated responses. As no specific antibodies to the IL-13 receptor chains were commercially available at the time, RT-PCR was carried out on RNA collected from H292 cells to confirm expression of IL-4Rα, IL-13Rα1 and IL-13Rα2. PCR using primers for the specific receptor chains and agarose gel electrophoresis revealed bands of 450bp, 555bp and 427bp for IL-4Rα, IL-13Rα1 and IL-13Rα2 respectively (figure 3.3). Minus-RT reactions and no template controls did not reveal any bands.

For detection of STAT6 in the H292 cells, SDS PAGE of whole cell lysates and Western blotting with a STAT6 specific mouse monoclonal antibody was used. Using this method, high levels of a single band with the same molecular weight as STAT6, 105kDa, was detected (figure 3.4). When the primary antibody was omitted and blots were stained with rabbit anti-mouse or swine anti rabbit immunoglobulins no bands were detected (data not shown, see chapter five, figure 3, panel C).

3.2.3 *Expression of STAT6 Variants in Bronchial Epithelial Cells*

A splice variant of STAT6 that negatively regulates function of STAT6, termed STAT6c, has been reported to be expressed in human lung [149]. Expression of this variant in murine B cells partially inhibited phosphorylation of endogenous STAT6, but its main mechanism of action was deduced to be inhibition of STAT6 dimerisation and binding to the promoters of target genes. Therefore expression of STAT6c could have important implications in the regulation of STAT6 signalling, and in the expression of pro-inflammatory genes in different cell types. Therefore experiments were carried out to investigate its expression in bronchial epithelial cells.

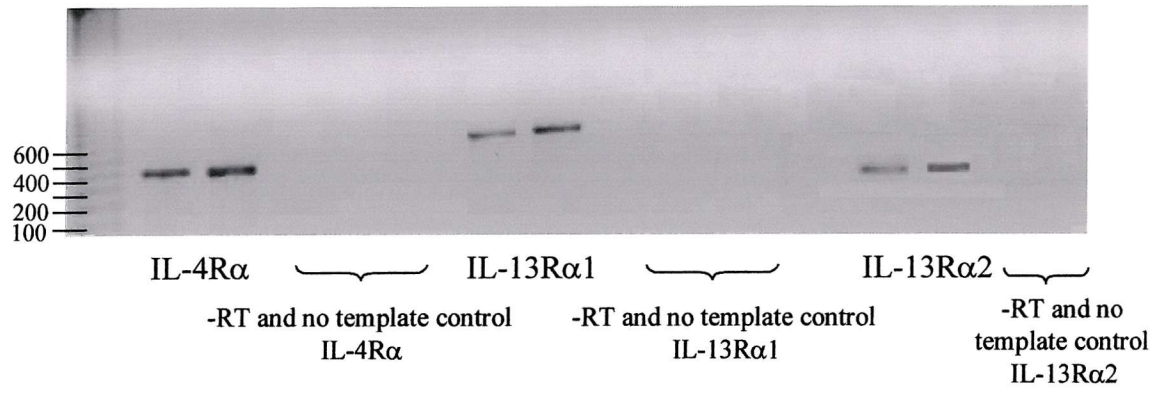


Figure 3.3 IL-4Rα, IL-13Rα1 and IL-13Rα2 mRNA Expression in H292 Cells.

RT-PCR using primers specific receptor chains was carried out on RNA collected from H292 cells. IL-4Rα1 was detected at 450bp, IL-13Rα1 was detected at 555bp and IL-13Rα2 was detected at 427bp. Duplicate samples were run in parallel along with –RT and –template controls (represented by gap between different receptor chain bands).

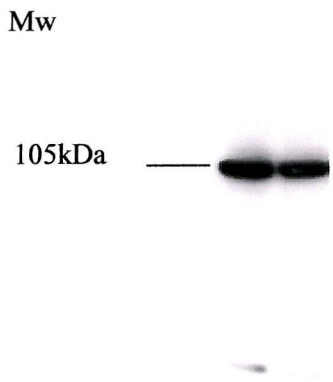


Figure 3.4 STAT6 in H292 Cell Lysates.
Western blotting of H292 cell lysates with a STAT6 antibody revealed high levels of a single band with a molecular weight of 105kDa,determined to be STAT6.

As little work had previously been carried out on this variant, a method was developed to analyse its expression in bronchial epithelial cells. To achieve this a single forward primer, which hybridised to STAT6 transcripts, was designed along with specific reverse primers to detect each isoform. Patel *et al* [149] reported that the dominant negative STAT6 (STAT6c) had an 86 base pair deletion within the SH2 domain of the molecule, therefore the sequence formed by the deletion was used to design real time TaqMan™ PCR primers (figure 3.5). Primers were also designed to detect full length STAT6 (termed STAT6 common) and transcripts with the SH2 domain sequence (termed SH2 domain STAT6). A single probe was used to detect all three isoforms. The necessary concentrations of primers, probe and template were optimised on RNA derived from various human tissues and these conditions were then applied to the H292 cells. From the experiments described below it was determined that the individual primer pairs did not amplify with equivalent efficiency. This was determined by subtracting the Ct values at each point on the standard curves for the two primer pairs being compared. If the different primers primed with equal efficiency this plot would be horizontal and the gradient less than 0.1. A comparison between STAT6 common Ct values and STAT6c Ct values gave a gradient of 0.78, STAT6 common and SH2 domain STAT6 gave a gradient of 0.26 and SH2 domain STAT6 and STAT6c gave a gradient of 0.26. From this it was determined that the levels of the STAT6 PCR products obtained using the different primer pairs could not be compared directly.

Initial experiments revealed that all the transcripts were detectable in H292 bronchial epithelial cells (figures 3.6 and 3.7). After optimising the primers and probe and preliminary experiments to confirm their expression in H292 cells, experiments were carried out to analyse their expression in primary bronchial epithelial cells. To confirm that a product of the correct size was amplified during the PCR reaction the PCR products were separated by agarose gel electrophoresis and visualised by staining with vistra green. This process revealed single bands for each of the products amplified using the different primer sets. The size of these products was as expected, 190bp for STAT6 common, 94bp for STAT6 and 83bp for STAT6c (figure 3.8). Whilst the products were the expected size, efforts were made to confirm their identity by direct sequencing. However their size prohibited this due to the problems associated with background 'noise' in the sequencing method used. It would have been possible to clone the products and them sequence but due to time restrictions this was not undertaken.

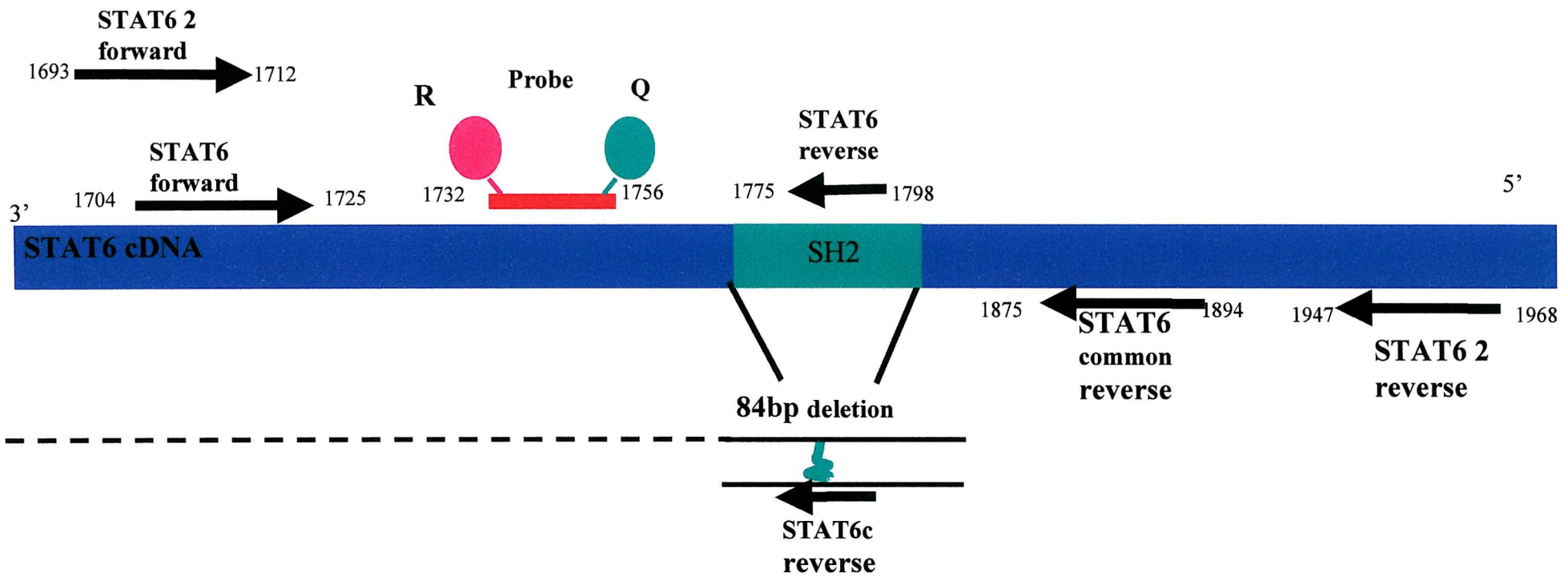
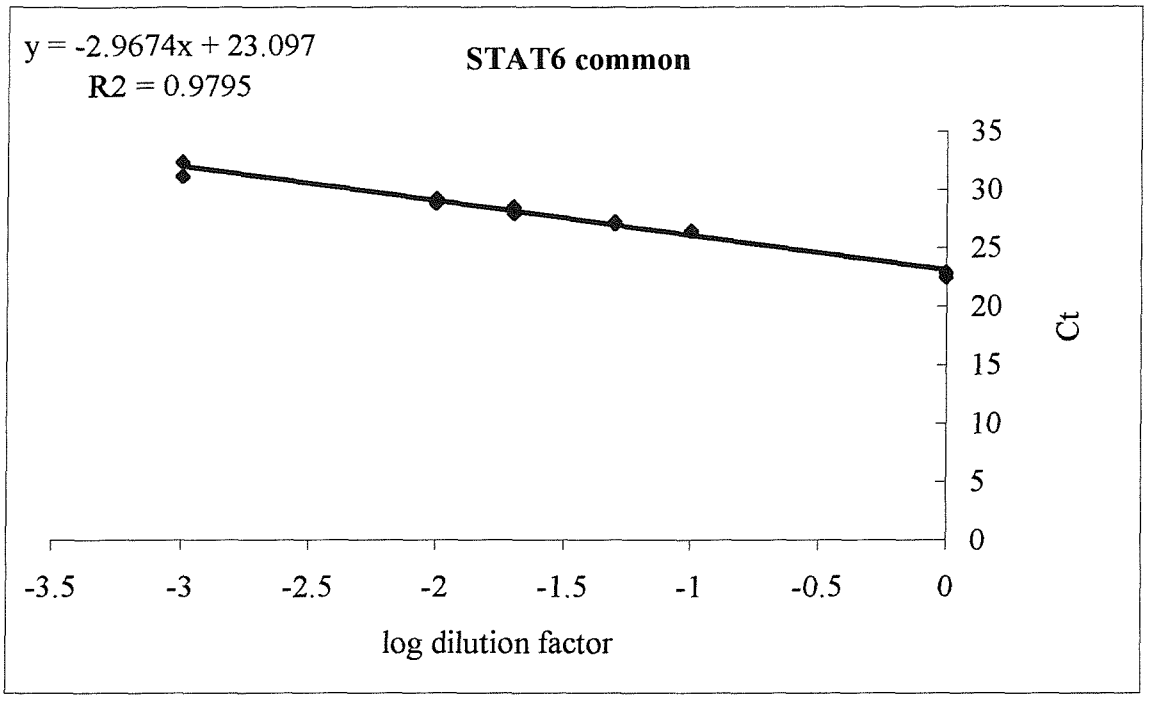


Figure 3.5 STAT6 Isoform Primer Design.
For analysis of STAT6 isoform expression a single forward primer and 5'FAM, 3' TAMRA labelled Taqman™ probe (R; reporter, Q; quencher common to all STAT6 transcripts was designed using the Perkin Elmer Primer Express program. Detection of the individual isoforms was achieved by designing reverse primers specific to the insoforms. STAT6 reverse primer hybridizes in the SH2 domain (STAT6 reverse) thus does not detect the dominant negative type. To detect this a primer was designed over the join created by deletion of 86 base pairs within the SH2 domain (STAT6c reverse). A reverse primer common to all full length STAT6 transcripts was also designed (STAT6 common). A second pair of primers were designed to detect full length STAT6 (STAT6 2)were also designed.



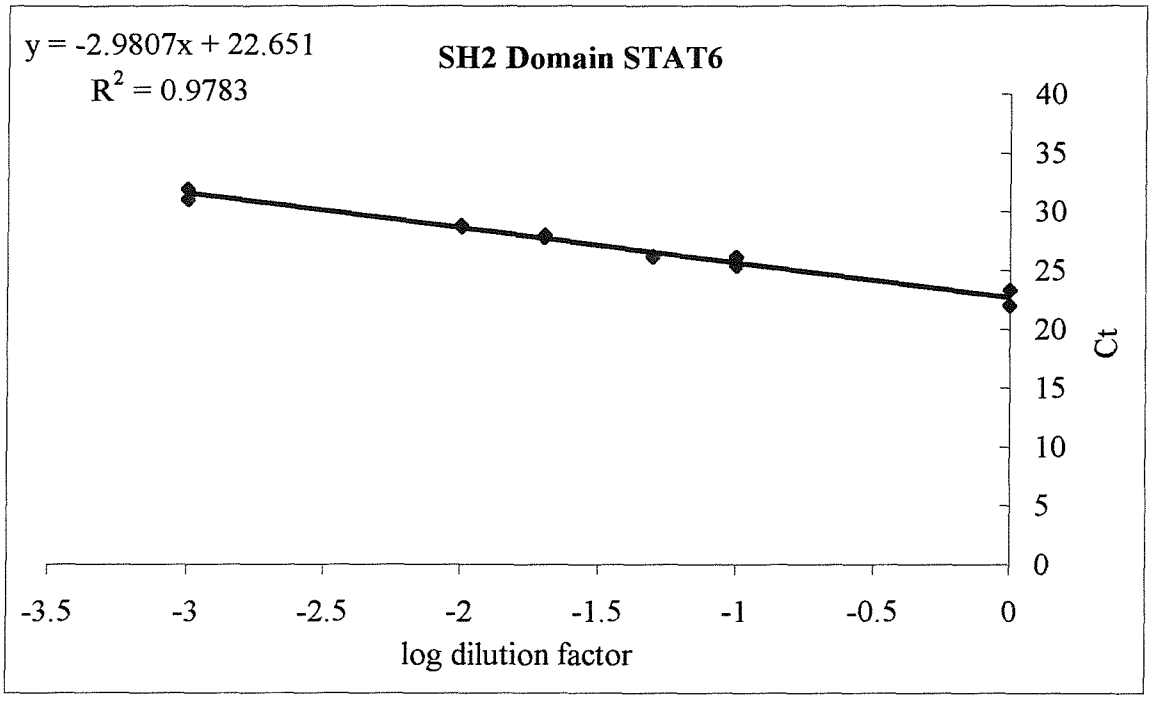


Figure 3.6 STAT6 Common and STAT6 Expression in H292 Cells.
TaqMan™ PCR primers were designed to amplify full length STAT6 (STAT6 common) and SH2 domain containing transcripts (STAT6). Both transcripts were detected at a range of concentrations of H292 cDNA (expressed as log of the dilution factor).

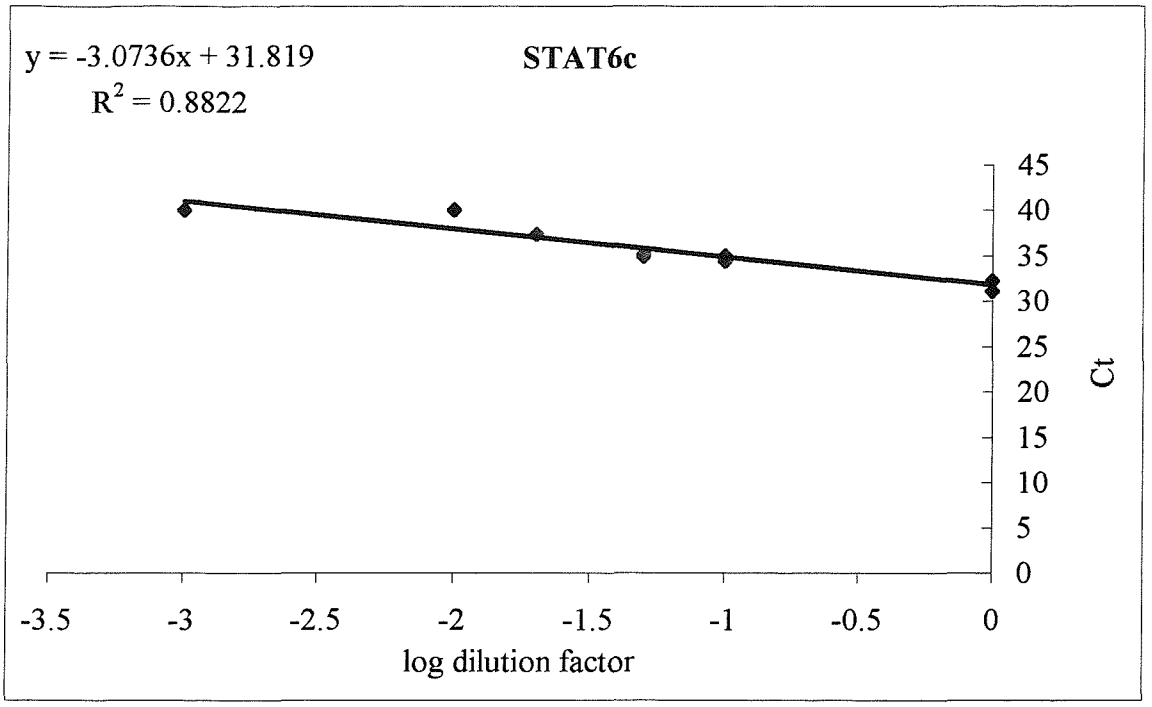


Figure 3.7 STAT6c (Dominant Negative) Expression in H292 Cells.
TaqMan™ primers were designed over the sequence formed by the deletion of 84 base pairs within the SH2 domain of the STAT6 molecule. The dominant negative STAT6 was detectable at a range of H292 cDNA concentrations (expressed as the log of the dilution factor).

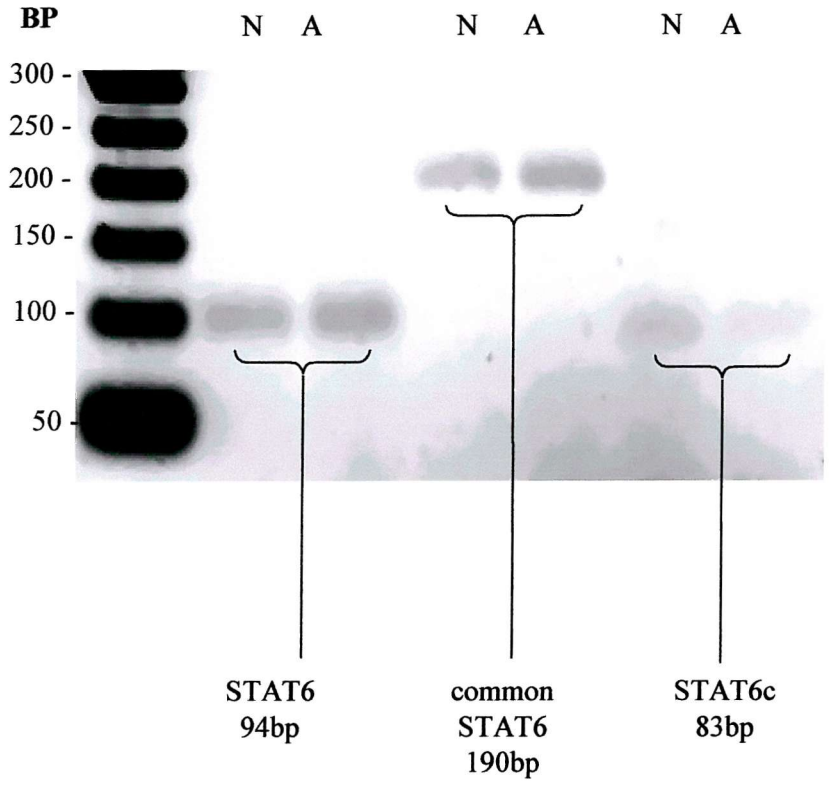
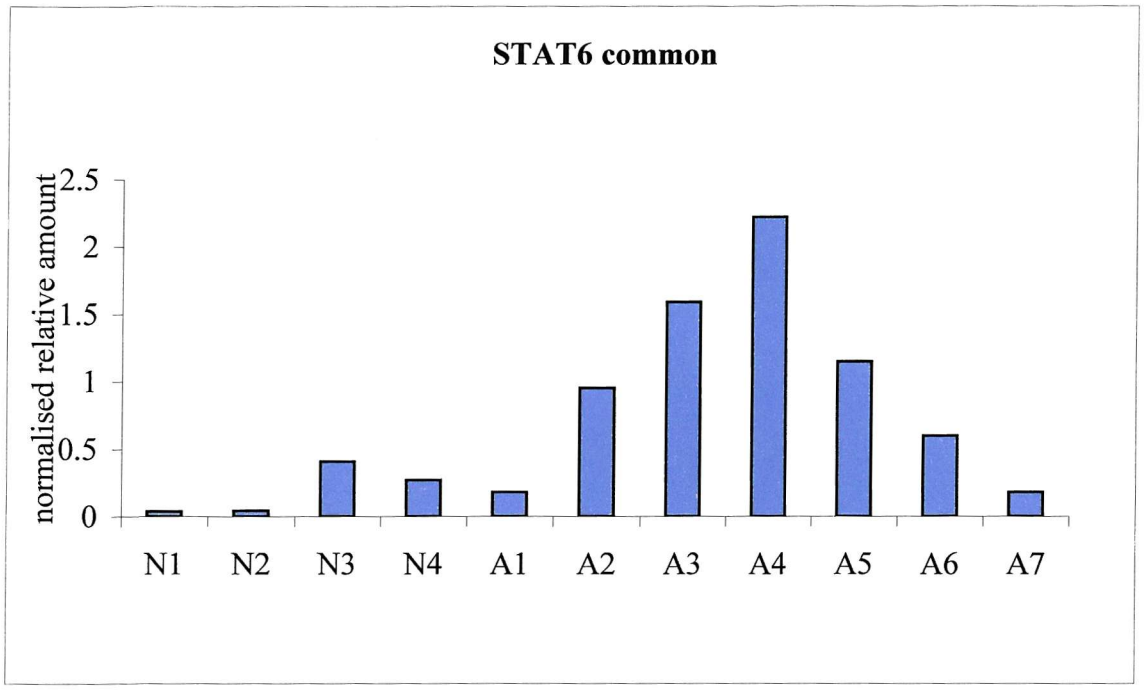


Figure 3.8 Detection of STAT6 Isoforms in Normal and Asthmatic Bronchial Epithelial Cells.
STAT6 transcripts containing or lacking the SH2 domain required for STAT6 dimerisation weredetected by a 35 cycle PCR followed by separation on a 3% agarose gel and visualisation with vistra green.
All three STAT6 isotypes were detected at the correct weight in both normal (N) and asthmatic (A) primary bronchial epithelial cells.

