UNIVERSITY OF SOUTHAMPTON

THE ROLE OF *DERMATOPHAGOIDES PTERONISSINUS* ALLERGEN AND TH2 CYTOKINES IN THE AIRWAY INFLAMMATION OF ALLERGIC ASTHMA

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ABSTRACT

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THE ROLE OF *DERMATOPHAGOIDES PTERONISSINUS* ALLERGEN AND TH2 CYTOKINES IN THE AIRWAY INFLAMMATION OF ALLERGIC ASTHMA

By Dr James Laurence Lordan

Atopic asthma is characterised by variable airflow obstruction and airway hyperresponsiveness on a background of diffuse bronchial inflammation. Asthma currently affects between 4% and 8% of the population, and the prevalence of asthma and allergy is increasing, although the reasons for this are unclear. The patho-physiological features of asthma are complex and include: allergen sensitisation with increased production of circulating allergen-specific IgE, airway infiltration by inflammatory cells, including Th2-type lymphocytes, eosinophils, mast cells and basophils. Th2-type $CD4^+$ and $CD8^+$ T cells play an important role in orchestrating the inflammatory process, by the release of Th2-type cytokines (IL-4, IL-5, IL-9, GM-CSF and IL-13) encoded on chromosome $5q_{31\text{-}33}$. A characteristic feature of asthma is the structural changes of airway remodelling, including epithelial cell damage, sub-epithelial fibrosis, smooth muscle hypertrophy, goblet cell metaplasia, and mucus hypersecretion.

Optimum T cell activation by antigen presenting cells requires co-stimulatory signalling by the interaction of CD80 or CD86 with CD28 on T lymphocytes. The study presented in chapter 3 has applied an integrated bronchial explant tissue culture system to investigate the hypothesis that CD-28 mediated T cell costimulation is required for cytokine production in moderately severe asthma. Allergen stimulation of bronchial explant cultures from mild asthmatics induces the production of IL-5 and IL-13 that is reduced by the fusion protein, CTLA-4Ig, which inhibits CD28-mediated costimulation. In chapter 3, I have shown that *Dermatophagoides pteronissinus (Der p)* allergen stimulates the production of IL-5, but not IL-13, by bronchial explants of moderately severe asthmatics. However, in contrast to similar explant studies in mild asthma, allergen-induced IL-5 production was not inhibited by CTLA-4Ig. Allergen stimulation of peripheral blood mononuclear cell cultures from these subjects resulted in increased production of IL-5 and IL-13, which was inhibited by CTLA-4Ig. This suggests that IL-5 production in the airways of moderately severe asthmatics is less dependent on CD-28 mediated co-stimulation. The difference in requirements for CD28-

mediated costimulation in PBMC cultures compared to explant cultures suggests that the tissue micro-environment influences the pulmonary inflammatory response in severe asthma.

It is increasingly recognised that the structural elements of the airways, such as the bronchial epithelium, are capable of responding to environmental stimuli and Th2 mediated inflammation with the release of cytokines, chemokines, and pro-inflammatory mediators. In chapter 4, I have optimised a reliable technique for the culture of confluent monolayers of primary bronchial epithelial cells from bronchial brushings of the airways of both normal and atopic asthmatic subjects. This was applied to test the hypothesis that the bronchial epithelium can respond to environmental stimuli such as *Der p* allergen or Th2 cytokines (IL-4 or IL-13) to promote airway inflammation and remodelling in asthma by the release of pro-inflammatory cytokines, chemokines and growth factors. I have shown that exposure of bronchial epithelial cells to *Der p* allergen or the pro-inflammatory cytokine tumour necrosis factor-α resulted in increased production of GM-CSF, IL-8, and RANTES. Addition of the corticosteroid, dexamethasone, significantly reduced cytokine production, although not completely to basal levels.

Recent *in vivo* and *in vitro* studies using animal models suggest that the Th2 cytokines, IL-4 and IL-13, are involved in goblet cell metaplasia and airway remodelling in asthma. The study in chapter 5 has applied reverse transcription-PCR to confirm the expression of mRNA by primary bronchial epithelial cells for hetero-dimer sub-units of the IL-4 and IL-13 receptors, including IL-4Rα, common γ_c, IL-13Rα1 and the recently characterised IL-13Rα2. Flow cytometry was also applied to show the presence of IL-4Rα and demonstrate for the first time the presence of IL-13Rα2 on primary bronchial epithelial cells of normal subjects and allergic asthmatics.

The study presented in chapter 6 has shown that exposure of bronchial epithelial cell cultures from normal subjects or allergic asthmatics to IL-4 or IL-13 for 24 hours leads to increased production of GM-CSF, IL-8 and RANTES, which can be reduced but not completely suppressed by the corticosteroid dexamethasone. A co-operative effect was noted for combined stimulation with IL-4 or IL-13 and *Der p* allergen for the production of GM-CSF, IL-8 and RANTES. IL-4 and IL-13 also stimulated the release of transforming growth factor-β, which promotes fibroblast proliferation and collagen deposition. Exposure of bronchial epithelial cells to either *Der p* allergen, IL-4, IL-13 or TNF-α led to increased release of the structurally unrelated transforming growth factor-α, which is a potent ligand for the epidermal growth factor receptor, and has been linked with goblet cell metaplasia and airway remodelling. Although there was no significant difference in the basal or stimulated production of GM-CSF and IL-8 by bronchial epithelial cells of normal or asthmatic subjects, the increased production of RANTES, TGF-β, and TGF-α was confined to bronchial

epithelial cells of asthmatic subjects. The ability of *Der p* allergen and Th2 cytokines to promote the release of cytokines, chemokines and growth factors by bronchial epithelial cells of asthmatic subjects provides a link between environmental allergen, Th2 mediated inflammation, and airway remodelling in asthma.

The development of a reliable method for the culture of primary bronchial epithelial cells of atopic asthmatics paves the way for dissecting the involvement of the bronchial epithelium in airway inflammation, remodelling and mucus production in atopic asthma.

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7.1 Dynamic role of the bronchial epithelium in the pathogenesis of airway inflammation and remodelling in asthma.

Abbreviations

AM	:	Alveolar Macrophage
APRT	:	Adenosine phospho-ribosyl-transferase
AP-1	:	Activator Protein-1
APC	:	Antigen Presenting Cell
BAL	:	Bronchoalveolar Lavage
BHR	:	Bronchial hyperresponsiveness
BEGM	:	Bronchial Epithelial Growth Medium
b-FGF	:	basic-Fibroblast Growth Factor
bp	:	base pair
cDNA	:	complementary DNA
COPD	:	Chronic Obstructive Pulmonary Disease
CRA	:	Corticosteroid Resistant Asthmatics
CSA	:	Corticosteroid Sensitive Asthmatics
CTLA-4	:	Cytotoxic T Lymphocyte Antigen-4
DC	:	Dendritic Cell
DEPC	:	Diethyl-pyrocarbonate
Der p	:	*Dermatophagoides pteronissinus*
Der f	:	*Dermatophagoides farinae*
Der m	:	*Dermatophagoides microceras*
dNTPs	:	deoxynucleoside triphosphates
Eur m	:	*Euroglyphus maynei*
EAR	:	Early Allergic Response
ECP	:	Eosinophil Cationic Protein
ELISA	:	Enzyme Linked Immunosorbent Assay
EMTU	:	Epithelial Mesenchymal Trophic Unit
EPO	:	Eosinophil Peroxidase
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptor
ET-1	:	Endothelin-1
FcεR I	:	High Affinity IgE Receptors
FcεR II	:	Low Affinity IgE Receptor

FEV_1	:	Forced Expiratory Volume (in one second)
GCM	:	Goblet cell metaplasia
GINA	:	Global Initiative for Asthma
GM-CSF	:	Granulocyte Macrophage-Colony Stimulating Factor
Gro α	:	Growth related oncogene alpha
HETE	:	Hydroxyeicosatetranoic acid
HLA-DR	:	Human Leukocyte Antigen DR
HB-EGF	:	Heparin Binding-Epidermal Growth Factor
ICAM-1	:	Intercellular Adhesion Molecule-1
ICOS	:	Inducible co-stimulator signalling
IFN	:	Interferon
Ig	:	Immunoglobulin
IGF-1	:	Insulin derived Growth Factor
IL	:	Interleukin
ISH	:	*In situ* hybridisation
KGF	:	Keratinocyte Growth Factor
LAR	:	Late Allergic Response
LTC_4	:	Cysteinyl Leukotriene C_4
MBP	:	Major Basic Protein
MCP-1	:	Monocyte Chemotactic Peptide-1
MC_{CT}	:	Chymase positive Tryptase positive Mast Cell
MC_T	:	Mast Cell tryptase only phenotype
MHC	:	Major Histocompatibility Complex
MIP-1α	:	Monocyte Inflammatory Protein-1α
MMP-9	:	Matrix-Metalloproteinase-9
mRNA:		messenger Ribonucleotide
NF-κB	:	Nuclear transcription Factor kappa-B
NGF	:	Nerve Growth Factor
PAF	:	Platelet Activating Factor
PAR	:	Protease Activated Receptor
PBEC	:	Primary Bronchial Epithelial Cell
PBMC	:	Peripheral Blood Mononuclear Cell
PBS	:	Phosphate Buffered Saline