Real time PCR was then carried out on cDNA prepared from primary bronchial epithelial cells obtained from four normal and seven asthmatic subjects, the clinical details are shown in table, 4 chapter 2, to evaluate the levels of expression of STAT6 common, STAT6c and SH2 domain STAT6, as shown in figure 3.9 and 3.10. STAT6 common was expressed in all the samples assayed and there appeared to be increased expression in asthmatic subjects compared to normals, (P=0.023), although more samples would need to be assayed for this to be confirmed. In contrast, the level of expression of STAT6c was much more variable and no clear pattern was apparent between the subject groups. The levels of SH2 domain containing STAT6 transcripts were much more constant between the subjects with no striking differences between the two groups. There was no apparent relationship between the amounts of the three transcripts within each subject, which is probably due to variation in the efficiency of the different primers. Neither was there a disease related difference in the expression of STAT6c. In contrast the finding that full length STAT6 (STAT6 common) was increased in asthma is consistent with the immunohistochemical analysis presented in this chapter, which shows that STAT6 protein expression was increased in asthmatic subjects. To verify the findings with the STAT6 common primers, a second set of primers was designed to detect all known STAT6 isoforms (figure 3.5). As shown in figure 3.11 the pattern of expression of full length STAT6 was found to be very similar when amplified using both the STAT6 common and the STAT6 2 primer pairs. However insufficient RNA was available from the normal and asthmatic primary cells to confirm analysis.

3.3 Discussion

These studies confirm that STAT6 is expressed in the bronchial epithelium and demonstrate for the first time that there is higher expression of STAT6 in severe asthma when compared to mild asthma or normal controls. Thus, STAT6 joins a variety of other transcription factors including STAT1 [198] NFκB [64] and AP-1 [199], whose levels are upregulated in the asthmatic bronchial epithelium. Increased expression of STAT6 within the airway epithelium provides a potential mechanism for enhanced IL-4 and IL-13 regulated responses that are apparent in the asthmatic airways. The finding that STAT6 was strongly expressed in the goblet cells of the epithelium is of significance as IL-4 has been shown to induce mucus release from bronchial epithelial cells [25]. Also STAT6 knockout mice are protected from antigen-induced increases in mucus containing cells [153]. Thus, together with increased expression of IL-4, increased expression of STAT6 in



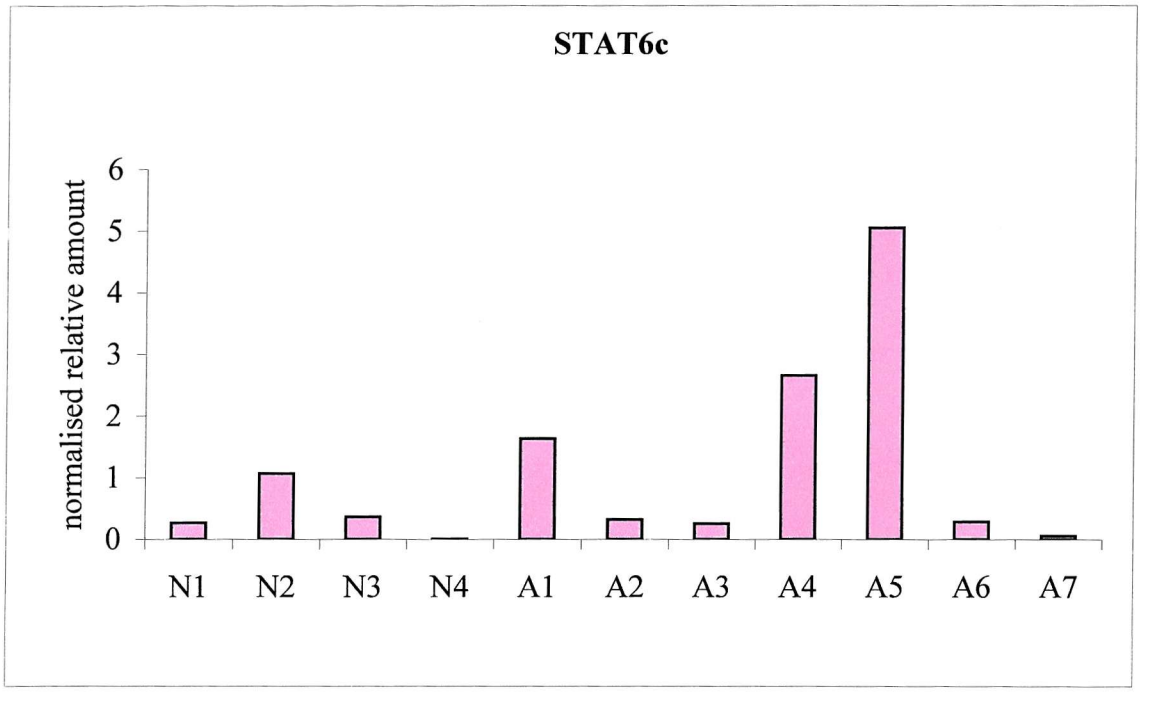


Figure 3.9 Full Length STAT6 and Dominant Negative STAT6 (STAT6c) Expression in Primary bronchial Epithelial Cells.
To quantify the level of expression of STAT6 common and STAT6c in primary cells Taqman™ PCR was carried out. All results were normalised to the amount of 18s RNA within each sample.STAT6 common was detected in all of the subjects but the level of expression was greater in asthmatic (A) than normal (N) subjects. The expression of STAT6c mRNA was detected in 9 out of 11 subjects but showed no clear pattern of expression between the subject groups.

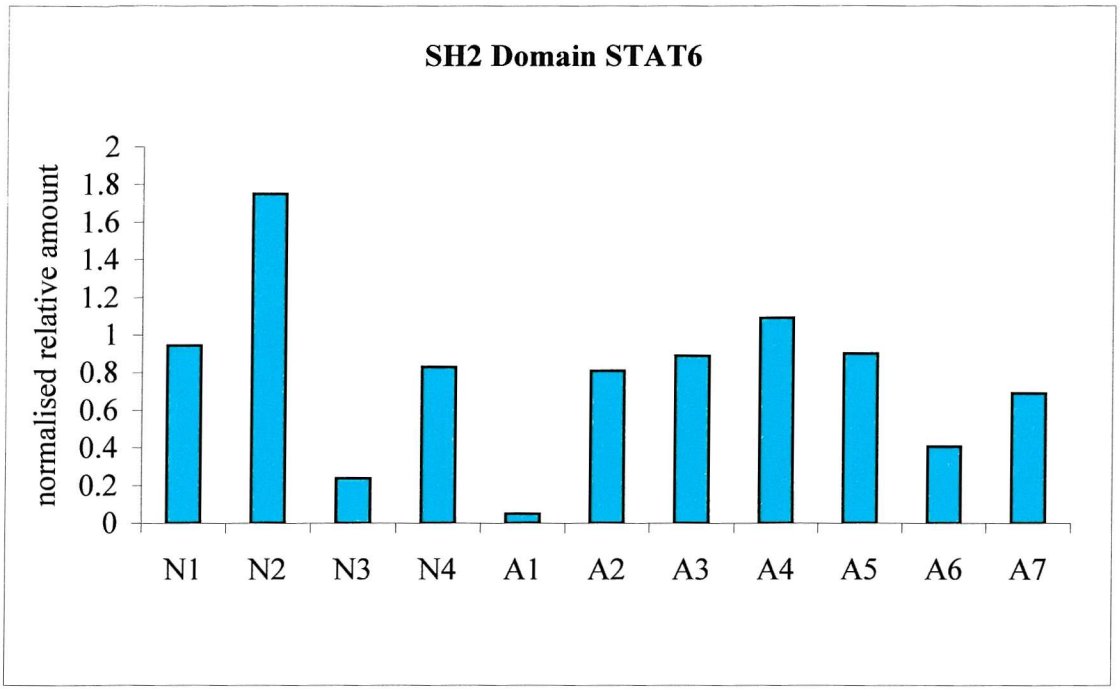


Figure 3.10 STAT6 (SH2 domain containing transcripts) Expression in Primary Bronchial Epithelial Cells.

To quantify the level of expression of STAT6 transcripts which contain an SH2 domain in primary cells Taqman™ PCR was carried out using primers designed over the SH2 domain. Cells from 4 normal (N) and 7 asthmatic (A) subjects were used.

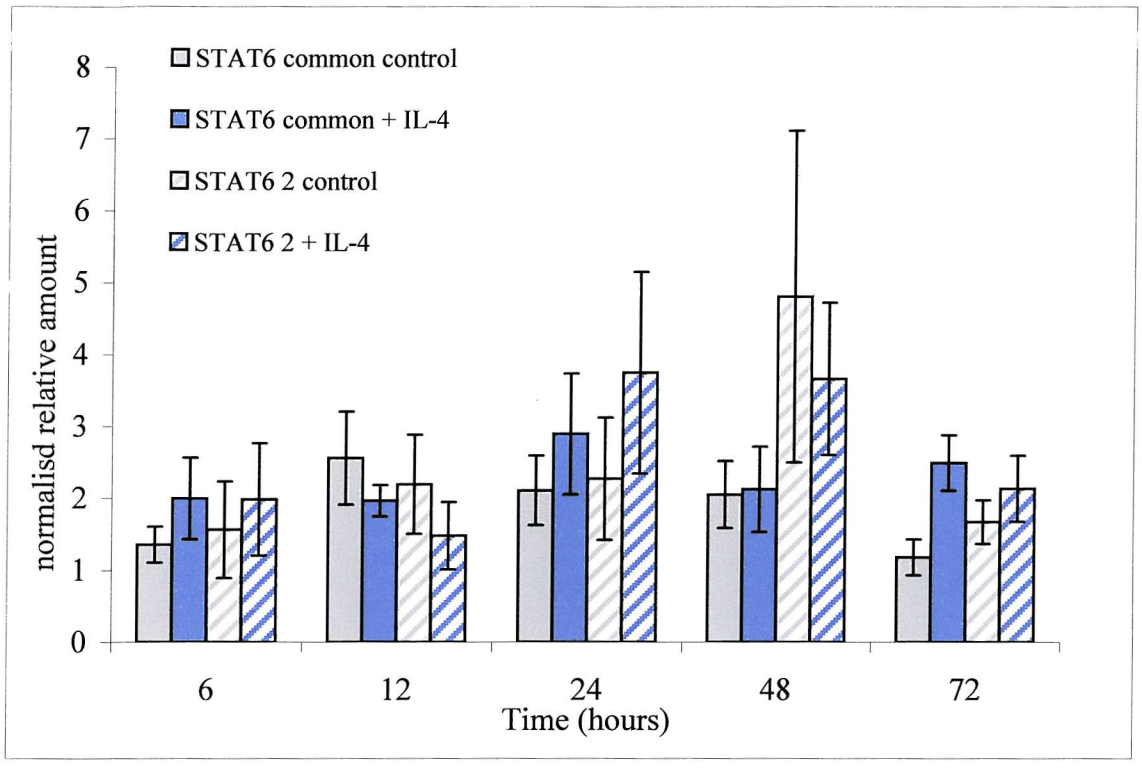


Figure 3.11 Full Length STAT6 Expression in H292 Cells.
Real Time PCR was performed on RNA derived from H292 Cells over 72 hours and the expression of STAT6 assessed using two different sets of primers which both amplify full length STAT6 .

asthmatic epithelium provides a potential mechanism for excessive mucus production, a characteristic of asthma which is poorly controlled with current therapeutic strategies and may be under the control of STAT6.

Although STAT6 expression in the submucosa was not quantified in the present study, a previous study has provided evidence that STAT6 is more strongly expressed in infiltrating cells in the submucosa of atopic and non-atopic asthmatics compared to controls [200]. Furthermore in allergic rhinitic subjects, STAT6 immunoreactive cells are increased after allergen challenge and correlate with levels of infiltrating CD3+ cells [201]. Thus as indicated in animal models, STAT6 appears to play an important role in regulating IL-4 mediated responses in several different cell types within the airways.

Inactive STAT6 is thought to reside in the cytoplasm of the cell and upon activation it translocates into the nucleus to initiate transcription. In some of the sections stained for STAT6, a nuclear ring pattern of staining was observed suggesting that a proportion of the transcription factor was active at the time of biopsy. In support of the possibility that a proportion of the total STAT6 detected was active is that one of the upstream activators of STAT6, JAK1 was found to be present in the columnar cells of the epithelium. However, use of a phospho-specific STAT6 antibody to confirm activation of STAT6 in the airway tissue did not reveal any positive immunoreactivity. Lack of immunoreactivity to the anti-phospho STAT6 antibodies may be due to only small levels of STAT6 being activated at a given point in time and is thus below the level of detection. It may also simply be due to the chosen antibody not being suitable for this application. Thus by using immunohistochemistry I was unable to determine differences in the activation status of STAT6 between the subject groups, and it is this which will determine the transcription of IL-4 and IL-13 responsive genes. To further investigate STAT6 activation it would be possible to use SDS page and western blotting of epithelial brushings to try and detect phospho STAT6 *in vivo*. Since some of the sections of bronchial biopsies used for immunohistochemistry were cut tangently through the epithelium, cytospin preparations might provide a much clearer view of the nucleus, making quantification easier and more accurate. This approach has been used previously to investigate activation of STAT1 in human bronchial epithelial cell brushings [198]. Alternatively in a study by Hart *et al*, the activation of NF-κB in asthma, was analysed using nuclear extracts prepared from asthmatic and normal subjects and the amount of the transcription factor in them analysed by gel shift assay [64]. Thus there are a number of methods that could be used in the future to quantify the amount of active STAT6 between the different disease groups.

As stated above one of the upstream activators of STAT6, JAK1, was detected in both normal and asthmatic bronchial biopsies. As with STAT6, the main site of expression was the bronchial epithelium with a ring pattern of staining around the cell membrane been apparent in some sections. The intensity of the staining for JAK 1 was much lower than that observed for STAT6 and due to this low intensity it was not possible to quantify the levels of JAK1. Whether the low levels of JAK1 compared to STAT6 was due to differences in the reactivity of the two antibodies or a true difference in the levels of expression cannot be confirmed. However it is possible that due to the catalytic function of JAK1, it may be expressed at much lower levels than STAT6. The signalling pathway which leads to STAT6 activation is highly regulated and very little is understood regarding the role the JAKs or the regulation of STAT6 activation in relation to airway disease.

After establishing that STAT6 was over expressed in severe asthmatic subjects one of the aims of this thesis was to investigate how this transcription factor might be involved in regulating IL-4 and IL-13 mediated responses relevant to asthma. Thus preliminary experiments were carried out to select a suitable model cell line one which to base these experiments. Previous studies by Dabbagh *et al* [25] showed IL-4 induces mucin gene expression in the NCI-H292 cell line. Therefore this cell line was chosen for its potential to serve as model for investigating the effects of IL-4 and IL-13. Obviously, the presence of IL-4 and IL-13 receptors on the chosen cell line was an important factor for future experiments and RT-PCR revealed that IL-4Rα, IL-13Rα1 and IL-13Rα2 mRNA was present in H292 cells. Whilst the presence of mRNA does not always relate to protein expression, the lack of commercially available antibodies for the IL-13 receptor chains at the time made RT-PCR the most appropriate method. Identification of IL-13 receptors was necessary in view of the finding that over expression of this cytokine in mice [116], as in IL-4 transgenic [114], resulted in many of the characteristics of asthma and blockade of IL-13 with a neutralising antibody abolished them [120]. Thus it was important to assess the effects of IL-13, as well as IL-4, on epithelial cells. The necessary antibodies have since become available and expression of the IL-13 receptors on primary bronchial epithelial cells has been confirmed [181] [180]. Had there been more time available, FACS analysis would have been performed to quantify the proportions of the specific receptor chains in the H292 cells. STAT6 was highly expressed in this cell line, which also was obviously an important issue. Taken together these data suggested that the H292 cell line was suitable for use as model on which to carryout future experiments to investigate how IL-4

and IL-13 interact with the epithelium to contribute to sub epithelial fibrosis and the role of STAT6 in any effect observed.

As described in chapter one, the IL-4/IL-13 signalling pathway is highly regulated with little being understood about the separate and combined effects at each point of regulation. A dominant negative isoform of STAT6 has previously been described and unfortunately it cannot be specifically detected with commercially available antibodies. Therefore a method was developed to measure its expression using real-time PCR. Primers were designed over the splice site formed by deletion of 86 base pairs in the dominant negative molecule. Alternatively this variant also has a 68 base pair insertion, which may also have served as a sequence for specific detection. Analysis of the expression of the dominant negative was carried out for two main reasons; to analyse its expression in the H292 cells, as this could have been of relevance to the ability of the cells to respond to IL-4 and also to investigate whether its expression could be related to disease status, which also would have implications in responses relevant to asthma.

Dominant negative STAT6 was detected in H292 cells and in primary bronchial epithelial cells, thus introducing another point at which IL-4/IL-13 signalling might be regulated. The antibody used to detect STAT6 in the H292 cells is directed against a portion of the molecule common to both full length STAT6 and STAT6c. STAT6c has been reported to have a molecular weight of 102kDa, slightly smaller than full length STAT6 (105kDa) but only one band was detectable on the western blot. It is possible that two bands were not apparent because STAT6c is expressed at very low levels, as observed by Patel *et al* [149]. or that the difference in molecular weight is not sufficient, as such a strong band is observed with the antibody. It is possible that the expression of STAT6c protein in bronchial epithelial cells could be determined by using 2-D gel electrophoresis. Whilst the different levels of the different STAT6 transcripts amplified could not be directly compared, there was a order of magnitude of difference between the Ct values obtained with the STAT6c primers and the other STAT6 primers. It is unlikely that this difference could be due solely to poor primer efficiency as all primers were designed using the appropriate software. Based on this it is likely that STAT6c was expressed a much lower levels than the other STAT6 transcripts.

The results in this chapter show that there was a substantial degree of variation in the expression of STAT6c mRNA between the subjects. Further work would need to be carried out to determine whether this variation contributes to the variability observed

between individual responses to IL-4 by examining the expression of STAT6 mRNA in relation to mediator release and STAT6 activation in a range of subjects.

There was no pattern for the expression of the different isoforms within any given subject. As a result of the differing primer efficiency I was unable to directly relate the levels of the transcripts containing the sequence for the SH2 domain and those without it, to the total levels of STAT6 in the samples. The data obtained with the STAT6 common primers were consistent with the finding that STAT6 protein is expressed at greater levels in asthma. When a second set of primers which also amplify full length STAT6 (STAT6 2) were used on a number of H292 cell samples the pattern of expression detected was mirrored with that obtained with the STAT6 common primers. This data suggests that the values obtained with the STAT6 common primers in the primary bronchial epithelial cells are an accurate representation of STAT6 mRNA expression in H292 cells. Unfortunately there was insufficient RNA from primary cells for these primers to be tested on a range of subjects.

In summary, the results contained within this chapter highlight the potential role for STAT6 in asthma and describe for the first time one of the many potential regulators of STAT6 function, STAT6c. With regard to the latter a much more detailed study would need to be carried out to assess the relevant contribution of this isoform to regulation of IL-4/IL-13 signalling and indeed asthma.

CHAPTER 4

4 IL-4 AND IL-13 INDUCED CYTOKINE RELEASE FROM BRONCHIAL EPITHELIAL CELLS

4.1 Introduction

The bronchial epithelium has long been regarded as merely a passive barrier serving to protect the airways from inhaled components of the environment. However, as described in chapter one it is now considered to make an important contribution to asthma pathology. It has the capacity to release pro-inflammatory mediators upon damage and stimulation by other mediators and cytokines including IL-4 and IL-13, which could help propagate the ongoing airways inflammation in the airways. More recently it has been shown to release several pro-fibrogenic growth factors in response to damage, and these have been shown to enhance proliferation of underlying mesenchymal cells which may contribute to structural changes observed in asthmatic airways [65 61 169].

One of the most potent profibrogenic growth factors released from epithelial cells is TGFβ. It has the capacity to induce proliferation and differentiation of fibroblasts into myofibroblasts, with the subsequent secretion of extracellular matrix proteins. It also stimulates the release of growth factors such as ET-1 and VEGF from fibroblasts, which can contribute to increased proliferation of smooth muscle and the microvasculature [93]. Expression of $TGF\beta_2$ is increased in the epithelium of asthmatic subjects [92] and studies have shown that bronchial epithelial cells have the capacity to release TGFβ under basal conditions and that this is increased in response to damage [61, 65]. The role of TGFβ in the epithelium following damage is probably to aid the initial stages of epithelial repair, which involves cell migration over the wound site so that a temporary barrier is formed. During this phase of repair, TGFβ also inhibits epithelial cell proliferation which must occur at a later stage, after restoration of barrier function [202].

Recent studies have indicated that IL-4 induces TGFβ release from human eosinophils [192] and animal studies have indicated a role for IL-13 as a mediator of subepithelial fibrosis [116]. Thus, the aim of this chapter was to characterise IL-4 and IL-13 stimulated TGFβ release from a model human bronchial epithelial cell line and to elucidate potential mechanisms by which these responses were occurring.

4.2 Results

4.2.1 *TGFβ$_2$ Release From Human Bronchial Epithelial Cells*

Initial experiments were undertaken to determine whether IL-4 and IL-13 affected cell growth as this could have important implications in any increase in TGFβ release observed. However, neither IL-4 nor IL-13 had any significant effects on bronchial epithelial cell growth as shown in figure 4.1. After 72 hours of treatment with 1.5 nM IL-4, there was slight increase in cell density compared to control, but this failed to achieve statistical significance (n=10, P=0.07).

TGFβ is produced as a latent inactive precursor, which is proteolytically cleaved to produce the biologically active growth factor. However in initial experiments the levels of active TGFβ$_2$ were below the limit of detection. Therefore, all results are presented as total TGFβ$_2$ release (i.e. active and inactive) and to show that effect of IL-4 and IL-13 on TGFβ release was not due to differences in cell number, the results were corrected to TGFβ$_2$ pg/4x10^5 cells. In initial experiments, where cells were plated down at 5x10^4 ml, a small increase in total TGFβ$_2$ release was observed at 24, 48 and 72 hours after exposure. Further investigation revealed that when cells were seeded at a lower density of 2.5x10^4 ml, a greater increase in TGFβ$_2$ release was observed than at the higher density as shown in figure 4.2. Thus, experiments proceeded using a seeding density of 2.5x10^4 cells /ml.

Initially, to take into account multiple testing, the effect of increasing doses of IL-4 or IL-13 on TGFβ$_2$ release was analysed using an ANOVA test. However due to variation at the lower concentrations of IL-4 and the strict criteria for this test a P value greater than 0.05 was obtained. Thus the results were subsequently analysed using a paired students t-test. At 48 hours, IL-4 stimulated TGFβ$_2$ production in a dose dependent manner with 0.5 nM and 1.5 nM significantly increasing total TGFβ$_2$ release from 69± 10 pg/4x10^5 cells to 88±11 (n=7, P=0.009,) and 118±27 (n=8, P=0.034) respectively (figure 4.3). The release after 72 hours was greater than that at 48 hours with untreated cells releasing 103±23 pg/4x10^5 cells at baseline. Again, TGFβ$_2$ release increased in a dose dependent manner and this was significant from cells treated with 0.16 nM (151±24 pg/4x10^5 cells (n=7, P=0.046)), 0.5 nM (138±27 pg/4x10^5 cells (n=7, P=0.05) and 1.5 nM (173±29 pg/4x10^5 cells (n=8, P=0.03)) IL-4, (figure 4.3).

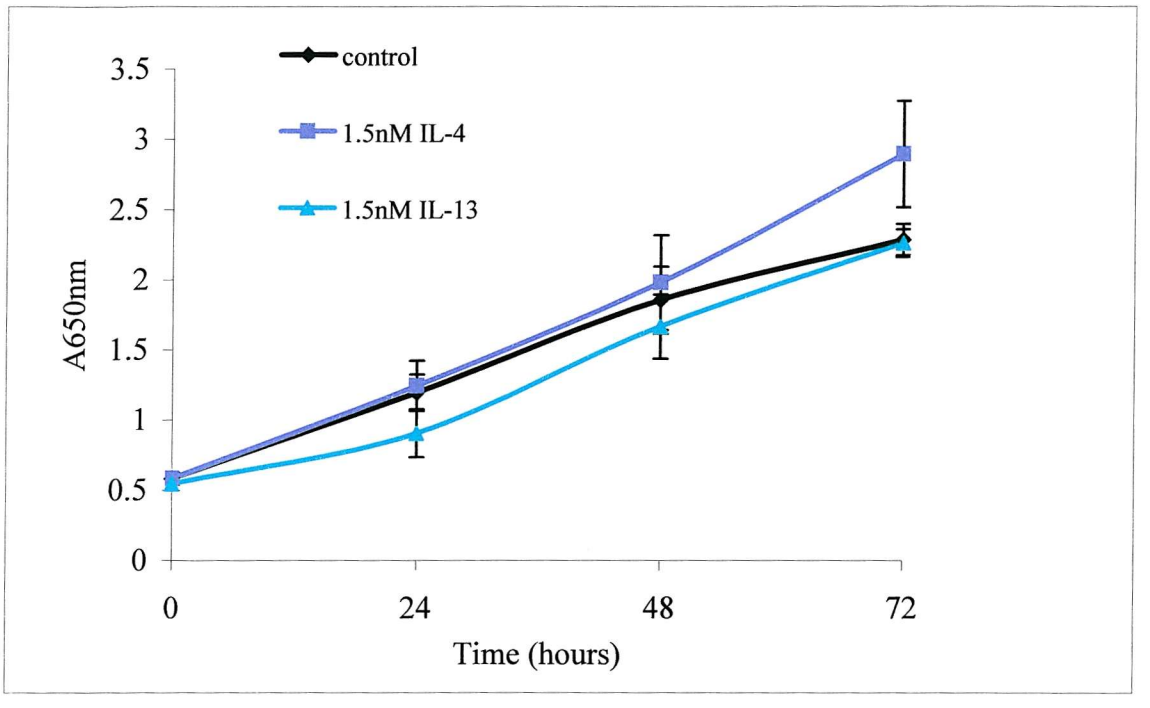


Figure 4.1 The Effect of IL-4 and IL-13 on Cell Density.
H292 cells were cultured in the absence or presence of IL-4 or IL-13. At the times indicated they were fixed and cell number assessed using a methylene blue assay as described in chapter two. At all time points neither IL-4 (n=10) nor IL-13 (n=8) caused a significant change in cell density compared to control (n=10) .

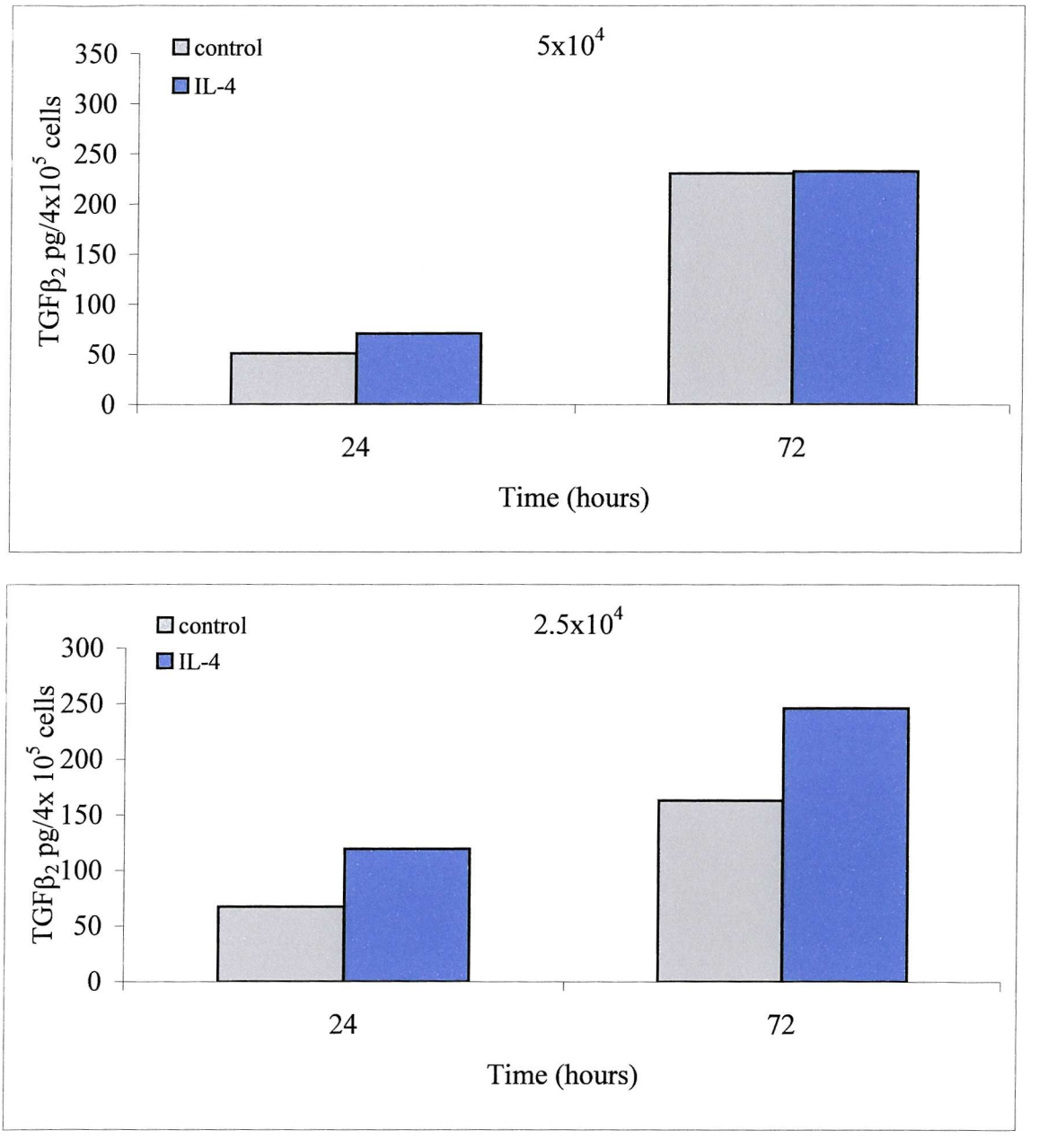
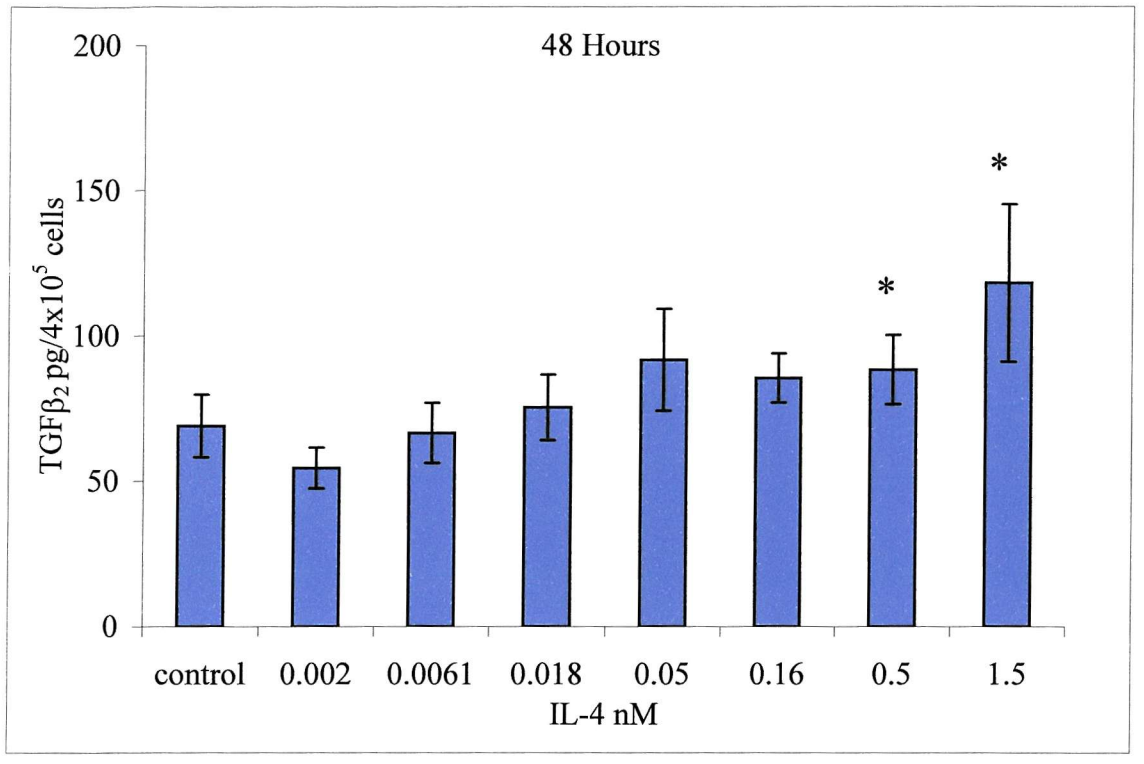


Figure 4.2 The Effect Of IL-4 On TGFβ_2 Release From H292 Cells Plated at High And Low Density.

Cells were plated at either $5x10^4$/ml or $2.5x10^4$/ml and the effect of 1.5nM IL-4 on TGFβ_2 production at 24 and 72 hours analysed (n=2) as described in chapter two. A greater increse in TGFβ_2 release was observed from cells seeded at the lower density.



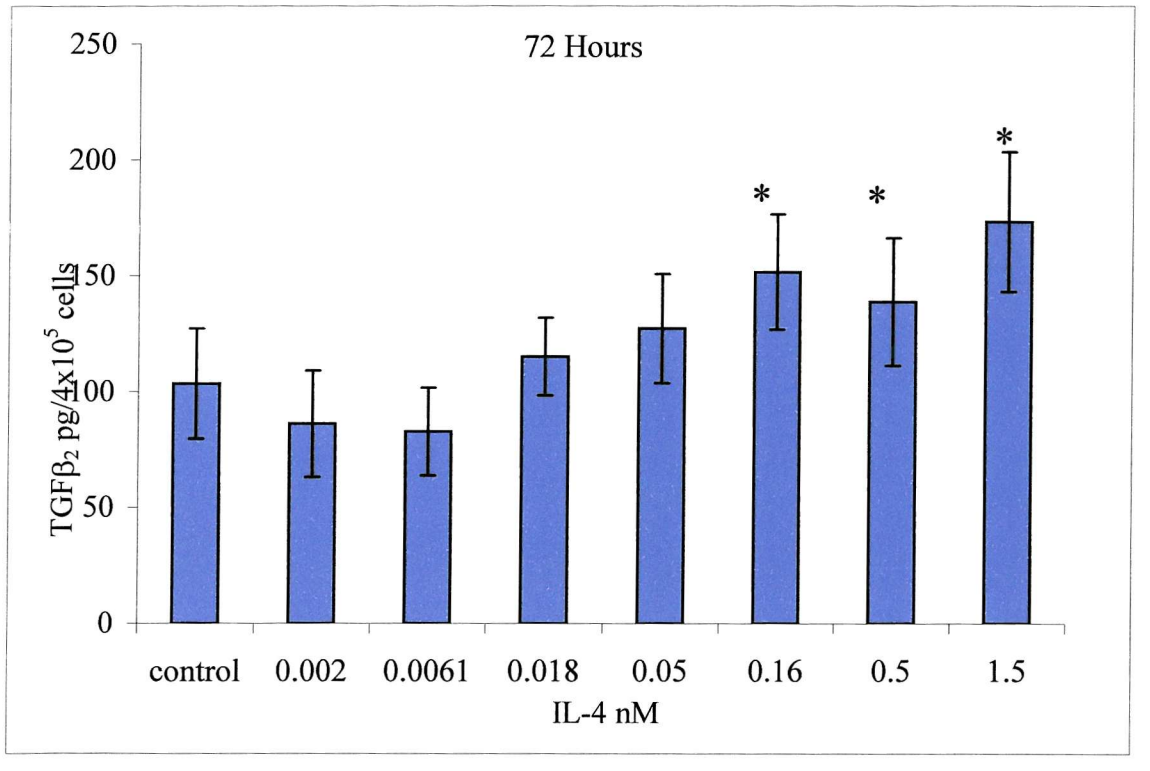


Figure 4.3. IL-4 Induced TGFβ_2 Release From H292 Cells .
H292 cells were treated with IL-4 for 48 or 72 hours, the conditioned media collected and assayed in duplicate for total TGFβ_2. * $P \leq 0.05$, versus control as determined using a students paired *t* -test.

It was apparent from the raw data of the experiments described above that there was a degree of variation in the amount of TGFβ$_2$ released from the H292 cells between the individual experiments. Based on the methylene blue data and the earlier finding that TGFβ$_2$ release is dependent on cell density, I postulated that just slight changes in cell density might affect release. Figure 4.4 shows the relationship between cell density, as determined by the methylene blue assay, and fold stimulation of TGFβ$_2$ release at 48 hours. From this plot, there was a trend for the fold stimulation to decrease with increasing cell density but this was not significant.

Unlike IL-4, neither 1.5, 0.5, 0.15 nor 0.015 nM IL-13 significantly increased TGFβ$_2$ release at 48 or 72 hours in four experiments with either of the statistical tests used, as shown in figure 4.5. In fact at the lower concentrations of IL-13 caused a decrease in TGFβ$_2$ release, although this did not achieve statistical significance. This dip in TGFβ$_2$ release at low concentrations of IL-13 was also apparent in cells treated with low concentrations of IL-4 although to a much lesser extent (figure 4.3). Induction of STAT6 phosphorylation in primary bronchial cells (Chapter 5, figure 5.3) by IL-13, confirmed that the lack TGFβ$_2$ release described above was not due to the molecule being inactive.

4.2.2 *TGFβ$_2$ mRNA Expression in Bronchial Epithelial Cells*

To establish whether the IL-4 induced increase in TGFβ$_2$ release was accompanied by an increase in gene transcription, and might thus be potentially STAT6 dependent, Taqman™ PCR was performed. For these experiments shorter time points were initially carried out on the assumption that transcription would precede protein synthesis and release. As described in chapter two, in these experiments cells were grown in a 57cm^2 petri dish, but in order to maintain consistency between the two experimental protocols, the number of cells/cm^2 adjusted to be equivalent to the experiments performed on 24 well plates. It became apparent that the amount of TGFβ$_2$ released from the cells cultured in the 57cm^2 petri dishes was lower than that observed for the 24 well plate, although every care was taken to ensure consistency between the two protocols. However the magnitude of response was similar even though the absolute amounts were different.

Initial time course experiments (n=3), where cells were treated with 1.5 nM IL-4 for 6, 12 and 24 hours, did not reveal any increase in TGFβ$_2$ mRNA production or any change in its levels over time (Figure 4.6 A). In contrast, conditioned medium from the same cells treated with 1.5 nM IL-4 showed a significant increase in TGFβ$_2$ concentration from

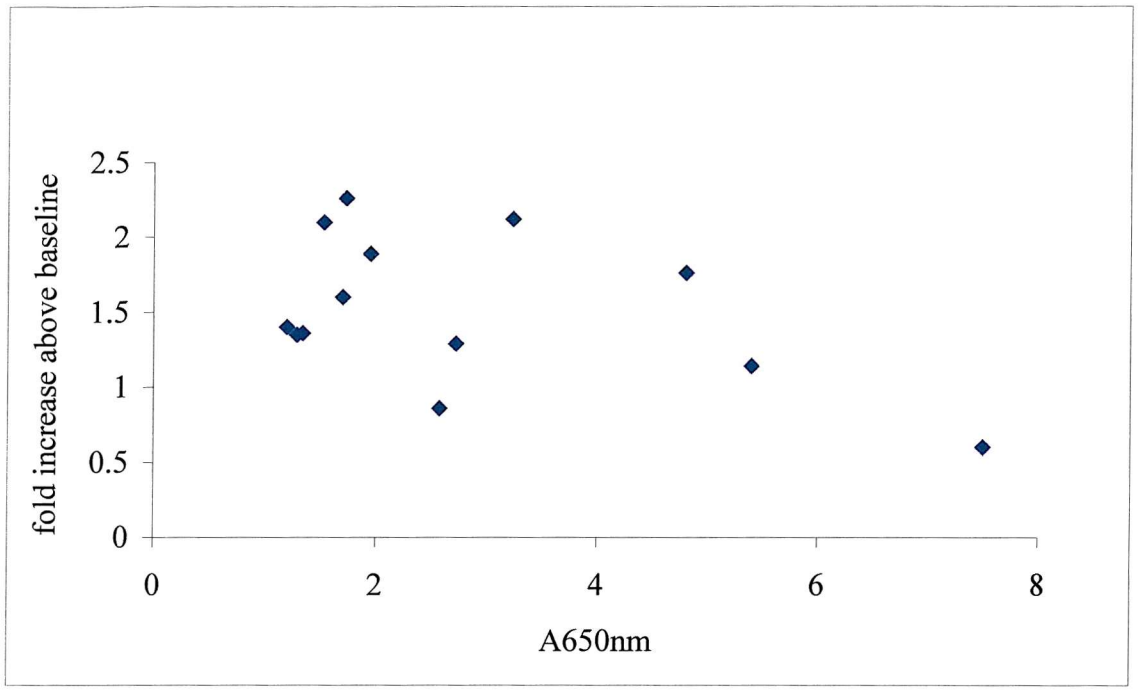
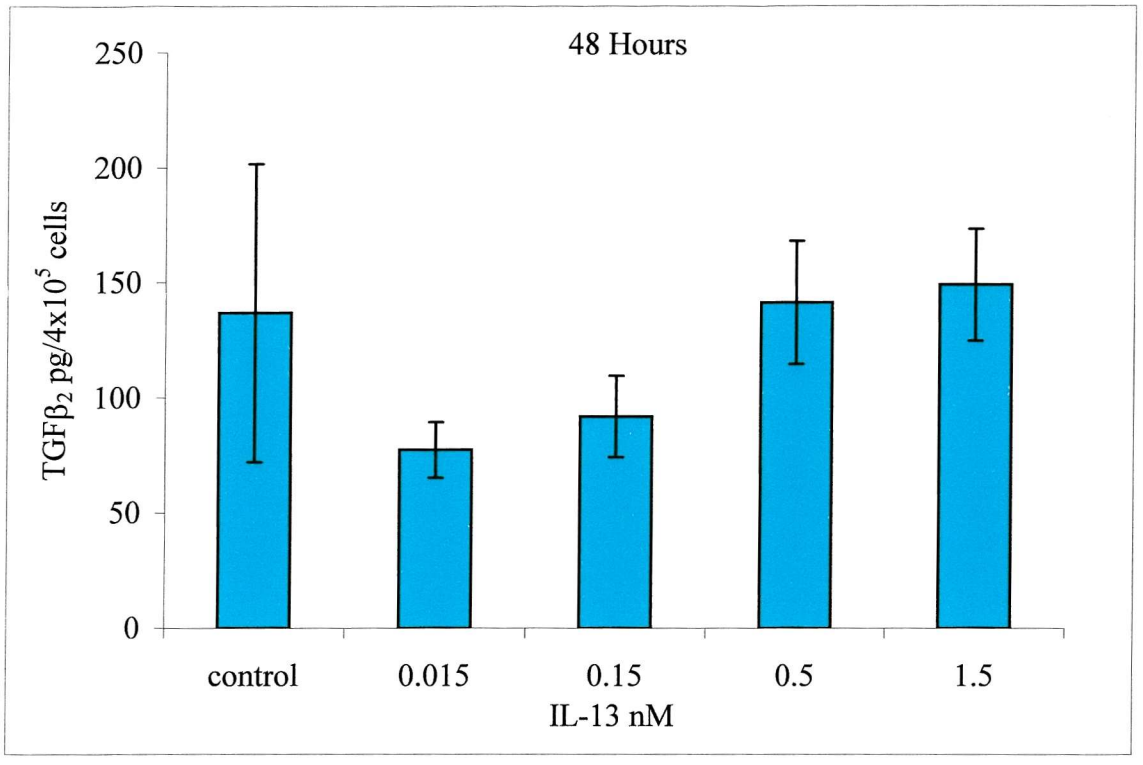


Figure 4.4 Relationship Between Fold Increase Above Baseline and Cell Density.

This figure shows how the fold increase above baseline upon treatment with 1.5nM IL-4 for 48 hours varies with cell density. As determined using uptake of methylene blue and measurement of absorbance at 650nM as a measure of cell density.