PCNA	:	Proliferating Cell Nuclear Antigen
PCR	:	Polymerase Chain Reaction
PDGF	:	Platelet derived Growth Factor
PGE_2	:	Prostaglandin E_2
PEFR	:	Peak Expiratory Flow Rate
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse Transcription-Polymerase Chain Reaction
RANTES	:	Regulated upon Activation Normal T cell Expressed and Secreted
SMAD	:	Sma- and Mad-related proteins
SQ	:	Standardised quality
STAT-1	:	Signal Transduction and activator of transcription-1
TACE	:	Tumour Necrosis Factor-α converting enzyme
TBS	:	Tris buffered saline
TCR	:	T Cell Receptor
TGF -α,β1,β2	:	Transforming Growth Factors -α,β1,β2
Th	:	T helper
TIMP	:	Tissue inhibitor of metalloproteinases
TNF-α	:	Tumour Necrosis Factor-α
TRAF	:	TNF-receptor associated family
TxB_2	:	Thromboxane B_2
UHQ	:	Ultra high quality
VCAM-1	:	Vascular Cell Adhesion Molecule-1
WHO	:	World Health Organisation

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PUBLICATIONS

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The research work in this thesis was presented at the following National and International meetings:

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1. Leckie MJ, ten Brinke A, Lordan J, Khan J, Diamant Z, Walls CM, Cowley H, Hansel TT, Djukanović R, Sterk PJ, Holgate ST, Barnes P. SB240563: A Humanised Anti-IL-5 Monoclonal Antibody. Initial Single Dose Safety and Activity in Patients with Asthma. Am J Resp Crit Care Med 1999; 159 (3): A624. (Study later published in manuscript form in *The Lancet* 2000; 356: 2144-2148).
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1. Recipient of Travel fellowship to attend the American Academy of Allergy Asthma & Airway Inflammation conference, 2000.
2. Recipient of UCB Institute of Allergy Travel Fellowship to attend European Respiratory Society meeting 2000.
3. Recipient of 2nd Prize Poster presentation, Postgraduate Conference, University of Southampton 2000.
4. Recipient of British Society of Allergy & Clinical Immunology/ Schering Plough Travel Fellowship to attend AAAAI meeting 2001.
5. Achievement of certificate of completion of specialist training in respiratory and general internal medicine December 2002.
6. Gojo-Ryu Nedan 2002.

CHAPTER ONE

Introduction

1.1 Introduction

Atopic asthma is characterised by widespread, variable and reversible airflow obstruction, which is either spontaneous or pharmacologically induced. The underlying patho-physiological feature of asthma is increased airway responsiveness (BHR), which develops on a background of diffuse bronchial inflammation. The prevalence and severity of asthma and other allergic diseases are increasing worldwide, despite improved treatment, which has resulted from a more comprehensive understanding of its pathogenesis [1]. In most countries asthma affects between 4 and 8% of the population, with a trend towards an increase in morbidity as reflected by an increase in asthma-related symptoms, medication usage, and a rise in hospital admissions for asthma [2, 3 4]. The reasons for this are unclear, but environmental factors such as indoor and outdoor air pollution and changes in lifestyle are considered to be amongst the contributing factors. The cost implications of treating asthma are substantial, with over half being spent on hospital care, and 80% of the costs attributable to the 20% of patients with more severe disease [5, 6].

1.2 Pathological features of asthma

Asthma is characterised by increased numbers of airway mucosal and submucosal inflammatory cells, including eosinophils, mast cells and T lymphocytes [7-9]. Other prominent features of asthma include variable damage to the bronchial epithelium and hypertrophy and hyperplasia of airway smooth muscle, basement membrane thickening, mucosal oedema and excessive secretion of mucus, all of which contribute to airway narrowing [10].

In severe exacerbations both large and small airways show gross damage and shedding of the epithelium, and the airways may become occluded by inspissated mucus and cellular debris which form tenacious plugs. There have been differences noted in the type of inflammation depending on the time-course of asthma exacerbation, with infiltration by eosinophils being noted in slow-onset fatal asthma, and an excess of neutrophils being the prominent feature of sudden acute asthma deaths and chronic severe asthma [11].

1.3 Early life origins of asthma

Family aggregation studies support an important role for genetic factors in the development of atopy and asthma. Asthma is a complex genetic disorder, with studies suggesting a polygenic inheritence pattern, which needs to interact with environmental factors in order to express the full clinical and pathophysiological phenotype [12]. There is linkage of

atopy and BHR to chromosome $5q_{31-33}$, which contains the genes for many cytokines considered to play an important role in asthma (Interleukin (IL)-3, IL-4, IL-5, IL-9, IL-13, and GM-CSF) [13].

It is likely that other factors in early life such as childhood infections, *in utero* and postnatal allergen exposure, dietary factors, and exposure to indoor and outdoor pollution may be involved in the subsequent development of the atopic phenotype. It is clear that even in early life, a Th2 associated cytokine immune response is established, which may persist in predisposed individuals with the later development of atopy and asthma in susceptible offspring of atopic mothers [14].

A recent bronchial biopsy study has noted eosinophilic airway infiltration, epithelial cell damage, and increased deposition of collagen in the basal lamina of children as young as two years of age up to four years prior to the onset of asthma [15]. This suggests that the airway features of asthma and the characteristic features of airway 'remodelling' are well established in early life, even prior to the development of symptoms of clinical asthma.

1.4 Asthma severity

The severity of asthma can vary considerably among affected individuals, from mild intermittent symptoms of chest tightness, cough, or wheeze, through shortness of breath interfering with activities of daily living, to extremely severe life threatening attacks. The baseline severity of asthma may not correlate with the potential risk of fatal outcome. Even subjects with mild or moderate disease may develop an acute severe or even fatal attack of asthma, as witnessed in the epidemics of acute severe asthma documented in Barcelona [387]. Increased asthma fatalities in the Barcelona region were linked to episodic exposure to soya bean dust originating from the docks of Barcelona, as consignments of soya beans were being unloaded from ships.

A number of criteria have been adopted by the International consensus Report on Asthma Diagnosis and Management (Global Initiative for Asthma guidelines) to define subgroups of asthmatics in terms of disease severity (Table 1.1)[16]. Ideally, the classification of asthma severity should be assessed prior to commencing treatment. The British Thoracic Society has also issued asthma treatment guidelines for the management of asthma, but also includes maintenance asthma treatment requirements in the assessment of disease severity [17].

Table 1.1: Asthma classification of disease severity.

Asthma classification
Mild asthma PEF > 80% predicted FEF 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, and nocturnal asthma symptoms more than twice per month. Daily requirement for inhaled β_2 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.

Due to the subjective assessment of symptoms by individual patients or attending clinicians, the inclusion of objective lung function parameters is particularly useful in defining subgroups of asthma severity.

1.5 Mechanisms of asthma severity

Despite the considerable improvement in the understanding of asthma pathogenesis, the mechanisms that determine disease severity remain poorly understood. The degree of sputum, bronchoalveolar lavage (BAL) and blood eosinophilia and, to a lesser extent, levels of eosinophil cationic protein (ECP) in blood have been found to broadly relate to disease severity. T cell activation and expression of Th2 type cytokines is found in all degrees of asthma but, as discussed above, it is most prominent in severe disease that is poorly controlled by corticosteroids [18]. Physiological indices of asthma severity such as methacholine responsiveness are related to the number and activity of eosinophils and activated $CD4^+$ T cells in BAL from atopic asthmatics [19, 20]. BHR but not symptom scores has been shown to be inversely related to the number of activated eosinophils and mast cells in bronchial biopsies from asthmatics not treated with corticosteroids [21]. In asthmatics treated with inhaled corticosteroids, BHR, but not symptom score or lung function, is inversely related to the number of infiltrating mast cells, activated eosinophils, $CD8^+$, and CD45 RO^+ T cells in bronchial biopsies from these patients [20].