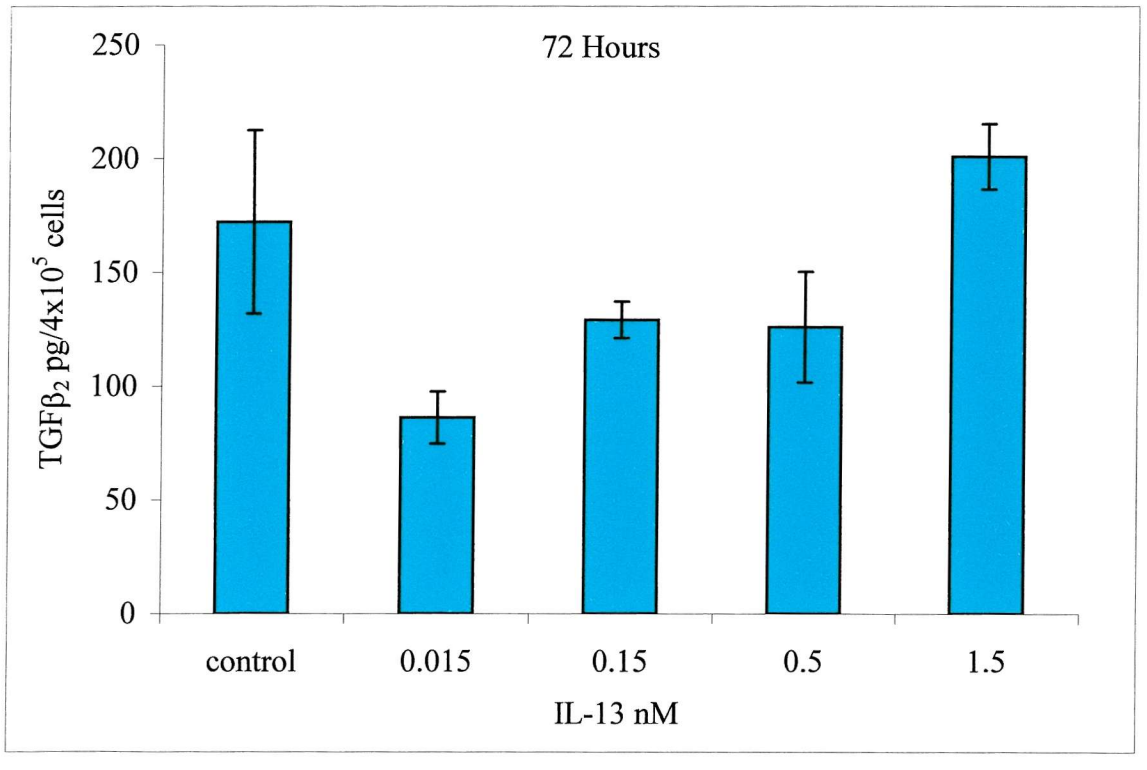
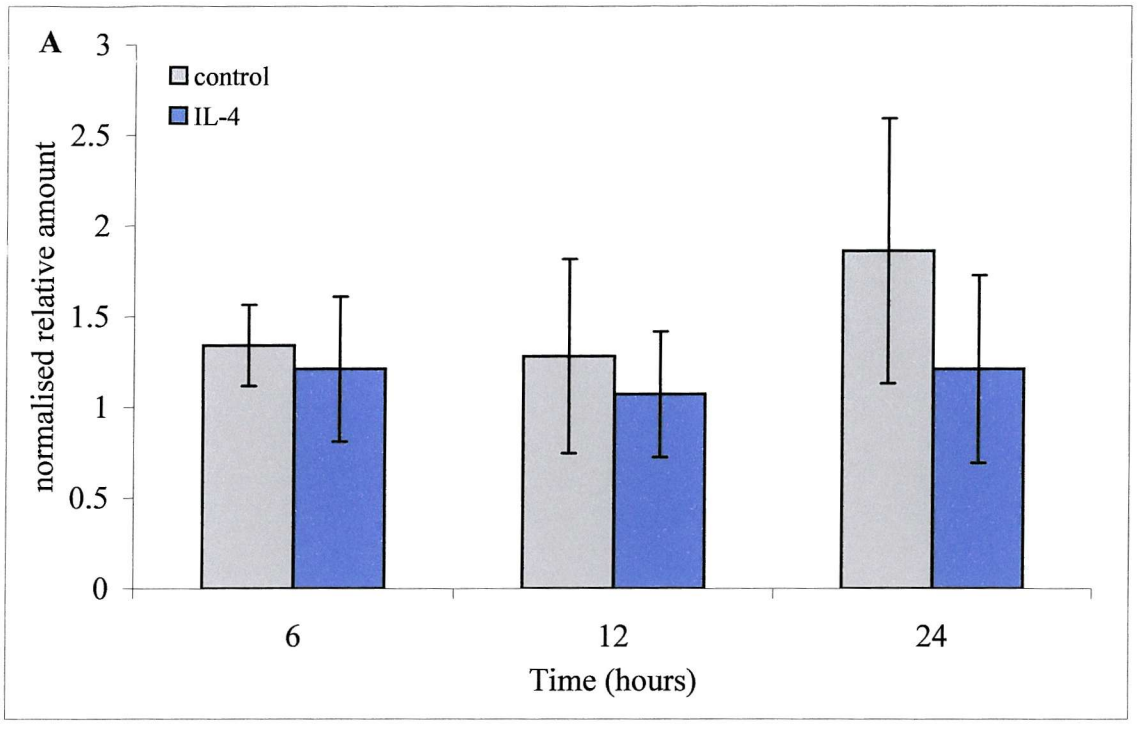


Figure 4.5. IL-13 Induced TGFβ_2 From H292 Cells.
H292 cells were treated with IL-13 for 48 or 72 hours, the conditioned media was collected and assayed for total TGFβ_2. Results (n=4) were compared using a students paired t-test and no significant differences were found.



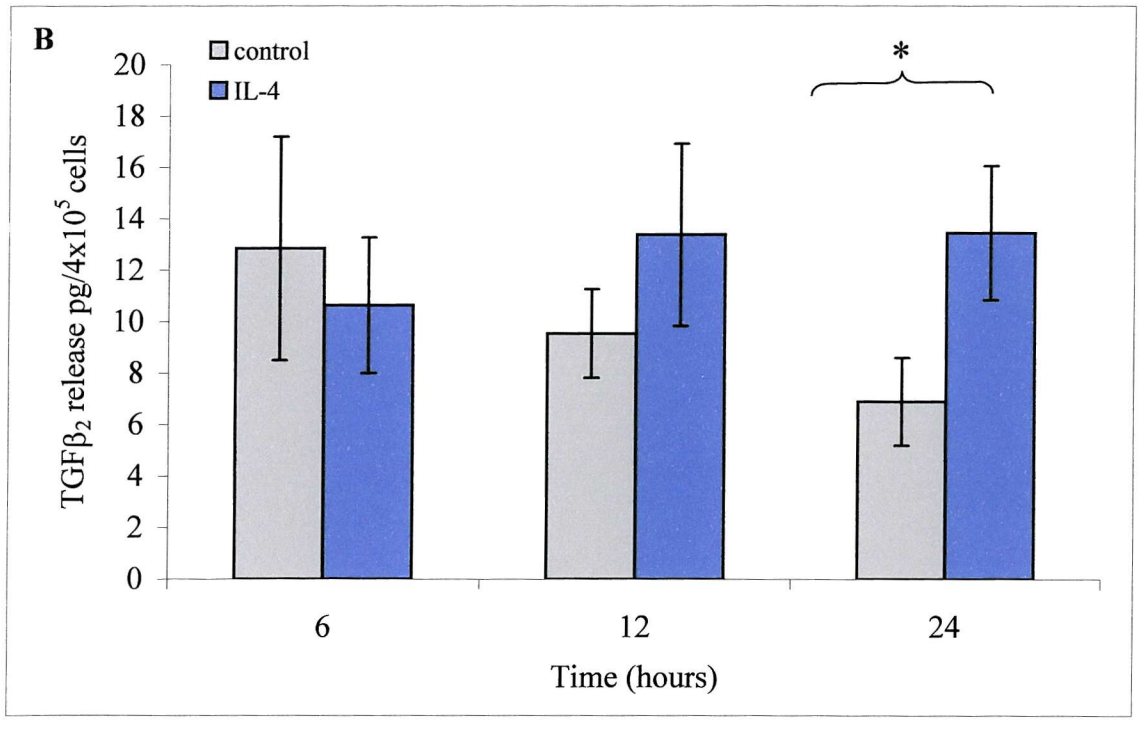


Figure 4.6 TGFβ Gene Expression and Protein Production Over 24 Hours.

A) H292 cells were treated with 1.5nM IL-4 and the effect on TGFβ transcription investigated using real time RT-PCR, $n=3$.

B) Conditioned medium was harvested from the same cells and assayed for TGFβ protein production (corrected to $4x10^5$) cells by ELISA $n=3$.

*=$P \leq 0.05$ as determined by a students paired t -test

6.89±1.69 to 13.45±2.6 pg/ml (n=3, P=0.02) at 24 hours (figure 4.6 B). However in these experiments no obvious time course for release was apparent, rather it appeared that in the control samples the levels of TGFβ_2 release decreased over time and this decrease was prevented in the presence of IL-4. There was however, no significant difference between the control samples at 6 and 24 hours. In view of the lack of gene activation at 24 hours it was hypothesised that IL-4 may indirectly induce TGFβ_2 release with the consequence that the time taken for induction of gene transcription might be delayed. Thus, the time for which the cells were exposed to IL-4 prior to collection of RNA and conditioned medium was extended to 72 hours (measurements taken at 6, 12, 24, 48 and 72 hours, n=3). As in the previous experiments there was no significant change in the level of transcription at 6, 12 or 24 hours post stimulation. In contrast to the three experiments carried out previously, at these early time points there was no decrease in the TGFβ_2 released from the cells upon treatment with 1.5 nM IL-4. However, at 48 hours TGFβ mRNA production increased from 1.98±0.416 in untreated cells to 2.62±0.46 when stimulated with IL-4. Similarly an increase from 1.65±0.4 to 2.53±0.82 was observed after 72 hours of IL-4 treatment as shown in figure 4.7 panel A. In parallel with the transcription data, TGFβ_2 levels in the conditioned medium harvested from these cells was increased from 13.82+3.54 pg/4x10^5 cells to 20.23+6.55 pg/4x10^5 cells at 48 hours and from 11.91±1.22 to 18.35±1.26 at 72 hours (n=2, P= NS) (Figure 4.7 panel B). Whilst this data did not achieve statistical significance, there was a trend for increased gene TGFβ gene transcription at the later time points.

4.2.3 *Regulation of Full Length STAT6 and STAT6c in H292 Cells*

It was postulated that the increase in TGFβ_2 protein release and gene transcription was driven either directly or indirectly by STAT6 and thus the slow induction may have been preceded by an increase in STAT6 mRNA or suppression of the dominant negative STAT6c variant. As shown in figure 4.8 panel A, over the 72 hour time course the level of STAT6 common mRNA remained relatively constant, with IL-4 having little effect. After 72 hours of treatment, there was a slight increase in STAT6 gene transcription but this failed to achieve statistical significance. Similarly IL-4 treatment had no effect on transcription of the STAT6c gene at any of the time points. However, as shown in figure 4.8 panel B, the level of STAT6c did gradually decrease over the 72 hours in culture. STAT6c levels were significantly reduced from 3.21±0.27 in untreated cells at 6 hours to

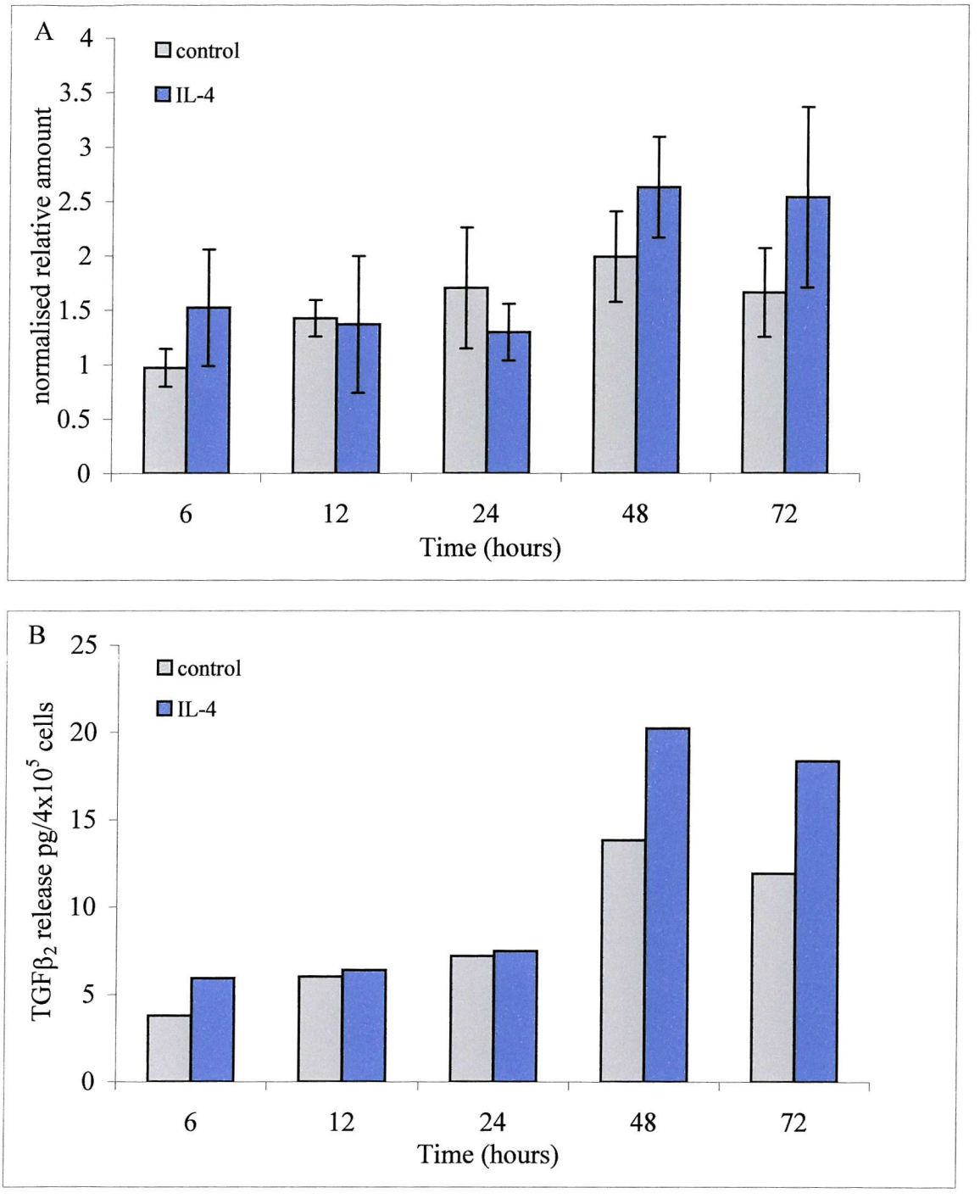
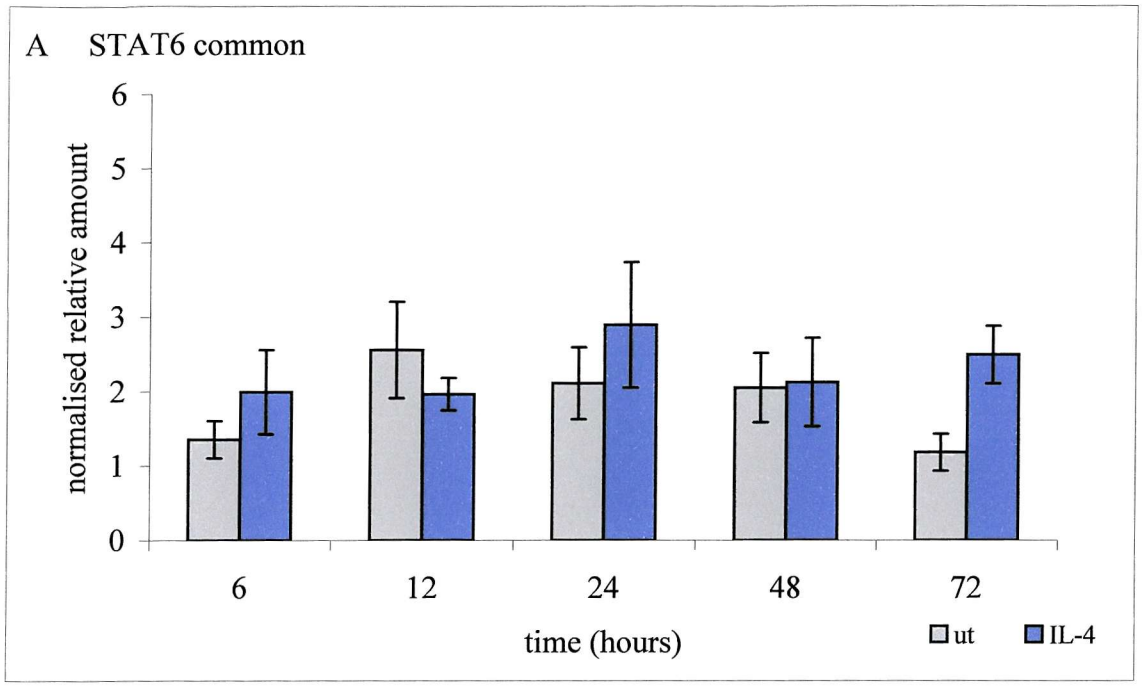


Figure 4.7 TGFβ Gene Expression and Protein Production Over 72 Hours.
A) H292 cells were treated with 1.5nM IL-4 for up to 72 hours and the effect on TGFβ transcription investigated using real time PCR. Results are normalised to 18s ribsomal RNA expression. n=3
B) Condtioned medium was harvested from the same cells and assayed for TGFβ protein production (corrected to $4x10^5$ cells). Results results are expressed as the mean of 2 experiments.



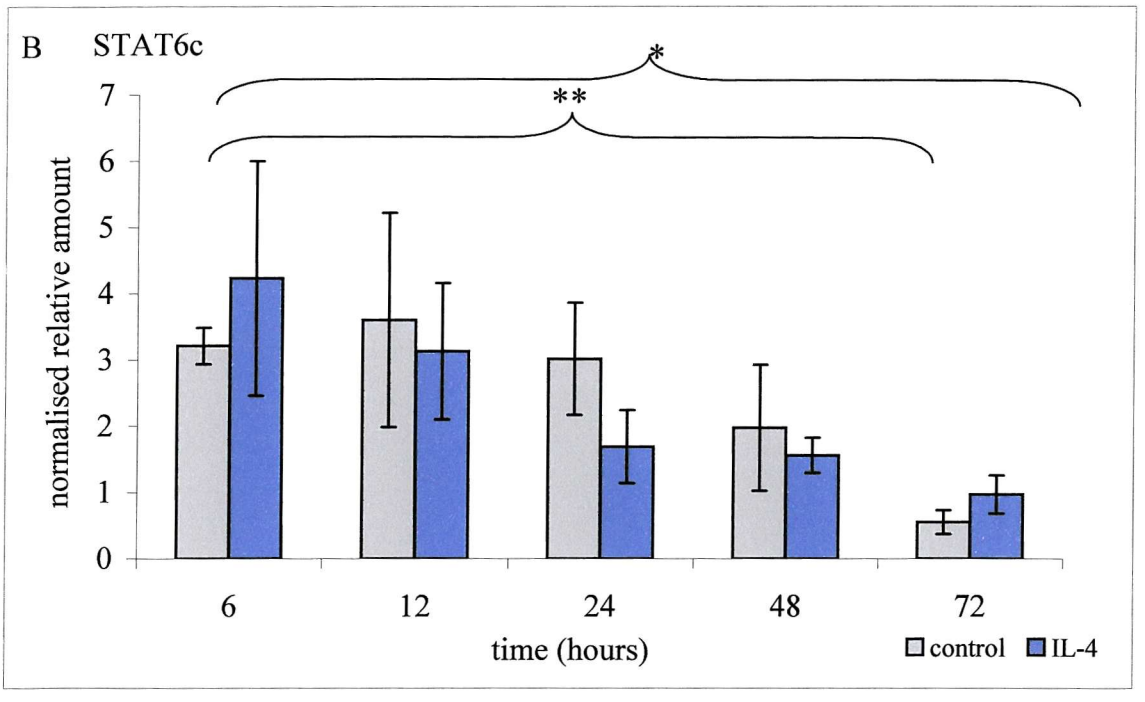


Figure 4.8 The Effect of IL-4 on STAT6 Common and STAT6c Expression in H292 Cells.

Cells were treated with 1.5nM IL-4 for up to 72 hours the RNA harvested and the expression of STAT6 common and STAT6c investigated using real time PCR.

A) STAT6 common normalised relative amount, n=3.

B) STAT6c normalised relative amounts, n=3,**P=<0.01,*P=<0.05 as determined by a paired students t-test.

0.533±0.17 (n=3, P=0.004) and 0.97±0.28 (n=3, P=0.03) in untreated and IL-4 treated cells at 72 hours respectively.

4.2.4 *Effect of MMP Inhibitor on TGFβ mRNA Expression in Bronchial Epithelial Cells*

Whilst the induction of TGFβ mRNA at 48 and 72 hours did not achieve statistical significance it warranted further investigation. Thus, more experiments were performed where cells were treated with 1.5 nM IL-4 for 48 hours. Also, to determine whether $TGF\beta_2$ release and the slow induction of TGFβ gene transcription was due to induction of TGFβ activating enzymes by IL-4, cells were treated in the absence or presence of an hydroxamate MMP inhibitor (N-hydroxy-1,3-di-(4-methooxybenzenesulfonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide) (MMPI, 5 μM). The results from each of these experiments showed a consistent increase in TGFβ transcription with a mean increase from 0.817±0.12 in untreated cells to 1.41±0.19 in IL-4 stimulated cells, (n=5, P=0.013) figure 4.9 panel A. In the presence of the MMPI there was no increase in TGFβ gene transcription when IL-4 was added 1.10±0.14 versus 1.09±1.09, figure 4.9 panel A. However treatment with the MMPI slightly increased TGFβ gene activation compared to untreated cells but this did not reach statistical significance. The accompanying ELISA data also showed a significant increase in $TGF\beta_2$ release from cells treated with IL-4 compared to the controls, (32±5.3 pg/4x10^5cells compared to 24±4.7 pg/4x10^5cells) (n=4, P=0.04). Treatment with MMPI increased the baseline release of $TGF\beta_2$ to 40±8pg/4x10^5 cells (n=4 P=0.04) but there was no further increase when IL-4 was added (39±4.2 pg/4x10^5 cells, n=4 P<0.007) figure 4.9 panel B.