The involvement of neutrophils in the airway inflammatory response of more severe forms of asthma has recently been confirmed by Wenzel and co-workers [22]. This study showed that whilst mucosal eosinophil numbers may or may not be raised, a significantly higher neutrophil count is seen in bronchial and transbronchial biopsies of severe, corticosteroid-dependant asthmatics compared with moderate asthmatics and normal controls [22]. Other studies have also documented increased numbers of neutrophils in induced sputum in severe asthma [23], during exacerbations [24], and in the circulation post allergen challenge [25]. *Post mortem* studies have shown distinct pathological features in cases of sudden onset asthma death, with a paucity of airway eosinophils but marked neutrophilic infiltration. By comparison, cases of slow onset asthma death [11, 24] are characterised by eosinophilic airway infiltration, gross epithelial shedding, airway oedema and airflow obstruction with inspissated mucus plug formation. Some studies have shown that corticosteroids can enhance neutrophil function through increased leukotriene and superoxide production and inhibition of apoptosis

[26]. Lipid-derived and mast cell derived mediators remained elevated in severe asthmatics despite treatment with high dose corticosteroids. It is possible that corticosteroids may exacerbate the neutrophil mediated inflammation in severe asthma, despite a reduction in lymphocyte and eosinophil mediated inflammation [22].

1.6 Asthma and Atopy

Asthma has been closely associated with atopy, the ability to generate an IgE response to environmental allergens which is characterised by elevated circulating allergen specific IgE and positive skin prick responses to allergen testing [27, 28]. Approximately eighty percent of asthmatics are sensitised to specific environmental allergens, such as house dust mites, pollens, and animal danders [29]. Although atopy is the single strongest risk factor for asthma, and increases the risk of asthma ten to twenty fold, only about one fifth of atopic subjects develop clinical asthma [30]. Moreover, in adults, at the severe end of the spectrum, atopy is seen to diminish as a risk factor for asthma. This suggests that other factors must be operating to focus the allergic process upon the airway mucosa.

Recent epidemiological and immuno-pathological studies have increased our understanding of the involvement of allergen sensitisation and IgE-mediated mechanisms in the genesis and persistence of chronic airway inflammation in asthma. Strong epidemiological associations have been shown between asthma and atopy, as assessed by total serum IgE or positive skin-prick tests in childhood [31] and in adults [32]. Data also suggests that local IgE mediated mechanisms may contribute to the chronic airway inflammation of "intrinsic", or non-atopic asthma, where allergen sensitisation has not been identified [33].

Studies have confirmed associations between the level of indoor allergen exposure, including house dust-mite allergen (*Der p* 1) and cat allergen (*Fel d* 1), and the degree of IgE sensitisation, serum IgE levels, and the frequency and severity of asthma symptoms [34-37] and airway hyperresponsiveness [32, 38-40]. Domestic exposure in early life to *Der p* 1 levels of greater than 2 μg per gram of dust has been shown to significantly increase the risk of initial allergen sensitisation and the development of asthma, while exposure to levels over 10 μg per gram of dust increases the risk of developing acute exacerbations of pre-existing asthma [41-43].

Whilst the presence of allergen specific IgE has been considered to be central to allergic responses in human allergic disease, studies using IgE-deficient (IgE^-/IgE^-) mice,

subjected to repeated inhalation of allergen extract of the mold *Aspergillus fumigatus*, have demonstrated that bronchial eosinophilic inflammation and BHR can occur in the absence of IgE [44]. To what extent these observations are relevant to human disease remains to be elucidated. Clinical studies using a humanised monoclonal antibody to IgE have noted a dramatic depletion of IgE from the circulation with the prevention of the early and late phase response to allergen challenge, and have also resulted in clinical improvement in severe allergic asthmatics [45]. These studies suggest that IgE is likely to be contributing to the acute inflammatory response to inhaled allergen in asthma, and also to the ongoing airway inflammation. However, other allergen-driven immune mechanisms may also be operating in parallel to perpetuate the eosinophilic airway inflammation and BHR of chronic asthma.