4.3 Discussion

Epithelial damage and repair is a characteristic feature of asthma and many cytokines and growth factors have the capacity to enhance the proliferation of bronchial epithelial cells. However neither IL-4 nor IL-13 significantly altered proliferation of H292 bronchial epithelial cells. The only effect was observed at 72 hours, where IL-4 was found to have a slight stimulatory effect which is consistent with the findings of Rose *et al.*, where a moderate increase in proliferation was observed with IL-4 but not IL-13 [203]. Therefore it

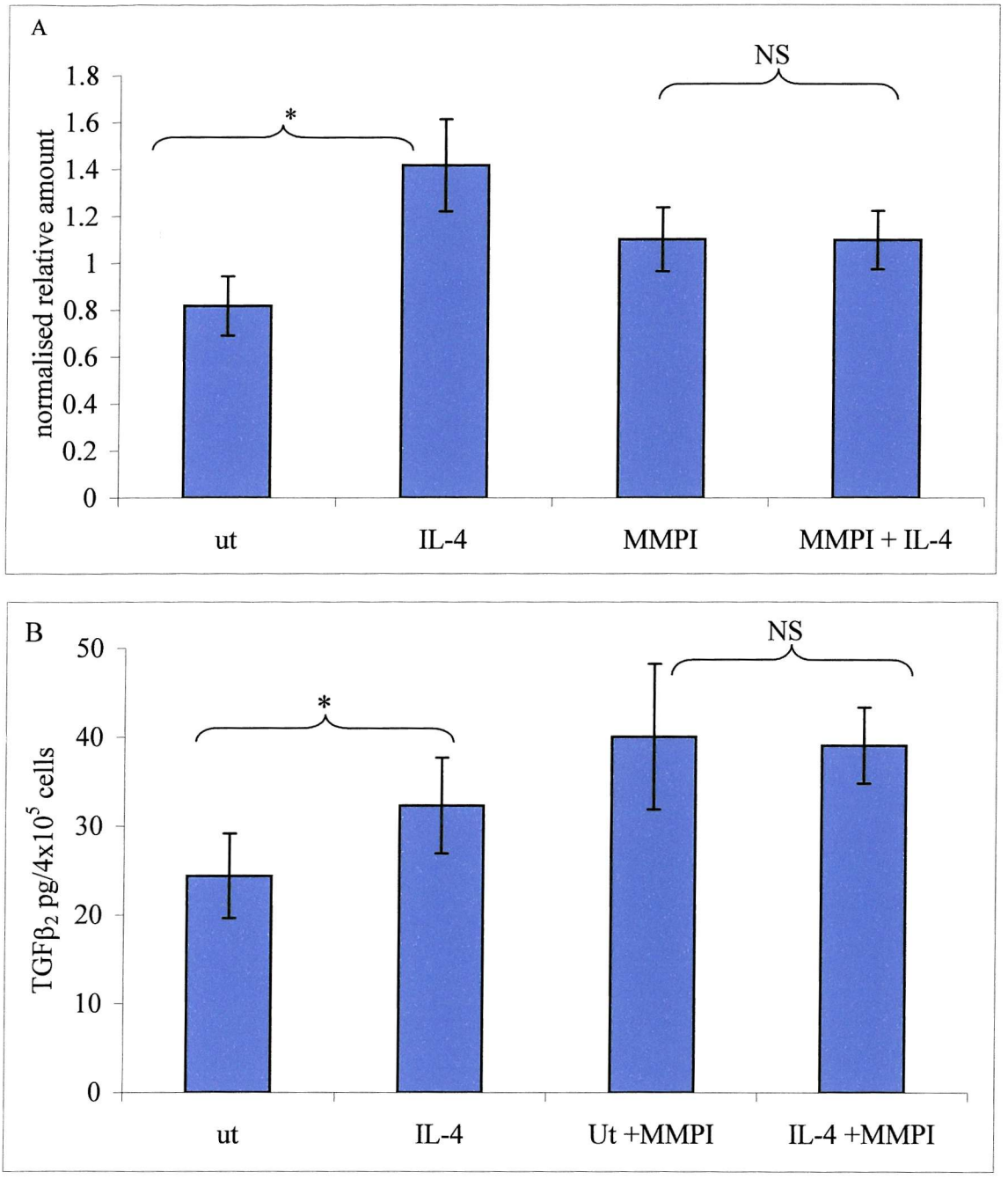


Figure 4.9 IL-4 Induced TGFβ Gene Expression and Release in the Presence and Absence of MMPI.

H292 cells were treated with 1.5nM IL-4 in the presence and absence of 5μM MMPI and MMPI alone for 48 hours and the effect on TGFβ gene expression and protein release assessed by real time PCR and ELISA respectively.

A) TGFb mRNA levels normalised to 18S ribosomal RNA expressed as mean ± SE n=5. *=P<0.05 as determined by a students paired t-test.

B) Conditioned medium was harvested from the same cells as which RNA derived and assayed for $TGF\beta_2$ protein production by ELISA, n=4 *<0.05 compared to control as determined by a paired students t-test . NS = not significant.

seems unlikely that the Th2 cytokines contribute to epithelial proliferation, which is a part of the repair process that results from epithelial damage.

The effects of IL-4 on TGFβ_2 release were shown to be dependent on density, being more pronounced at lower cell densities where the cells had not reached confluence rather than from a confluent monolayer. This is supported by the findings that the variation between the individual experiments may be related to just small differences in cell density. A previous study on bovine bronchial epithelial cells revealed that those cultured at low density produce more TGFβ_2 on a per cell basis than cells plated at higher densities [204]. A similar effect has also been demonstrated in human airway smooth muscle cells [42]. However in the experiments described in this chapter, the overall amount released was not greater at low density, rather the stimulation of TGFβ release by IL-4 was more pronounced. It is possible that this enhanced stimulation is due to changes in the cells responsiveness to IL-4 under certain conditions, as is the case with some T-cell clones [205] where the cells become unresponsive to IL-4 when grown at high density. For example, it is possible that the expression of IL-4 or IL-13 receptors is altered when the cells approach confluence. This could be determined using flow cytometry, to obtain receptor numbers on the cells at different cell densities, and also to assess the effect of IL-4 on receptor expression. These studies at least show the confounding effects of cell density when studying TGFβ.

The effects of IL-4 on a sub confluent monolayer have implications with regard to the asthmatic phenotype as it may serve as a model of epithelial damage. Epithelial damage is a prominent feature of asthmatic airways along with increased numbers of inflammatory cells [4]. TGFβ is released from damaged epithelium [65], and further release due to IL-4 provided by the inflammatory cells may contribute to the amount of TGFβ, which could affect epithelial proliferation and subepithelial fibrosis. In contrast, in a normal non atopic subject with an intact epithelium and much lower levels of IL-4, TGFβ levels would be lower and thus less likely to affect the underlying mesenchymal cells.

The observation that IL-4 can stimulate TGFβ_2 release from bronchial epithelial cells is consistent with the findings of Richter *et al* using primary bronchial epithelial cells [93]. This suggests that components of the Th2 inflammation characteristic of asthma may indirectly impinge on the epithelial-mesenchymal trophic unit to contribute to airway wall

remodelling, through induction of TGFβ. However, the results contained within this chapter show that IL-13 did not significantly induce $TGF\beta_2$ where as in primary bronchial epithelial cells it does. It has also been shown that, in animal models of asthma, IL-13 transgenic mice display sub epithelial fibrosis whilst no such effect was apparent in IL-4 transgenic mice. Again this may highlight the differences between animal models and humans and in this case the difference between cell types. One possible explanation for the differences between these two cell types is differential expression of IL-13 or IL-4 receptors. For example H292 cells may express greater levels of IL-13Rα2 than primary cells and this may act as a decoy receptor to prevent activation of intracellular signalling pathways by IL-13 [130]. It is also possible that H292 cells express lower levels of IL-13Rα1 which is necessary for IL-13 signal transduction. Alternatively it is possible that the cell line secretes greater amounts of a soluble IL-13 binding protein which prevents IL-13 from binding to its receptors [206].

The results contained within this chapter show that IL-4 increases $TGF\beta_2$ release from H292 cells in a dose related manner, however the kinetics are complex. The results shown in figure 4.6 suggest that the effect of IL-4 in these experiments was to prevent the decrease in $TGF\beta_2$ accumulation in untreated cell supernantants. These data suggests that IL-4 regulation of $TGF\beta_2$ release may be complex and that a simple cause and effect response is not occurring in this system. However this effect was not reproduced in later experiments where a time related increase in basal release was apparent (figure 4.7). It is likely that this effect was due to differences in cell passage number and possibly the cell density between the sets of experiments. As described in section 4.2.2, when the experiments were carried out in the $57cm^2$ petri dishes the absolute amount of $TGF\beta_2$ released was much lower than that observed when the cells were cultured in the 24 well plates. It is possible that having more cells present increases the amount of extracellular matrix proteins, (see below) relative to TGFβ such that it binds to them and is less able to be released into the culture medium.

The lag period between treatment and observing any effect of IL-4 suggested that it might indirectly induce TGFβ gene transcription and protein release by acting on an intermediary molecule, which in turn brought about the observed effects. As one of aims of this thesis was to establish a role for STAT6 in epithelial function, the levels of STAT6 and dominant negative STAT6 were measured in the same samples from which the TGFβ data was derived. This was carried out to determine if an increase in STAT6 gene

expression was required for the response to be evoked. It was found that at the time points in which TGFβ message goes up the level of STAT6c has been reduced whilst full length STAT6 remains unchanged. Whilst this effect is not IL-4 dependent, it is possible that this change in the ratio STAT6c to STAT6 over time allows the cells to become more responsive to IL-4 enabling them to evoke a response. It is unclear why the levels of STAT6c mRNA decline in this way, and it is possible that STAT6c transcription has ceased due to an accumulation of protein in the cell.

As described in chapter one, TGFβ regulation occurs at all levels from synthesis to release to activation. These factors make it difficult to dissect out the particular mechanism by which IL-4 induces increased TGFβ release, and this may be further confounded by other downstream effects on TGFβ utilisation. There are many potent activators of TGFβ transcription including active TGFβ itself [207]. The mechanism that was investigated in this study was that IL-4 induced an enzyme that could activate TGFβ by proteolysis, which in turn induced its own transcription and subsequent protein synthesis. It has been shown *in vitro* that MMP-9 can activate TGFβ [208] and animal studies have shown that IL-13 associated increases in TGFβ production were decreased by breeding IL-13 transgenic mice with MMP-9 null mice [209]. In view of this, the effect of an MMPI on IL-4 mediated transcription and release of $TGF\beta_2$ was investigated. The presence of the MMPI blocked any increase in TGFβ mRNA production by IL-4, but the results were complicated by an effect of the MMPI alone. When compared to untreated cells, IL-4 in the presence of the MMPI significantly increased TGFβ transcription, although not to the same extent as IL-4 alone. This suggests that maximal TGFβ gene activation by IL-4 is at least partly dependent on activation of MMPs. Similar results were obtained when protein release under these conditions was assessed, where the MMPI alone significantly increased $TGF\beta_2$ release. However, as with the gene activation data, whilst the MMPI did cause an increase there was no further increase in the presence of IL-4. This suggests that IL-4 induced release is dependent on MMPs but that other mechanisms that control $TGF\beta_2$ release are negatively regulated by the MMPs. For example, the inhibitor used in these experiments was not specific for MMP-9 and inhibits a range of MMPs some of which may negatively regulate TGFβ.

From the data presented, further work would need to be carried out to confirm a role for epithelial MMP-9 in IL-4 induced TGFβ transcription and release. Recent studies have indicated that the subepithelial fibrosis observed in IL-13 transgenic mice is mediated by

TGFβ and this effect is MMP-9 dependent [209]. However, in this study the major source of TGFβ was lung macrophages. Thus the results contained within this chapter provide another potential source of TGFβ, which could contribute to airway wall remodelling. To begin to investigate the role of MMP-9 in TGFβ release from H292 cells one would initially investigate any involvement of IL-4 in induction of MMP-9, as has been shown in IL-13 transgenic mice [210]. This might involve using RNA from the samples from which the above data was derived or by measuring activation of MMP-9 by IL-4 using zymography. One could also use specific inhibitors for this enzyme or possibly use MMP-9 null cells to confirm its involvement. Alternatively treatment of cells with a range of MMPs would enable a broader assessment of their effect on $TGF\beta_2$ release. TGFβ has, in many cell types, been implicated in the inhibition MMPs so it is possible that negative feedback mechanisms also occur to inhibit $TGF\beta_2$ release [97]. As no active $TGF\beta_2$ could be detected in the H292 culture medium cells, the availability of active $TGF\beta_2$ also needs to be considered. Its low levels may be due to concentrations being below the sensitivity of the assay, or maybe all of the active protein binding to its receptors once formed [89]. To further investigate the involvement of TGFβ activation in IL-4 induced $TGF\beta_2$ release and transcription one could carry out the experiments described in this chapter in the presence of a TGFβ receptor antagonist or blocking antibody.

In addition to MMP-9, a number of other enzymes are capable of activating TGFβ including plasmin, angiotensin II, thromboxane, and thrombospondin. Angiotensin II is able to induce $TGF\beta_1$ gene activation in endothelial cells [211] but there is no data linking IL-4 to angiotensin. Nonetheless the possibility that it may regulate TGFβ should not be excluded. Also, in endothelial cells, IL-4 has been shown to stimulate expression of urokinase-type-plasminogen activator, which in turn has the potential to activate plasmin which could activate TGFβ [212]. These examples highlight the complexity of the cytokine networks within cells and tissues. To further investigate such a complex system it might be possible to use affymetrix gene chips, which contain probe sets to identify activation of a very large number of genes in cells. This method has recently been used to investigate some of the genes that are activated by IL-13 in human bronchial epithelial cells, human lung fibroblasts and human smooth muscle cells [213]. This study by Lee *et al* [213] has highlighted the effect IL-13 has on the expression of many genes as well as the diversity of gene expression within the three different cell types, as there was virtually no overlap in the genes that it induced. Of interest was the finding that IL-13 induced the expression

of the extracellular matrix TGFβ binding protein, biglycan. TGFβ can associate with matrix proteins such as decorin and biglycan which in turn can modulate TGFβ bioactivity [85] by storing it in the extracellular matrix. Thus induction of proteins such as biglycan by IL-4 could explain why no active $TGF\beta_2$ was observed. Similarly, down-regulation of a TGFβ binding protein could also alter the expression of TGFβ especially with regard to TGFβ auto-induction. There are many factors which can influence the actions of TGFβ, and whilst an *in vitro* system will exclude the confounding effects of other cells, other factors are present which must be considered. In summary, TGFβ regulation is a highly complex process and the work in this chapter shows that IL-4 is capable of inducing its gene activation and release although the mechanism is not fully understood.

In summary the results contained within this chapter show that IL-4 can significantly increase TGFβ transcription and release from H292 bronchial epithelial cells. However the lag period and the effect observed in the presence of an MMPI suggests that this response is not direct. In support of the hypothesis that IL-4 indirectly induces TGFβ release and transcription is the finding that the $TGF\beta_2$ promoter does not contain an optimal STAT6 consensus sequence. However, as described in the introduction to this chapter, STAT6 is thought to be involved in the majority of IL-4/IL-13 mediated responses. However, there is as yet no information regarding the role of this transcription factor in potentially pro-fibrogenic responses. Therefore further work was carried out to assess the involvement of STAT6 in IL-4 induced $TGF\beta_2$ release.

CHAPTER 5

5 INVOLVEMENT OF STAT6 IN IL-4 INDUCED TGFβ RELEASE FROM BRONCHIAL EPITHELIAL CELLS

5.1 Introduction

As described in chapter four, IL-4 can stimulate cytokine release from bronchial epithelial cells. Engagement of IL-4 receptors activates at least two distinct intracellular pathways: IRS signalling and STAT6 signalling [126]. IRS-1 activates signalling molecules such as Grb-2 and PI-3 kinase which are involved in mitotic responses [166]. Activation of STAT6 causes it to dimerise and translocate to the nucleus where it binds to specific consensus sequences on the promoter of target genes. Studies using STAT6 deficient mice have suggested that despite the multiple signalling pathways activated by IL-4, STAT6 signalling is essential for mediating most responses of lymphocytes to IL-4 [151]. In the $STAT6^{-/-}$ mice, expression of neither CD23 nor MHC class II molecules in B cells was enhanced in response to IL-4. IL-4 induced B cell proliferation was also abolished and the production of Th2 cytokines from T cells was profoundly reduced. More recently several studies have highlighted the necessity of STAT6 for the development of many of the features associated with human asthma for example AHR and mucus production [153]. The features of STAT6 knockout mice are strikingly similar to mice in which the IL-4 gene has been disrupted highlighting the critical role of this transcription factor in IL-4 activity [214 119].

In chapter three, the expression of STAT6 in human airways, and in H292 cells was described and *in vitro* studies carried out by other groups have shown that STAT6 is activated in IL-4 and IL-13 treated bronchial epithelial cells [183 190]. Studies have also confirmed the involvement of STAT6 in specific functional responses of epithelial cells, for example eotaxin transcription and release. In these studies STAT6 was found to bind to its specific consensus sequence of the eotaxin promoter and mutation of this sequence abolished the IL-4 induced eotaxin gene activation and the release of protein [190]. More recent work has shown that IL-4 and IL-13 induced eotaxin release from bronchial epithelial cells is enhanced in cells transfected with wild type STAT6 and abolished in cells transfected with the dominant negative STAT6 [191]. In chapter four, it was demonstrated that IL-4 could induce the release of TGFβ from H292 cells. In view of the

studies described above it hypothesised that STAT6 was involved in this response. Thus the aim of this chapter was to establish whether STAT6 was necessary for IL-4 induced TGFβ gene activation and release, and to characterise the activation of STAT6 in the H292 cell line.

5.2 Results

5.2.1 *STAT6 Activation in Bronchial Epithelial Cells*

To begin to ascertain whether STAT6 is functional in H292 cells and thus potentially involved in IL-4 induced $TGF\beta_2$ release, experiments were carried out to investigate its activity. Initially experiments were carried out to investigate changes in the levels of tyrosine phosphorylation on proteins when the cells were treated with IL-4 using a pan phosphotyrosine antibody. These experiments showed that treatment of H292 cells with 0.75, 1.5 and 3 nM IL-4 for 5 minutes resulted in an increase in the levels of tyrosine phosphorylation on proteins with a molecular weight comparable to that of STAT6 (figure 5.1). However, these changes were very slight and thus not always readily detectable. A similar experiment was carried out on 16HBE cells with the assumption that if these cells readily responded, conditions could be optimised for the H292 cells. Treatment of 16HBE cells with 3 nM IL-4 for 5, 10 and 15 minutes resulted in an increase in the levels of tyrosine phosphorylation on proteins with molecular weights of 100-110kDa and 170kDa (figure 5.1). These are comparable with the molecular weights of STAT6 and IRS-1/2 respectively. As with the H292 cells the changes in the 16HBE cells were slight and not always readily detectable, thus further experiments focused on H292 cells. Immunoprecipitation of H292 cell lysates with anti-phosphotyrosine antibodies did not improve detection of phosphorylated STAT6 (PY-STAT6) (figure 5.1). However, in the same experiment treatment with EGF resulted in increased levels of tyrosine phosphorylation on proteins with a molecular weight comparable to that of the EGF receptor (figure5.1).

As with the pan phosphotyrosine antibody, western blotting using an antibody raised against the phosphorylated form of STAT6 also showed levels of PY-STAT6 to be low in H292 bronchial epithelial cells, whilst high levels of STAT6 were always detected. To optimise epithelial cell protein retention, proteins were transferred onto PVDF membrane.

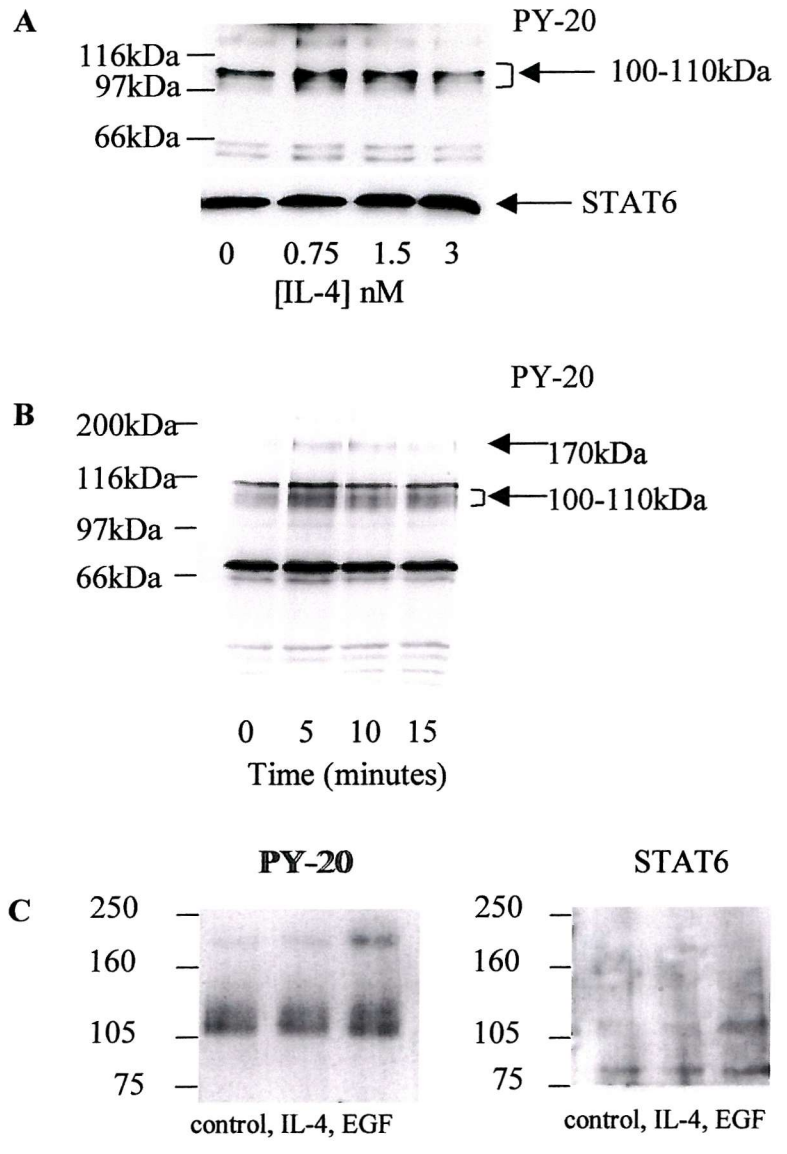


Figure 5.1 Tyrosine Phosphorylation in IL-4 Treated H292 and 16HBE Cell Lysates.

A. H292 cells were incubated with the indicated concentrations of IL-4 for 5 minutes. Probing the Western blot with a PY-20 antibody revealed increased levels of tyrosine phosphorylation on proteins with a molecular weight of 100-110KDa. Duplicate Blots were also probed for STAT6 to confirm equivalent loading.

B. 16HBE cells were incubated with 3nM IL-4 for the times shown. This treatment caused increased levels of tyrosine phosphorylation on proteins with molecular weights comparable to that of STAT6 (105kDa) and IRS1/2 (170kDa).

C. Phospho-tyrosine immunoprecipitation of H292 cells revealed increased levels of tyrosine phosphorylation on a protein of approximately 170kDa in cells treated with 6ng/ml EGF. Probing a duplicate blot for STAT6 did not reveal any phosphorylated STAT6 in samples treated with 1.5nM IL-4 or EGF.

Using this method, slight changes in the levels of PY-STAT6 were detectable in H292 cells following treatment with 0.75 nM IL-4 for 10 and 30 minutes, (figure 5.2). Similar results were obtained from 16HBE cells (data not shown). PVDF membrane was used from then on and increasing the primary antibody concentration did not aid detection. In view of the finding that the time taken for induction of TGFβ transcription was 48 hours, the time for which the H292 cells were exposed to IL-4 prior to collection of lysates was increased accordingly. Lysates were collected at 1, 4, 8, 12, 24 and 48 hours post treatment but no PY-STAT6 was detectable at any of these time points (data not shown).

To confirm that the PY-STAT6 antibody was working, whole cell lysates from primary airway cells were prepared and probed. In three experiments treatment of primary bronchial epithelial cells with 1.5nM IL-4 caused a sustained increase in STAT6 phosphorylation from 15 to 120 minutes. Phosphorylated STAT6 was also detectable in four experiments where cells were treated with 0.4 and 0.75 nM IL-4 and 0.75 and 1.5nM IL-13 for 1 hour (figure 5.3). From these experiments it was also determined that none of the bands visualised were due to non-specific binding of the secondary antibodies. Similarly, in two experiments treatment of primary human lung fibroblasts with 3 nM IL-4 caused a substantial phosphorylation of STAT6 from 5 to 60 minutes (figure 5.4).

5.2.2 *Transfection of H292 Cells with Wild Type STAT6 and Dominant Negative STAT6*

Whilst only low levels of PY-STAT were detectable in the H292 cells further experiments involving transfection of cells with a dominant negative STAT6, which can inhibit the actions of endogenous STAT6 [191] were carried out to assess the involvement of this transcription factor on IL-4 induced $TGF\beta_2$ release. H292 cells were transiently transfected with a tyrosine 641 mutant of STAT6, which acts as a dominant negative and wild type STAT6 (STAT-WT), both a kind gift from Tularik Inc. Cells were also transfected with a basic empty plasmid as a control. Both the dominant negative and wild type STAT6 were expressed in pcDNA3 but the empty plasmid was not supplied and is no longer available commercially. Thus, as described in chapter two a similar plasmid pRc/CMV was used as an empty plasmid control. Following transfection, cells were treated with 1.5 nM IL-4 for 48 hours after which time RNA and conditioned medium were collected for analysis of TGFβ gene expression and $TGF\beta_2$ release respectively. Cell density was also assessed at this time point using the methylene blue assay. In

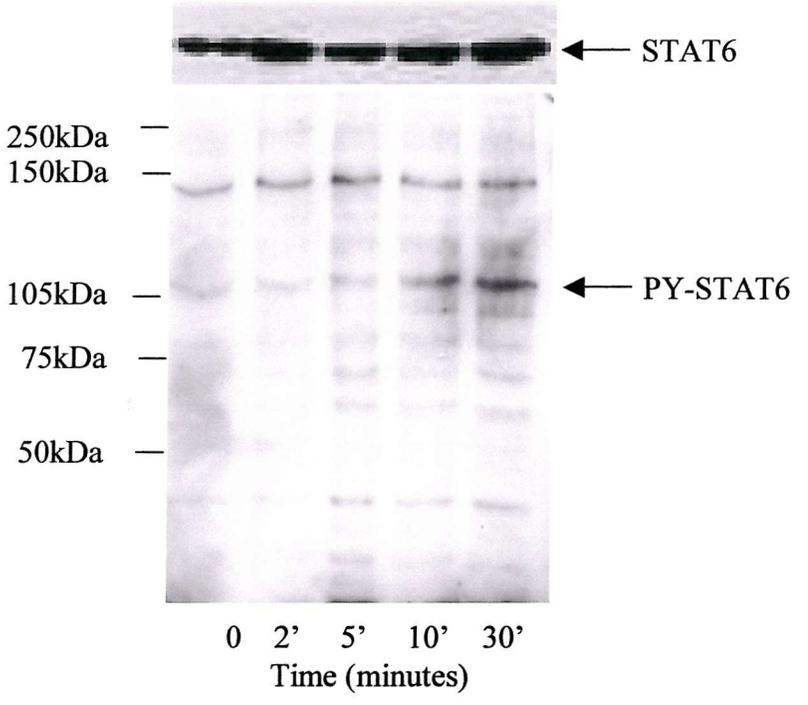


Figure 5.2 STAT6 Phosphorylation in H292 Cell Lysates.
H292 cells were treated with 0.75nM IL-4 for the time periods indicated. Western Blotting onto PVDF membrane and detection using a PYSTAT6 antibody revealed phosphorylation of STAT6 upon treatment with IL-4. An equivalent blot was probed with a pan STAT6 antibody to determine equivalent protein loading.

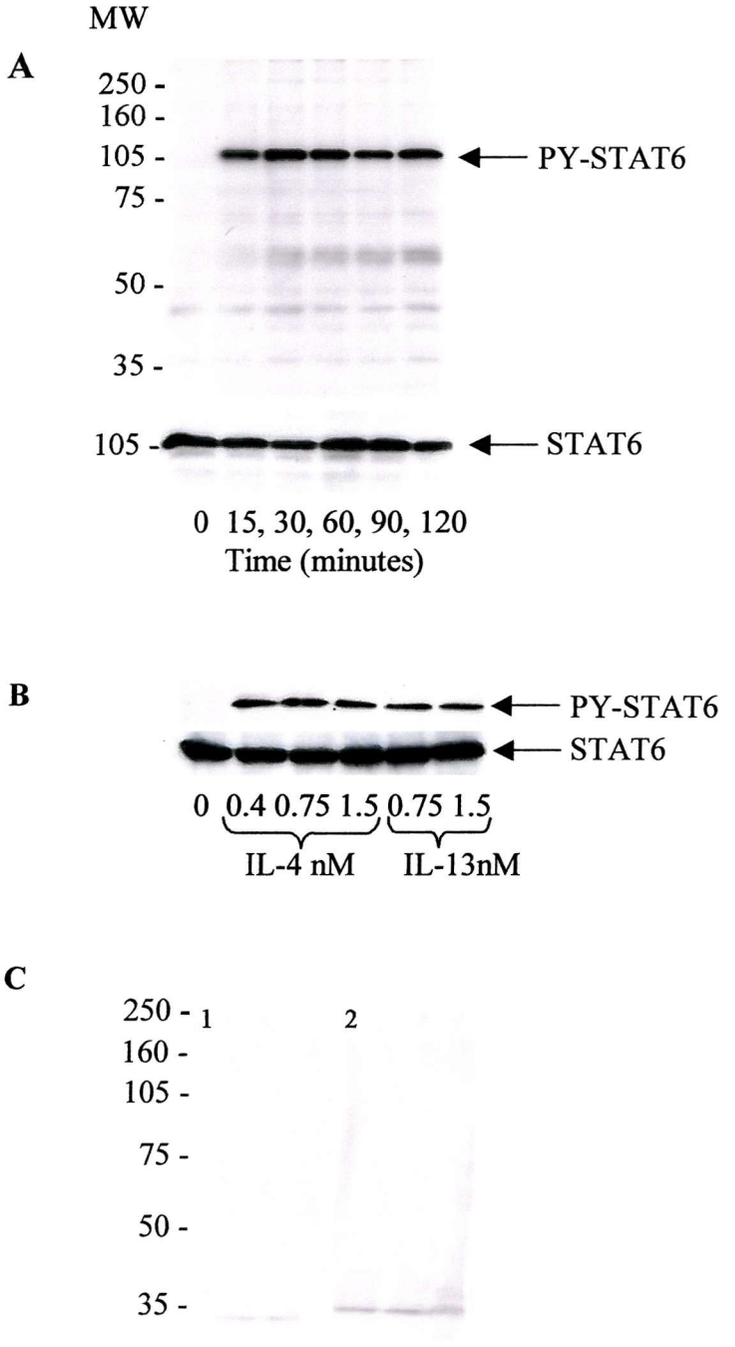


Figure 5.3 STAT6 Phosphorylation in Primary Bronchial Epithelial Cells.
A) Cells were incubated with 1.5nM IL-4 for the times indicated and STAT6 phosphorylation determined using an anti-phospho STAT6 antibody. A pan STAT6 antibody was used to determine equivalent protein loading (n=3).
B) Cells were incubated with the indicated doses of IL-4 or IL-13 for 1 hour and phospho-STAT6 detected as in (A) (n=4).
C) 1. Membranes probed with rabbit anti-mouse HRP secondary antibody only.
2. Membranes probed with swine anti-rabbit HRP secondary antibody only.

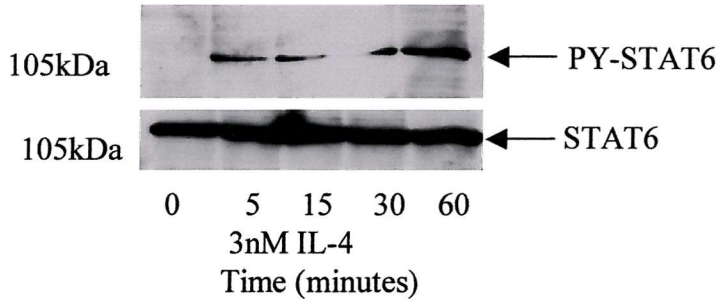


Figure 5.4 STAT6 Phosphorylation in Primary Human Lung Fibroblasts.

Primary Human Fibroblasts were treated with 3nM IL-4 for the times shown. Whole cell lysates were prepared and Western blots were probed for PY-STAT and STAT6 to confirm equivalent protein loading (*n*=2).

addition, in parallel experiments, cells were treated with 1.5 nM IL-4 for 60 minutes and whole cell lysates prepared to investigate changes in STAT6 expression and activation.

5.2.3 *Cell Viability in Transfected H292 Cells*

As demonstrated in chapter four, IL-4 did not alter significantly alter cell growth. Upon visualisation under a light microscope throughout the course of the experiment the transfected cells, especially those transfected with the empty plasmid, appeared to be growing at a slower rate and more dead cells and cellular debris were apparent in the medium compared to untransfected cells. The detrimental effect of transfection on the H292 cell viability became more apparent when a methylene blue assay was carried out on the cells at the end of the 48 hour time course experiment. As shown in figure 5.5, the density of the transfected cells at the end of the 48 hour time course experiments were significantly lower than in untransfected cells. The mean absorbance at 650nM (a measure of cell density) was reduced from 3.15±0.19 in untreated untransfected cells to 1.89±0.34 (n=3 P= 0.01) and 2.66±0.29 (n=3 P=0.03) in untreated cells transfected with STAT6-WT and dominant negative STAT6 respectively. The wild type expression vector was more toxic than the dominant negative and the cell density was significantly reduced from 2.66±0.29 in the latter to 1.89±0.34 (n=3 P=0.02) in the former. The empty control plasmid also significantly reduced cell density compared to untransfected cells (3.15±0.19 reduced to 0.97±0.22 (n=3 P=0.01) with the cell density. The density of cells transfected with the empty plasmid was also significantly lower than cells transfected with dominant negative STAT6 (P=0.02) and was lower than cells transfected with STAT6-WT, but this failed to achieve statistical significance (P=0.06). From these results it was apparent that the empty plasmid, whilst nearly structurally identical to pcDNA3 was toxic to the cells. A similar finding has been observed with the pEGFP-C2 plasmid, which is relatively similar in structure to the pRc/CMV plasmid [215]. Therefore the pRc/CMV plasmid was not suitable for use as a control. Thus the wild type STAT6 expression vector was used as a control for any effects observed in cells transfected with the dominant negative vector, as it was identical to the latter with exception of the tyrosine 641 mutation.

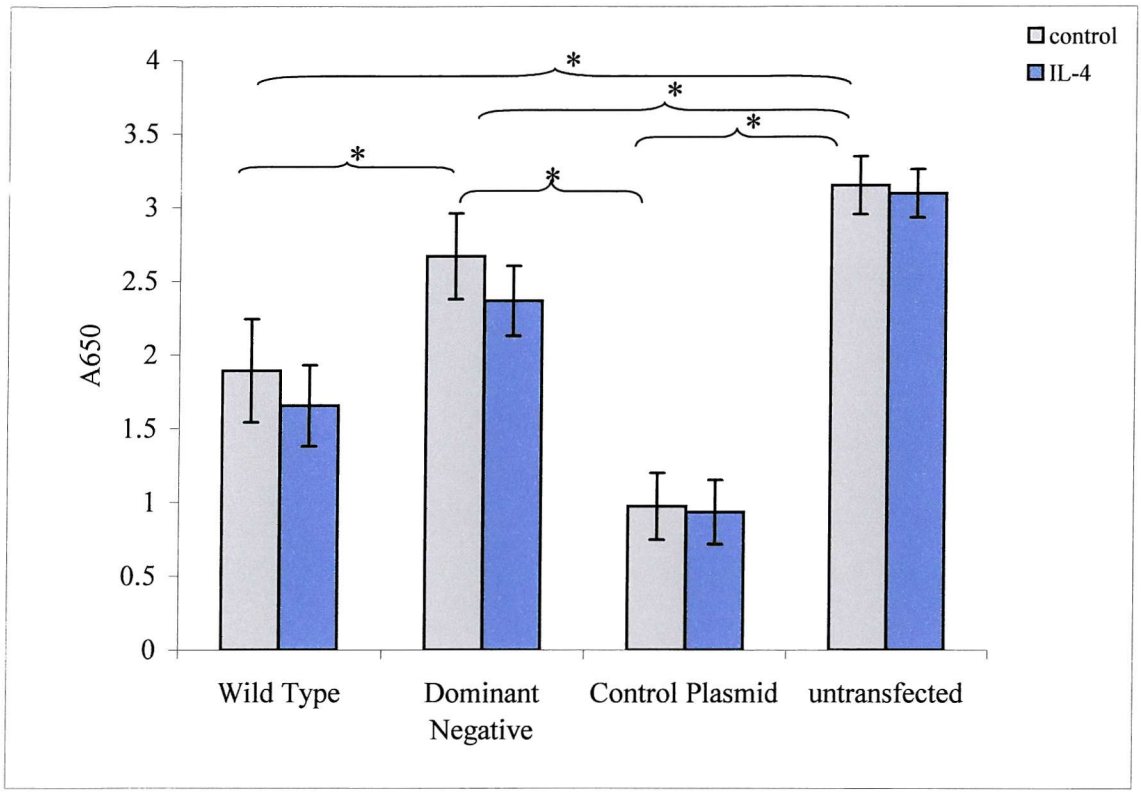


Figure 5.5 The Effect of Transfection on H292 Cell Denisty.
Methylene blue assays were carried out in H292 cells transfected with wild type STAT6 dominant negative STAT6 or an empty plasmid 48 hours post stimulation with 1.5nM IL-4, as described in chapter two. Parallel experiments were also carried out on untransfected cells. Results are based on three individual experiments incorporating 2 duplicate wells for each. Supernatant from each well was also assayed in duplicate. Results are expressed as the mean±SE, $*P < 0.05$ as determined by a paired students *t* -test.

5.2.4 *Wild Type STAT6 and Dominant Negative STAT6 Expression in Transfected H292 Cells*

To investigate whether transfection of H292 cells with wild type or dominant negative STAT6 caused an increase in expression of this transcription factor, whole cell lysates were prepared and probed for STAT6. In addition to this, samples were also probed for PY-STAT6 to investigate whether increased expression was accompanied by an increase in activation. As shown in figure 5.6, Western blotting of whole cell lysates prepared from cells transfected with STAT6-WT showed a slight increase in expression of this transcription factor. When normalised to levels of β-actin there was approximately a 30% increase in STAT6 expression. Similarly, there was also approximately a 30% increase in STAT6 expression in the cells transfected with dominant negative STAT6, using the STAT6 antibody, which does not distinguish between STAT6-WT and dominant negative STAT6. No difference in band intensity was apparent in cells transfected with an empty control plasmid. Transfection of cells with STAT6-WT did not aid detection of PY-STAT6 when cells were treated with 1.5 nM IL-4 (figure 5.6). RNA extracted from parallel samples showed no increase in the levels of STAT6 mRNA in either wild type or dominant negative STAT6 transfected cells when compared to the untransfected cells, although this was measured 48 hours post transfection (Figure 5.7).

5.2.5 *TGFβ$_2$ Release and Transcription in Transfected H292 Cells*

In the experiments described in chapter four, TGFβ$_2$ release from H292 cells was readily detectable at 48 hours post stimulation. Thus this time course was selected for transfection studies. Consistent with the findings in chapter four, at 48 hours 1.5 nM IL-4 significantly increased TGFβ$_2$ release from 80±7.88 pg/4x10^5 cells to 151±25.53 pg/4x10^5 cells from untransfected cells (n=3, P=0.05) (figure 5.8). When the cells were transfected with wild type STAT6, 1.5 nM IL-4 also significantly increased TGFβ$_2$ release from 101.63±19.02 pg/4x10^5 cells 188±39.7 pg/4x10^5 cells (n=3 P=0.05) (figure 5.8). The increase in the STAT6-WT transfected cells was not greater than in the control cells, nor was baseline TGFβ$_2$ release significantly different under the two conditions. In contrast to these results, transfection of cells with dominant negative STAT6 completely abolished the IL-4 induced TGFβ$_2$ release, whilst basal levels were not markedly affected. The IL-4 response was also abolished in the cells treated with the same amount of pRc/CMV plasmid (data not shown) although this is likely to be due to the toxicity described above.

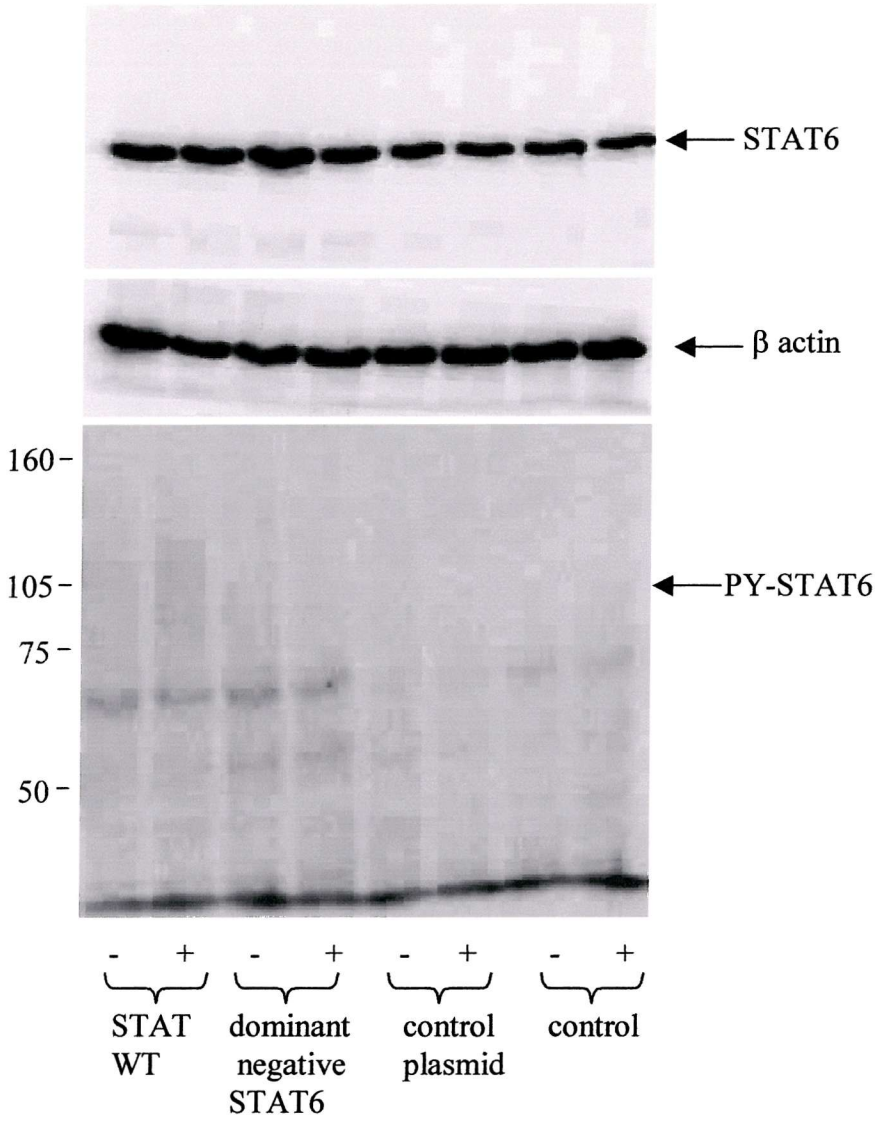


Figure 5.6 STAT6 Protein Levels in Transfected H292 Cells.
Cells were transfected with dominant negative STAT6, wild type STAT6 (STAT6 WT) or control plasmid and incubated in the presence (+) or absence (-) of 1.5nM IL-4 for 60 minutes and whole cell lysates prepared. Western blots were probed for STAT6 and β actin and PY STAT6 as indicated. The blots represented are typical of two independent experiments.