1.7 House dust-mite allergy and the development of asthma

Although it was known as early as 1920 that house dust causes allergic disease, it was not until 1964 that it became clear that mites of the genus *Dermatophagoides* are the main source of house dust mite allergen. It is now recognised that environmental exposure to allergens plays a major role in the initial development of asthma, and exacerbation of pre-existing asthma. *Dermatophagoides pteronissinus* is one of the most important environmental aero-allergens worldwide [46]. In some series, as many as 60-85 percent of asthmatics have had a positive skin prick test to *Der p*, as compared to 5 to 30 percent of the general population [29].

Exposure to significant levels of HDM in early life, particularly in the first two years of life, increases the risk of development of atopy and asthma in adulthood [37]. Exposure to greater than 2μg HDM / gram of dust increases the risk of sensitization, while exposure to greater than 10μg/gram is associated with a 4.8 fold relative risk of developing atopic asthma by the age of ten years [41]. Sensitization was associated with an odds-ratio of 19.7 of having asthma [47].

A number of sub-species of house dust mite have been shown to have allergenic potential in asthma, including *Dermatophagoides pteronissinus (Der p), Dermatophagoides farinae (Der f), Dermatophagoides microceras (Der m), and Euroglyphus maynei(Eur m).* The allergens of *Der p* and *Der farinae* have eighty to ninety percent homology, so there is unlikely to be any difference in the allergenicity of the homologous proteins from each species.

Application of molecular techniques has proven useful in characterising the individual protein constituents of house dust mite antigen, such as *Der p* 1 - 9 (Table 1.2) [48, 49]. Over 80% of HDM sensitive subjects have *Der p* 1 and *Der p* 2 specific IgE antibodies [48]. *Der p* 1, 3, 4, and 9 allergens have been shown to have enzymatic activity, *Der p* 1 has been shown to be a cysteine-serine proteinase, while *Der p* 3, 4, 6, and Der p 9 are serine proteinases, cleaving the insulin B chain at distinct and unrelated sites. It is suggested that polymorphisms of *Der p* 1 exist, and that the proteolytic activity of the mite allergens may be related to the allergenicity [50].

It is suggested that the proteinase activity of mite allergens may interact with viruses, disturb the IgE–synthesis regulatory network, and promote the deviation of the immune system towards an allergy-related Th2 phenotype. Proteinases are capable of acting as adjuvants for the release of IL-4 and IL-13 from basophils and mast cells. *Der p* 1 has also been shown to cleave membrane CD23 (low affinity IgE receptor) on the surface of B cells to increase the release of soluble CD23 [51]. As membrane bound CD23 acts as a negative feedback mechanism for IgE synthesis, this would promote the synthesis of IgE and augment immune responses.

The *Der p* protein molecules have been shown to have proteolytic properties distinct from their allergenic properties. *In vitro* studies by Wan *et al* have shown that the cysteinyl protease activity of *Der p* 1 is capable of causing damage to and detachment of the bronchial epithelium, by cleavage of the tight junction adhesion protein occludin, thereby facilitating the penetration of allergens to the sub-epithelial area [52]. This increased access to sub-epithelial antigen presenting cells facilitates penetration of allergen to the sub-epithelium, increasing the activation of the inflammatory cascade [49]. It has also been shown that allergens are capable of directly activating the bronchial epithelium by both protease-dependent and independent mechanisms resulting in increased release of cytokines, chemokines, and pro-inflammatory mediators [53, 54].

Allergen group	Enzymatic activity	IgE binding strength	IgE binding of serum (%)	Residues	Molecular Weight (kDa)	Concentration In extracts (μg/mg)
Group 1	Mixed Serine-Cysteinyl protease					
Der p 1		High	50-80	222-223	25	10-100
Group 2						
Der p 2	Epithelial secretion?	High	60	129	14	5-50
Group 3	Serine proteinase					
Der p 3	Trypsin-like molecule	High	16-100	233	30	<1
Group 4			46 adults			
Der p 4	Amylase	?	25 children	19	57	?
Group 5						
Der p 5	?	High	40	132	15	<1
Group 6	Serine proteinase					
Der p 6	Chymotrypsin	Moderate	60	231	25	?
Group 7						
Der p 7	?	High	50	215	26,29,31	<1
Group 8						
Der p 8	Glutathione-S-transferase	?	40	209	26	Low
Group 9	Collagenolytic	High				
Der p 9	Serine proteinase		90		30	?
Group 10		Variable				
Der p 10	Tropomyosin	High	50-95	284	37	Low
Group 11	Paramyosin	High	80	-	92, 98	High
Der p 11						
Group 12	?	?	50	-	14	?
Group 13	Fatty acid binding protein	Usually low	10	-	15	?
Mag 1	?	?	30	-	39	?
Mag 3 (M177)	?	High	70	-	177	0-30
Mag 29	hsp 70	?	10	-	-	?