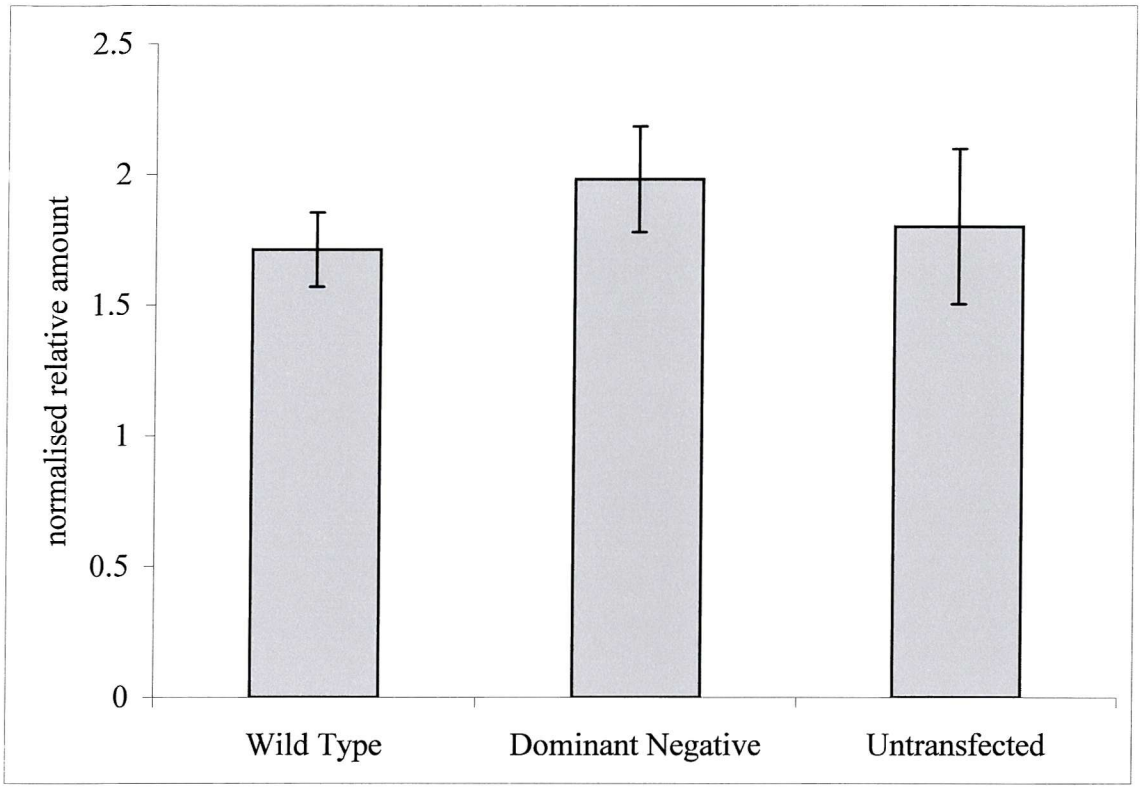


Figure 5.7 STAT6 Common mRNA Production in Transfected H292 Cells.
H292 cells were transfected with wild type STAT6 or dominant negative STAT6 and treated with 1.5nM IL-4 for 48 hours. The RNA was collected for real time Taqman™ PCR to measure STAT6 mRNA. In parallel untransfected were treated in the same way. Results are expressed as the mean±SE of three independent experiments.

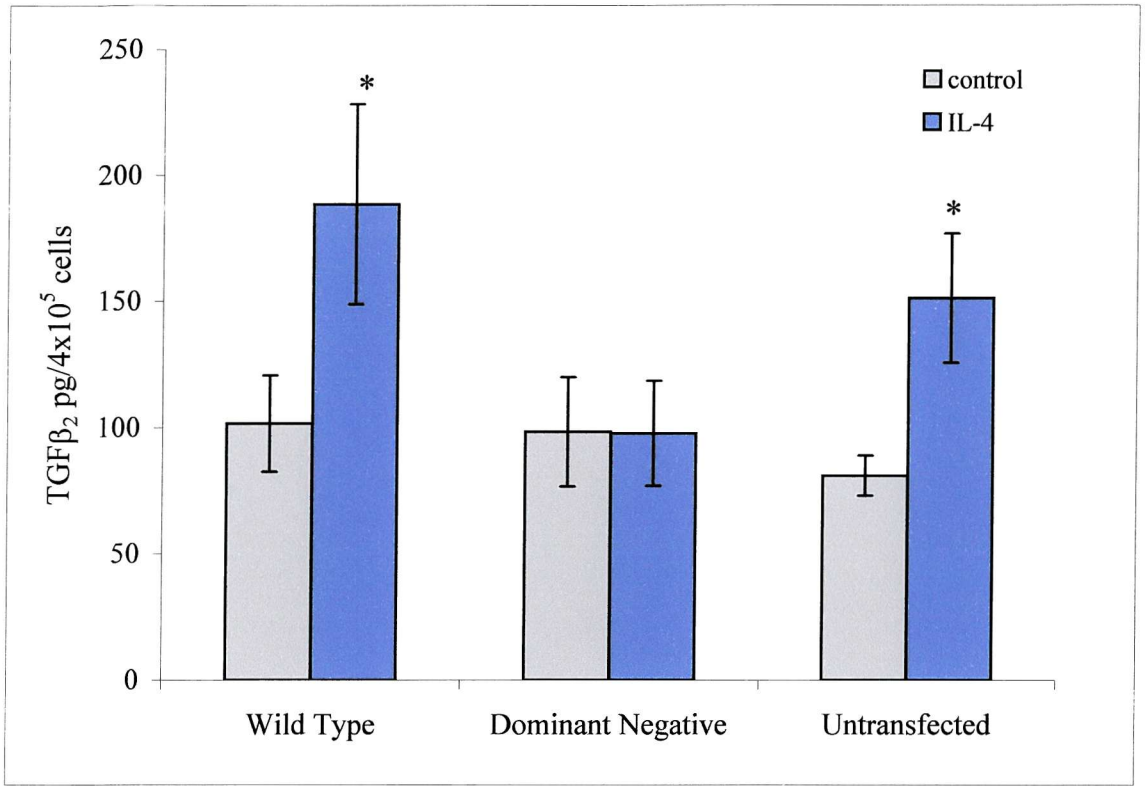


Figure 5.8 TGFβ_2 Release from Transfected H292 Cells.
H292 cells were transfected with wild type STAT6 or dominant negative STAT6 and treated with 1.5nM IL-4 for 48 hours and the conditioned medium harvested for ELISA. In parallel untransfected were treated in the sameway. Results are based on three individual experiments incorporating 2 duplicate wells for each condition. Supernatant from each well was also assayed in duplicate. Results are expressed as the mean$\pm$SE, *$P<0.05$ as determined by a paired students t-test.

RNA derived from the cells from which the conditioned medium was collected, was extracted and changes in TGFβ mRNA levels were investigated. In the untransfected cells there was a slight induction of transcription at 48 hours after IL-4 treatment, but this did not achieve statistical significance (figure 5.9). The levels of TGFβ mRNA were unaffected by 1.5 nM IL-4 in any of the transfected cells. As with the protein release, baseline TGFβ mRNA levels were not significantly altered in the transfected cells compared to the control cells.

5.3 Discussion

The role of STAT6 in mediating the pro-inflammatory effects of IL-4 and IL-13 has been well described in animal models and more recently studies have also been carried out *in vitro*. However there is little information regarding how this transcription factor might be involved in mediating epithelial responses that relate to airway wall remodelling. Therefore, the role of STAT6 in mediating IL-4 induced $TGF\beta_2$ release from bronchial epithelial cells was investigated, by assessing the phosphorylation of STAT6 in H292 cells and subsequently transfecting cells with a dominant negative STAT6 mutant.

Under a variety of conditions only low levels of STAT6 phosphorylation were detected in H292 bronchial epithelial cells lines compared to primary bronchial epithelial cells and primary lung fibroblasts. However using immunoprecipitation of tyrosine phosphorylated proteins from EGF treated H292 cells resulted in an increase in the detection of phosphorylated proteins, including one with a molecular weight comparable to that of the EGF receptor. This suggests that the experimental procedure used did not have any technical problems. Since H292 cells express high levels of STAT6, the finding that activation of this transcription factor is difficult to detect was surprising. Over expression of STAT6 in the H292 cells by transfection, did not aid detection of phosphorylated STAT6, suggesting that the cells were not able to phosphorylate more STAT6 than already present in the cells. Therefore it is likely that only very slight changes were occurring in the cell lines compared to the primary cells and that the methods used were perhaps not sensitive enough to detect them. The lack of STAT6 phosphorylation in the H292 cells compared to the primary cells could be due to differences in the numbers of IL-4 receptors expressed, or differences in the expression of negative regulators of STAT6 function. As discussed in chapter four, IL-13 failed to stimulate $TGF\beta_2$ release from H292 cells, whilst in primary cells a clear response can be evoked [93]. This clearly demonstrates that there are differences in the responses of primary cells and H292 cells to

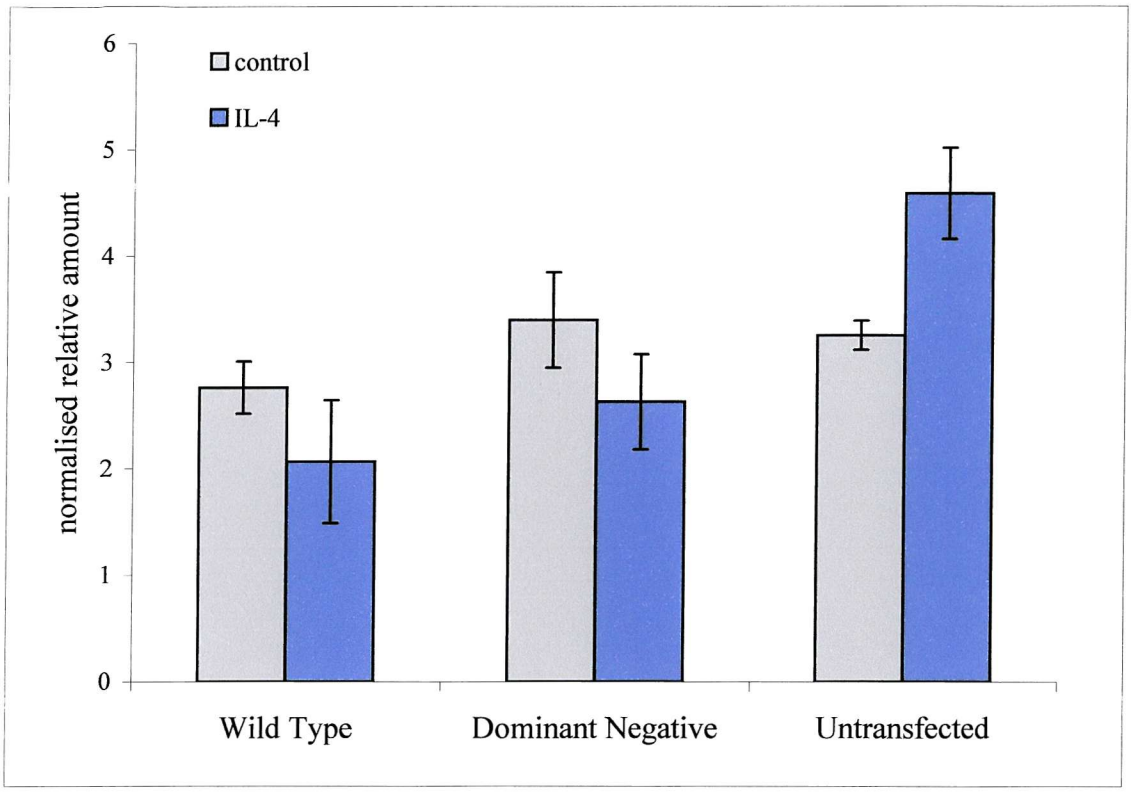


Figure 5.9 TGFβ mRNA Production in Transfected H292 Cells.
H292 cells were transfected with wild type STAT6 or dominant negative STAT6 and treated with 1.5nM IL-4 for 48 hours and the RNA collected for real time Taqman™ PCR. In parallel untransfected were treated in the same way. Results are expressed as the mean±SE of three independent experiments.

IL-4 and IL-13. Therefore it is possible that other components of their signalling pathways vary between the two cell types. Furthermore, the low levels of STAT6 phosphorylation in the H292 cells may also explain why the IL-4 induced TGFβ release was not as great as that observed by Richter *et al* using primary bronchial epithelial cells with a comparable concentration of IL-4 for the same duration [93]. As described in chapter three, STAT6 isoforms have been identified in bronchial epithelial cells and it is possible that expression of the dominant negative isoform is the cause of low levels of phosphorylation of STAT6 in H292 cells. If this were the case, one would expect that the ability of IL-4 to induce STAT6 dependent $TGF\beta_2$ release to be reduced. In addition to the dominant negative STAT6, another STAT6 variant has been identified in mast cells [150] so the possibility of variants, other than those described, regulating function in bronchial epithelial cells can not be ruled out. Discrepancies between primary cells and cell lines have been described previously in a study by Nakamura *et al* where both IL-4 and IL-13 increased GM-CSF release from primary bronchial epithelial cells but no effect was observed in the BEAS-2B cell line [188].

It is likely that the differences observed between the primary cells and the cell lines is due to the latter being virally transformed or derived from carcinomas. The phenotype of these immortalized cells vary quite considerably from the parent type in terms of their growth rate and expression of cell markers [216]. It has been reported that the SV-40-transformed airway epithelial cell line 16HBE14o$^-$ maintains many of the features of the native epithelium following transformation, including growth into monolayers, polarization and expression of tight junctions [194]. However these cells are still transformed and it is possible that there are differences within the IL-4/IL-13 signalling pathway, which could account for the variable results described above. It is also possible that differences within the cell line experiments are due to differences in cell line passage. As cells age, the number of genetic mutations within them is likely to increase. Therefore, efforts were made to ensure that all experiments were carried out on cells, within a range of 6 passages. Further experiments would need to be carried out to characterise the differences between the primary cells and the H292 cell line. Similarly further experiments would be required to confirm the activation of STAT6 in the H292 cells, for example by preparing nuclear extracts and Western blotting or by EMSA as has been done in other studies using bronchial epithelial cell lines [190].

In the transfection experiments the finding that the empty plasmid was toxic to the cells made it unsuitable for use as a control. The only difference between the two plasmids,

pcDNA3 and pRc/CMV is in the polylinker region so it is difficult to explain why the pRc/CMV was so toxic. However it has been reported previously that some plasmids can be toxic to certain cell types [215]. However in the study by Matsukura *et al* using the same STAT6 expression vectors to study the effect on eotaxin gene activation, adding the same amount of pcDNA3 did not influence their readouts [191]. Similarly in the experiments described above, transfection with WT-STAT6 did not significantly alter the IL-4 induced TGFβ response suggesting that the effect observed with the dominant negative is not due to the presence pcDNA3 alone.

The finding that transfection of H292 cells with wild type or dominant negative STAT6 results in an increase in protein expression is consistent with the findings of Matsukura *et al*, where the same vectors were used to transfect BEAS-2B cells, a human airway epithelial cell line transformed with adenovirus 12-simian virus 40 hybrid virus [191]. The increase in STAT6-WT expression was not as great as observed in the BEAS-2B cells but there are many factors that can influence transfection efficiency, including the degree of confluency and passage number. It is also possible that the differences may be due to the different transfection reagents used and slightly different concentrations of expression vector.

Whilst only low levels of tyrosine phosphorylation were detectable in the H292 cells, the finding that insertion of the dominant negative STAT6 completely abolished $TGF\beta_2$ release from IL-4 stimulated H292 cells implies that this transcription factor is in some way involved in this response. It also suggests that STAT6 phosphorylation in the H292 cells was sufficient for activation and that the methods used for detection were not sensitive enough as suggested above. However expression of STAT6-WT did not enhance the response when compared to control cells suggests that the response induced by IL-4 is optimal and cannot be increased further. It is possible that the reason increased levels of STAT6 mRNA were not detected in the transfected cells, was due to the cells having lost the plasmid or it no longer been functional after 72 hours (cells were serum starved for 18 hours post transfection, prior to incubation with IL-4 for 48 hours). This may suggest that STAT6 is involved at an early stage in the response.

A recent study has shown that delivery of a peptide derived from the STAT6 binding region of IL-4Rα into cells effectively inhibits IL-4 dependent STAT6 phosphorylation [217]. A protein of this kind could be used to inhibit STAT6 and confirm its involvement of STAT6. It would be of interest to carry out such experiments on the primary bronchial

epithelial cells where IL-4 has a more pronounced effect on $TGF\beta_2$ release and STAT6 phosphorylation. It might also be of interest to investigate the possible role of IRS-1 in the induction of TGFβ, possibly by using a fusion protein or by transfecting cells with mutant forms.

Whilst cells transfected with STAT6-WT showed an increase in $TGF\beta_2$ release of similar magnitude to untransfected cells, they did not show any increase in TGFβ transcription, although the response of the untransfected cells was as previously observed. It is possible that this is due to differences in the kinetics of each of the transfected cell types to induce TGFβ gene activation. It is possible that cells transfected with WT-STAT6 cause earlier induction of TGFβ transcription, especially if STAT6 is involved in an early response which indirectly brings this about, for example by inducing MMP-9, as discussed in chapter four. By only measuring one time point, one is unable to confirm this possibility, thus extended time course experiments would need to be carried out. Similarly, if it were found that IL-4 induces MMP-9 expression or activation as was hypothesised in chapter four, it would be important to determine whether this response were altered in transfected cells. In support of IL-4 inducing $TGF\beta_2$ release through an indirect mechanism, is the finding that the TGFβ gene does not have an optimal STAT6 consensus sequence. This makes it less likely that STAT6 binds directly to the TGFβ promoter to induce transcription.

Overall the results contained within this chapter indicate that IL-4 induced $TGF\beta_2$ release from H292 cells is mediated via a STAT6 dependent mechanism and that, whilst detection of STAT6 phosphorylation was difficult in these cells, the levels of activation must be sufficient for induction of $TGF\beta_2$ release. These results provide a potentially novel mechanism by which epithelial expression of STAT6 might contribute to airway wall remodelling. Thus, these results, combined with the finding that STAT6 is necessary for IL-13 induced eotaxin release by Matsukura *et al* [191], suggest that the increased expression of STAT6 *in vivo,* could have an effect on the release of pro-inflammatory and pro-fibrogenic mediators from the epithelium.

CHAPTER 6

6 FINAL DISCUSSION AND FUTURE WORK

IL-4 and IL-13 play a central role in the pathology of asthma, through their actions on inflammatory cells, where the effects of IL-4 on Th2 cell differentiation and the generation of IgE are well documented. However, more recently it has been suggested that dysregulation of the airway structural cells also confers susceptibility to asthma. With the finding that the bronchial epithelium can release many pro-inflammatory mediators and pro-fibrogenic growth factors, this cell type as become the focus of much attention through its potential to regulate airways inflammation and airways remodelling. Similarly, it has become apparent that the actions of the Th2 cytokines, IL-4 and IL-13, are not localised to the inflammatory cells. Bronchial epithelial cells express IL-4Rα [179] [182], IL-13Rα1 [181] and the decoy receptor IL-13Rα2 [180]. Many studies on both primary bronchial epithelial cells and bronchial epithelial cell lines have shown that IL-4 and IL-13 can act on this cell type to initiate the synthesis and release of many proinflammatory mediators, including GM-CSF [188], eotaxin [189] and IL-8 [186] [187] which all act as chemoattractants and survival factors for various inflammatory cells. Thus, it was apparent that IL-4 and IL-13 have the potential to influence the activity of both the airway structural cells, as well as the inflammatory cells to contribute to the inflammatory response in asthma

Binding of IL-4 and IL-13 to their cell surface receptors initiates a series of phosphorylation events which results in the activation of the down stream signalling molecules STAT6 and IRS-1. However this signalling pathway is highly complex due to presence of genetic polymorphisms and regulation at multiple points (figure 6.1). Several polymorphisms have been described in IL-4 and IL-13, as well as their receptors. A polymorphism in the promoter region of the IL-4 gene has been related to higher IgE levels in an American population [104]. Similarly a polymorphism in the IL-13 gene alters the charge of this molecule, which may alter ligand-receptor binding and hence up-regulate IL-13 signalling [104]. One of the polymorphisms in the IL-4Rα gene, Pro478, has been demonstrated to reduce activation of JAK1 and thus phosphorylation of STAT6, whilst the IRS1/2 pathway was unaffected [104]. In contrast, an Ile50Val mutation in the extracellular domain of the IL-4 receptor upregulates receptor responses to IL-4, leading to increased STAT6 activation and IgE synthesis [218]. In addition to the potential presence

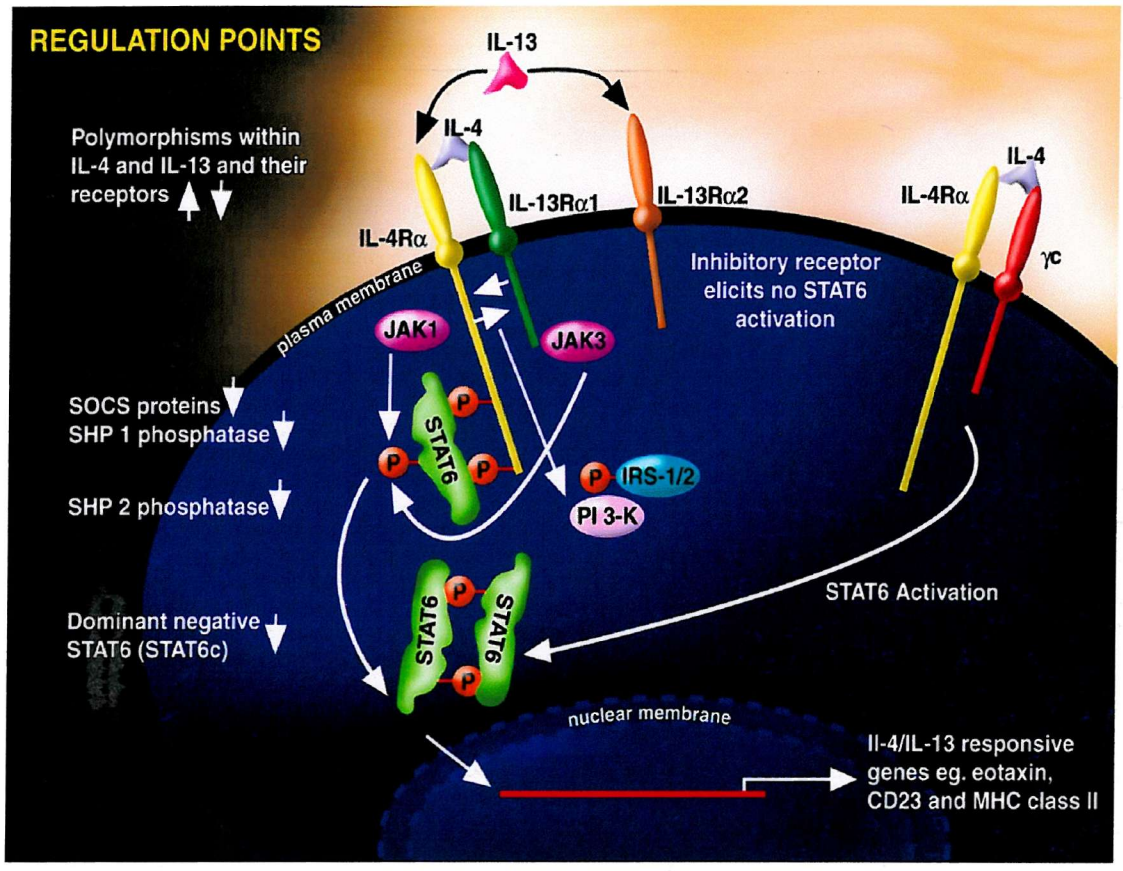


Figure 6.1 Regulation of IL-4/IL-13 Signalling.
Schematic representation of regulatory points of the IL-4 and IL-13 signalling pathways. Binding of IL-4 and IL-13 to their heterodimeric receptor complexes induces activation by phosphorylation of the associated tyrosine kinases, the JAKs. The phosphorylated receptor serves as a docking site for STAT6, which also becomes phosphorylated. This enables it to form a homodimer and to translocate to the nucleus to initiate transcription of IL-4/IL-13 responsive genes. The regulatory points in the signalling pathway are indicated on the left; *small arrows* (up or down) indicate their effect on signal transduction. Figure taken from Mullings *et al., J. Allergy. Clin. Immunol.* 2001:108:832-8.

of polymorphisms within IL-4/IL-13 receptors and their ligands, expression of the decoy receptor IL-13Rα2 on cells is likely to influence downstream signalling and thus any IL-13 induced response.