Table 1.2: Composition and proteinase activity of house dust mite allergens.

- House dust-mite allergens. Thomas WR, Smith W 1998 Allergy; 53: 821-832. Clin Exp Allergy 1999; 29:1583-1587.

1.8 IgE synthesis

Cytokines have been shown to control IgE synthesis by regulating isotype class switching in B cells. This requires the presence of IL-4 or IL-13 [55, 56], and involves gene re-arrangement and splicing to join segments determining antigen specificity (VDJ genes) with segments determining isotype (constant C genes for IgM, IgA, IgE, or IgG). T cell derived cytokines play a critical role in controlling this process (Figure 1.1).

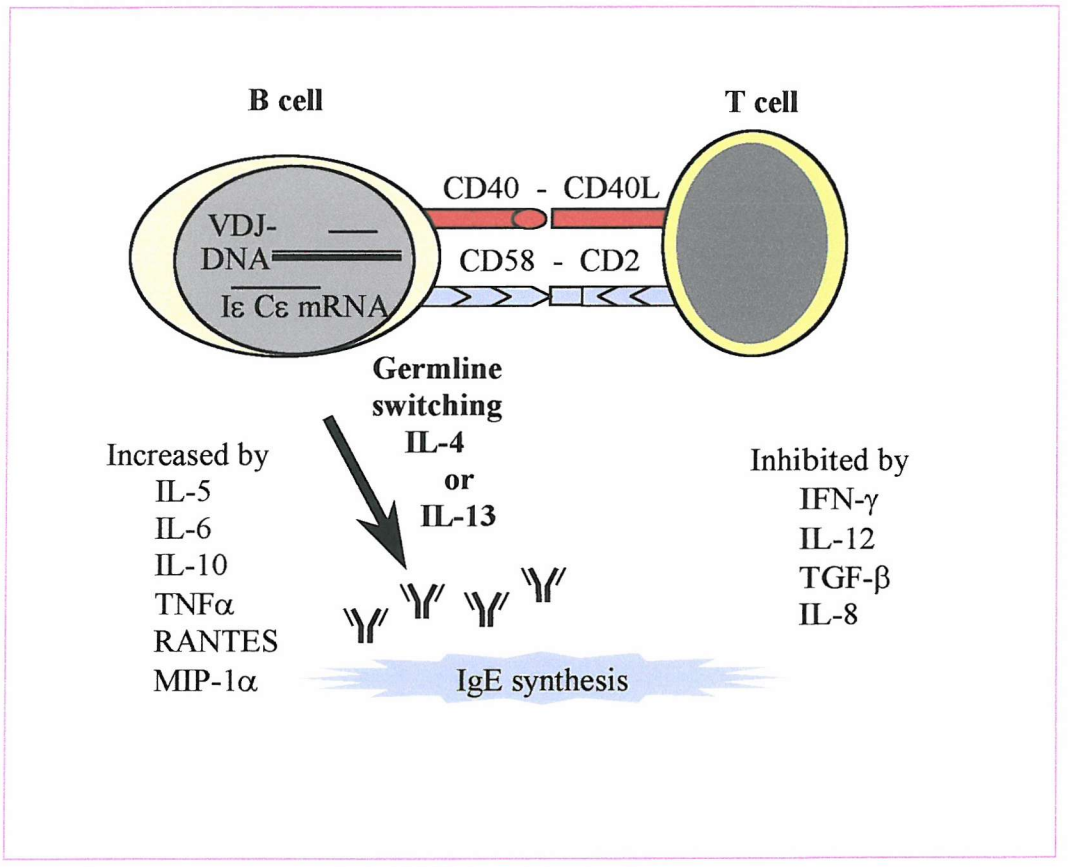
There is compelling evidence for a particularly dominant role for IL-13 in the induction of IgE synthesis [56-59]. Interleukin-13 is produced by activated Th2 type $CD4^+$ and Tc2 type $CD8^+$ T cells, basophils, and mast cells [60-62], and shows similar biological effects to IL-4, including upregulation of CD23 and MHC class II expression on B cells and monocytes, and induction of ε germline transcription and isotype switching to IgE/IgG_4 [63, 64]. Although IL-13 and IL-4 share several functions, only IL-4 can act as a growth factor for T cells [65]. In humans, IgE synthesis also requires IL-6 (B cell differentiation factor), and is augmented by IL-5 (B cell growth factor), IL-10, TNF-α, MIP-1α (Macrophage Inflammatory Protein-1α) and RANTES (Released upon Activation Normal T-cell Expressed and Secreted) [66-70], but is inhibited by IFN-γ, IL-12, Transforming Growth Factor- β (TGF-β), and IL-8 [67, 71-74].