Once ligand binding activates the receptors, signalling occurs through STAT6 and IRS1/2. However there is much evidence to suggest that the former is responsible for mediating many of the biological effects of IL-4 and IL-13 relevant to asthma. Recently studies have been carried out to investigate the negative regulators of STAT6 function. As described in chapter one, SHP1 phosphatase, SOCS1 and SOCS3 have all been demonstrated to negatively regulate STAT6 function through dephosphorylation of the IL-4R [219] and inhibition of JAK activity respectively [176]. SOCS1 and SOCS have been detected in bronchial epithelial cells where they were strongly induced by both IL-4 and IL-13 [178]. However the expression of other negative regulators and their role in disease pathology remains to be determined. Also a splice variant of JAK3 has been detected in a range of cell types of both haematopoietic and epithelial origin. This variant lacks its carboxy terminal kinase domain and does not undergo auto-phosphorylation, thereby possibly altering downstream signalling[220]. Therefore, the existence of splice variants and polymorphisms within all the molecules involved in IL-4/IL-13 signalling must not be excluded.

In addition to the negative regulators of STAT6 function, several isoforms of this transcription factor have also been described. A dominant negative isoform of STAT6 (STAT6c) exists, which can act to inhibit the dimerisation and DNA binding actions of full length STAT6 [149]. I have shown for the first time that that mRNA for this isoform is expressed in bronchial epithelial cells obtained from both normal and asthmatic subjects. Whilst it was found that there was no relationship between STAT6c expression and disease severity, further studies would need to be carried out to elucidate the contribution that this isoform makes to control STAT6 function *in vivo*. A well-controlled study analysing the levels of expression in relation to STAT6 mediated responses from the same cells, in a large sample group would provide more information on this matter. Another isoform of STAT6 has been identified in human mast cells; this isoform lacks the carboxy terminus domain present in full length STAT6 [150], which is thought to influence transcriptional activity [140]. However the effect of this isoform on gene expression and the functions of full length STAT6 have not been determined. Also the STAT6 gene is assigned to chromosome 12q, at a site of genetic linkage to asthma [156]. A polymorphism

within the STAT6 gene on chromosome has been associated with atopy in a Japanese population [157].

Whilst it is apparent that there are many components of the IL-4/IL-13 signalling pathways the aim of this project was to focus on the involvement of STAT6 in responses relevant to asthma. Activation of STAT6 has been implicated in mediating many of the pro-inflammatory effects of IL-4 and IL-13, and the strikingly contrasting characteristics observed between mice over expressing these cytokines and STAT6 knockout mice highlight this. Both IL-4 and IL-13 transgenic mice display features such as increased numbers of inflammatory cells in BAL [114 116]. In contrast, STAT6 knockout mice do not manifest these features when challenged with antigen [153]. More recently, studies have shown that blockade of STAT6 signalling, by transiently transfecting cells with a dominant negative isoform, is essential for IL-13 induced eotaxin release [191]. In this thesis, the same dominant negative STAT6 expression vectors were used to show that IL-4 induced TGFβ release is also dependent on STAT6 activation.

To begin to rationalise studying the involvement of STAT6 in IL-4 and IL-13 driven responses in bronchial epithelial cells, its expression in bronchial biopsies was assessed. Initial experiments showed that there is abnormally high expression of STAT6 in severe asthma when compared to mild asthma or normal controls. As described in chapter one, the expression of many signalling molecules is increased in asthmatic bronchial epithelium, probably as a result of injury or activation. The factors that control STAT6 expression have not been extensively studied; most work has focused on the mechanisms that control its activation. In T and B lymphocytes, anti-CD40, anti-IgM and ionomycin can all augment STAT6 expression at the transcriptional level [221]. STAT6 expression is also increased in rabbit arterial tissue challenged by perivascular injury, and this increase precedes, and is thought to, stimulate smooth muscle proliferation [222]. The latter suggests that damage could be a stimulus for increased STAT6 expression in bronchial epithelial cells. In this thesis the expression of STAT6 in damaged areas was not assessed but, in the *in vitro* experiments investigating the effects of IL-4 on TGFβ release, different findings were observed from cells at high and low density. It is possible that the paracrine actions of mediators released from damaged areas are the cause of the increase. Further studies would need to be carried out *in vitro* to assess what mediators might stimulate STAT6 expression as has been done for NFκB. In the *in vitro* model described in this thesis, IL-4 did not alter STAT6 mRNA, which is consistent with the findings of Shen *et al* relating to STAT6 protein levels following IL-4 treatment [223]. However

mRNA levels do not always relate to protein and it is possible that as STAT6 was strongly expressed in the H292 cells under basal conditions, no further induction was necessary. As with full length STAT6, neither did IL-4 treatment alter the expression of the dominant negative splice variant, STAT6c. However the levels of STAT6c did decrease over time, which could possibly contribute to the increased responsiveness of the cells at later time points. Since *in vivo* conditions are so complex, a variety of factors are likely to influence STAT6 expression. For example there are likely to be factors, not yet described, which down-regulate STAT6 expression. Therefore, the cause of this increased expression may be disease specific failures of feedback mechanisms that control its expression.

In this study I was unable to assess changes in activity of STAT6 in bronchial biopsies although some nuclear staining was detected in normal and asthmatic biopsies, suggesting that a proportion of it was active. To assess whether increased STAT6 activation is associated with the asthmatic phenotype, lysates prepared from bronchial brushings could be probed for phospho-STAT6 using western blotting, as has been done to investigate the activation of NFκB [64]. If the STAT6 detected in the bronchial biopsies is active, its excessive expression in severe asthma could result from insufficient deactivation by the negative regulators such as the SOCS proteins described above.

Increased expression of STAT6 within the airway epithelium provides a potential mechanism for enhanced IL-4 and IL-13 mediated responses in asthma. For example excessive secretion of the leukocyte chemoattractants GM-CSF, eotaxin or IL-8 production, which are all regulated by IL-4, and are expressed at increased levels in asthma [188 187 191]. In humans, *in vivo* cellular sources of IL-4 and IL-13 are necessary for the activation of STAT6. These include Th2 lymphocytes, mast cells and eosinophils, all of which infiltrate the airway mucosa in both atopic and non-atopic asthmatic subjects [20]. Bronchial biopsies of atopic and non-atopic asthmatic subjects show an increased number of cells containing IL-4mRNA and protein [111], with the major site of IL-4 expression been $CD4^+$T lymphocytes, eosinophils and mast cells [112]. Similarly, increased levels of IL-13 mRNA has been observed in the bronchial mucosa of atopic and non-atopic asthmatics compared to healthy controls [113]. Furthermore greater concentrations of both IL-4 and IL-13 are recovered in BAL fluid collected from asthmatic subjects [12] following antigen challenge. Thus in the airways of asthmatics there are much higher levels of the cytokines necessary for the activation of STAT6, but whether greater concentrations of IL-4 and IL-13 result in increased activation of STAT6 *in vivo* remains to be determined.

In vitro experiments on primary bronchial epithelial cells, did not demonstrate a clear dose related response in the levels of phospho-STAT6, suggesting that the lowest dose induced maximum activation. Similarly in the cell lines very little phosphoSTAT6 was detectable, suggesting that very little phosphorylation is needed in these cells to fully activate the system. It has been shown that both IL-4 and IL-13 can induce expression of SOCS1 and SOCS3 to inhibit STAT6 DNA binding [178]. Therefore it is likely that increases in IL-4 and IL-13 mediated responses would be counterbalanced by this negative feedback loop. However in asthma, increased expression of STAT6 along with increased IL-4/IL-13 expression could result in amplification of inflammatory responses.

Inhibition of transcription factors is one of the mechanisms by which corticosteroids, commonly used to treat asthma, produce their anti-inflammatory actions. If the STAT6 in the biopsies studied were found to be active, the finding that STAT6 levels are increased in the severe asthmatics despite high doses of both inhaled and oral corticosteroids may suggest that control of STAT6 expression is steroid insensitive. In support of this possibility, is the finding that in primary bronchial epithelial cells IL-4 and IL-13 induced TGFβ release was not affected by dexamethasone [93], a response that work contained in this thesis has shown to be STAT6 dependent. However IL-4 induced IL-8 and GM-CSF release from primary bronchial epithelial cells can be significantly inhibited by the addition of dexamethasone [187]. It is possible that the differential effects of dexamethasone on IL-4 induced TGFβ and IL-8/GM-CSF release may be due to interactions between STAT6 and NFκB. These two transcription factors have been shown to directly bind to each other and act in synergy to induce transcription of particular genes, which contain both STAT6 and NFκB consensus sequences[223]. An example of where this type of synergistic interaction occurs is in the eotaxin promoter, where treatment with TNFα and IL-4, which activate NFκB and STAT6 respectively, causes an additive effect on eotaxin gene activation. This is due to the finding that the eotaxin promoter contains a putative STAT6 binding site which overlaps with a putative NFκB binding site [190]. Thus it is possible that the pro-inflammatory effects mediated via IL-4 also involve association with NFκB which is known to be a target for corticosteroids [224 152]. Whether epithelial STAT6 is a direct target for corticosteroids remains to be determined, but studies in fibroblasts derived from foetal lung have shown that the corticosteroid fluticasone, reduced IL-4 and abolished IL-13 induced STAT6 phosphorylation [225]. However this effect was not maintained upon passage of the cells

and no inhibition was apparent in partially and fully differentiated fibroblasts obtained from adult tissue.

As discussed, increased STAT6 expression in the bronchial epithelium could potentially amplify inflammatory responses in asthmatic subjects. It also has the potential to contribute to goblet cell hyperplasia and increased mucus secretion, which are features of remodelled airways. Mucin production can be induced by IL-4 and IL-13 [25] [185] and both IL-4 and IL-13 transgenic mice display these features, whilst they are absent in STAT6 knockout mice. While several effects of IL-4 and IL-13 have been characterised, there is very little information describing how IL-4 and IL-13 might interact with the epithelium to contribute to the subepithelial fibrosis observed in the *lamina reticularis* of asthmatic subjects.

The results contained within this thesis show that IL-4 stimulated the transcription and release of $TGF\beta_2$ from the model H292 bronchial epithelial cell line and that this stimulation was dependent on STAT6. The finding that IL-4 stimulated TGFβ release is consistent with the findings of Richter *et al*, using primary bronchial epithelial cells, where IL-4 caused a steroid insensitive increase in TGFβ release [93]. However, in contrast with the present findings, IL-13 also induced TGFβ release. One potential cause of this difference could be the receptor profile on the two cell types. For example H292 cells may express higher levels of the IL-13Rα2 chain, which does not activate STAT6 upon ligand binding and is thought to act as a decoy receptor [130]. Furthermore it is not known whether particular receptor combinations preferentially activate the STAT6 or IRS1/2 signalling pathway. Whilst mRNA for the three different receptor chains was detected in the H292 cells, this gives no indication of the relative expression of each. Therefore, further work would be required to investigate the numbers of IL-4 and IL-13 receptors on the H292 cells by comparison with primary bronchial epithelial cells. This would provide further phenotypic characteristics of this cell line, and its usefulness as a model for studying IL-4 and IL-13 mediated responses. Also, a soluble IL-13 binding protein [206] has been described and studies have shown, that it is a potent inhibitor of IL-13 receptor binding respectively. It is possible that secretion of this protein from the H292 cells accounts for the lack of induction of $TGF\beta_2$ by IL-13. Another difference between the H292 cells and the primary cell was the degree of STAT6 phosphorylation observed when the cells were treated with IL-4. This also may be due to differences in the expression of signalling molecules or simply the amount of active STAT6 necessary to evoke a

response. However taken together these results may suggest that if further work were to be carried out, efforts might be better spent developing a method to transfect primary bronchial epithelial cells. Indeed methods have been developed to culture differentiated bronchial epithelial cells [226;227], which may serve as a better model.

Overall, the finding that IL-4 can induce TGFβ_2 release, by a STAT6 dependent mechanism, provides a way by which the Th2 cytokines might contribute to the thickening of the *lamina reticularis* observed in asthma, in addition to their effects on inflammation through actions on epithelial and inflammatory cells (figure 6.2). The pro-fibrogenic effects of TGFβ are well documented and specifically it has been shown to induce the myofibroblast phenotype that is associated with asthma [77]. These cells synthesise extracellular matrix proteins such as collagen 1 and release ET1 and VEGF [93]. However, treatment of fibroblasts with IL-4 or IL-13 did not cause myofibroblast transformation, collagen secretion or pro-fibrogenic growth factor release [93]. The finding that IL-4 stimulated epithelial production of TGFβ_2 suggests that IL-4 has the potential to contribute to subepithelial fibrosis indirectly rather than by any direct effect on fibroblasts. Studies have shown that IL-13 transgenic mice display subepithelial fibrosis [116] and, it has been suggested that this fibrotic response is due to release of TGFβ from macrophages, as these cells were the major site of TGFβ expression in the transgenic animals [209]. The finding that a second mechanism involving epithelial production of TGFβ_2 provides another cellular source of this profibrogenic growth factor, which could potentially amplify the remodelling response.

Whilst IL-4 induced TGFβ_2 release from H292 bronchial epithelial cells in a STAT6 dependent manner, the time taken for induction of gene transcription and the lack of a STAT6 consensus sequence on the TGFβ promoter, suggested that this response was indirect. In the IL-13 transgenic mice described above, the activation of TGFβ *in vivo* was absent in MMP-9 null mice, suggesting an important role for this enzyme in IL-13 driven subepithelial fibrosis. In view of this, it was hypothesised that treatment of H292 cells with IL-4 caused an increase in MMP-9 expression (or activation), which activated TGFβ [208], which in turn induced its own transcription [207]. To begin to test this hypothesis, the effect of a broad range MMP inhibitor on IL-4 induced TGFβ gene activation and release was assessed. Whilst the presence of the inhibitor prevented IL-4 induced increases in TGFβ_2 release the results were complicated by the effect of the inhibitor itself. To confirm the involvement of active TGFβ in induction of its own expression,

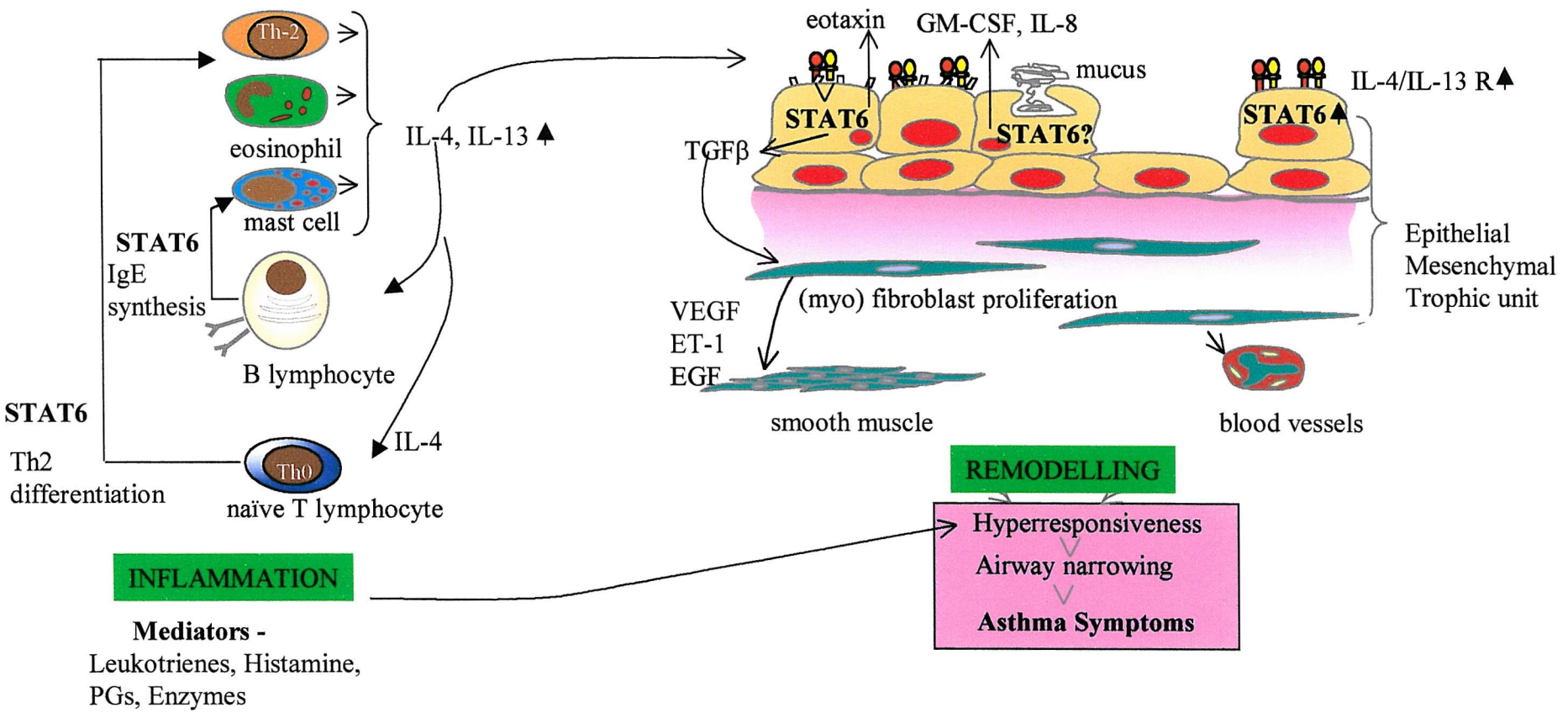


Figure 6.2 Schematic Representation of the Involvement of IL-4, IL-13 and STAT6 in Asthma.
This figure illustrates how IL-4 and IL-13 contribute to airways inflammation and remodelling through activation of STAT6. IL-4 is necessary for induction of the Th2 phenotype and both IL-4 and IL-13 can induce IgE synthesis from B lymphocytes, actions which are inhibited in STAT6 null mice. IL-4 and IL-13 can stimulate pro-inflammatory mediator release from bronchial epithelial cells, but only eotaxin release has been shown to be dependent on STAT6 activation. IL-4 can induce TGFβ release from bronchial epithelial cells and this too is dependent on STAT6. TGFβ can act on the underlying mesenchymal cells to cause airway wall thickening. Expression of IL-4, IL-13, their receptors and STAT6 are all expressed at increased levels in asthmatic subjects.

experiments using a TGFβ blocking antibody would be necessary, although samples treated with such an antibody would not be suitable for analysis by ELISA. To investigate the involvement of MMP-9 in IL-4 induced $TGF\beta_2$ release experiments using a more specific inhibitor would be required. At present no specific inhibitors of MMP-9 are available commercially, although inhibitors specific for MMP-9 and MMP-2 are, which may be more suitable for future work. It might also be possible to inhibit the actions of MMP-9 using anti-sense technology. Also the effects of IL-4 on TGFβ expression and activation would need to be investigated, possibly by TaqMan™ PCR, Western blotting or immunohistochemistry. If MMP-9 were found to be involved STAT6 dependent TGFβ release this could have important implications in asthma, as IL-4, STAT6 and MMP-9 are all expressed at increased levels in this disease [82]. Thus again there is the potential for an amplified fibrotic response in certain individuals. However TGFβ activation and release is a complex process and it is possible that IL-4 interacts with a number of molecules to bring about the increase in expression and release. It would be possible to begin to investigate what molecules may be involved by using cDNA or oligonucleotide arrays to investigate the genes activated by IL-4 in epithelial cells. Using this technique studies in primary bronchial epithelial cells have shown that IL-13 induces expression of the TGFβ binding protein, biglycan, which can sequester TGFβ in the extracellular matrix, [213]. A mechanism such as this might explain why active $TGF\beta_2$ could not be detected in culture supernatants.

In conclusion, the Th2 cytokines IL-4 and IL-13 are known to make an important contribution to the asthmatic phenotype, through their actions on airways inflammation. The present studies suggest a further role for IL-4 in airway wall remodelling and indicate the involvement of STAT6 in mediating these responses. The finding that a functionally altered splice variant is expressed in airway epithelium could be of significance with regard to IL-4/IL-13 driven responses. Also the finding that asthmatic subjects display increased levels of STAT6 provides the potential for IL-4 and IL-13 mediated responses to be amplified in asthmatic subjects, which could have important implications for future asthma therapies.

7 Appendix I

Determination of Relative Amounts of cDNA Following TaqMan™ Real-Time PCR.
All calculations were carried out using Microsoft Excel.

1. A standard curve was plotted, using Ct values obtained from known starting concentrations of cDNA on the y-axis, and the log of the dilution factor of the cDNA on the x-axis.

2. A linear trendline was then plotted through the data points and the equation of this line and the R^2 value calculated.

3. To determine the input amount for unknown samples, the equation of the trendline was used to calculate the log dilution factor for a given Ct value, i.e. **x = Ct value-c/m**, where c is the intercept on the y axis and m is the gradient of the line.

4. The anti-log of the log dilution factor for a given Ct value was then calculated. The resulting figure was defined as the relative amount of starting cDNA.

5. Steps 1-4 were carried out for each mean Ct value obtained for 18S ribosomal RNA and the gene of interest for each sample. The relative amount of the gene of interest, for example TGFβ, was then divided by the corresponding relative amount of 18S ribosomal RNA to give the normalised relative amount of cDNA within a sample.

8 Reference List

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