A second signal is necessary for definitive realignment of VDJ and Cε genes for IgE mRNA synthesis, which is delivered by CD40 ligand or CD2 on T-cells interacting with CD40 or CD58 molecules respectively on B-cells [75, 76]. *In vitro* studies show that soluble factors, such as hydrocortisone can also provide this signal [75, 76]. Human type 2 CD4+ T cell clones have been shown to support B-cell synthesis of IgE *in vitro* but other cells may also be involved. Basophils can produce IL-4 [77], express CD40L, and *in vitro* studies confirm isotype switching to IgE synthesis. Mast cells were seen to require exogenous IL-4 production for this process, while eosinophils did not induce class switching. The isolation of allergen-specific $CD4^+$ Th-2 type cells from asthmatic airways, and the demonstration of a Th-2 associated cytokine profile suggests that this process is operative *in vivo*.

The low affinity IgE receptor, FcεRII/ CD23 is thought to have a prominent role in the regulation of the IgE response. CD23 can be cleaved into soluble fragments of variable length (sCD23); these fragments have been suggested to have cytokine like activities [78]. Various roles for the membrane bound CD23 molecule have been found; the B cell CD23 molecule

has been shown to present allergen in the form of an allergen-IgE-CD23 complex to T cells; it has also been suggested that CD23 expression is required as a "third" component in the induction of IgE producing cells and for these cells to become memory cells [79].

Figure 1.1: Role of cytokines and co-stimulatory signalling from T cells in the switching of B cells to the synthesis of IgE.



1.9 Dendritic cells, IgE, and Facilitated antigen presentation in asthma

Antigen presentation of allergens to the immune system is central to the initiation and maintenance of airway inflammation in asthma. On exposure to antigen, T cells require the assistance of 'professional' antigen presenting cells (APC), such as dendritic cells, tissue macrophages, or in the case of secondary antigen presentation by B cells, for optimal activation [80]. The ability of dendritic cells to direct T-cell cytokine polarisation to a Th1 or a Th2 response suggests that two distinct groups of dendritic cells (DC_1 or DC_2) exist, or alternatively, naïve dendritic cells may differentiate in response to the local micro-environment [81]. It has been suggested that PGE_2 released by the epithelium may inhibit IL-12 production by dendritic cells, favoring a Th2 response [82]. Dendritic cells (DC) are believed to be the main APC in the airways, being strategically positioned in the bronchial epithelium for the uptake and presentation of allergen to T cells. Dendritic cell numbers are elevated in the mucosa of asthmatic individuals and are reduced by treatment with inhaled corticosteroids in association with improved disease control [83, 84].

It is increasingly considered that IgE may potentiate allergen-specific responses by binding to high-and low-affinity IgE receptors (FcεRI and FcεRII) on the surface of APCs, thereby facilitating the capture and internalisation of antigen, which can subsequently be processed and presented to T cells [85]. It has been demonstrated that IgE / FcεRI-mediated antigen uptake results in 100 to 1000-fold increased effectiveness of antigen presentation by APCs [86]. A recent bronchial biopsy study has shown that pulmonary dendritic cells, and mast cells stain positively for the α sub-unit of FcεRI, supporting the role of IgE-facilitated antigen presentation in the induction and maintenance of chronic inflammation in asthma [87].

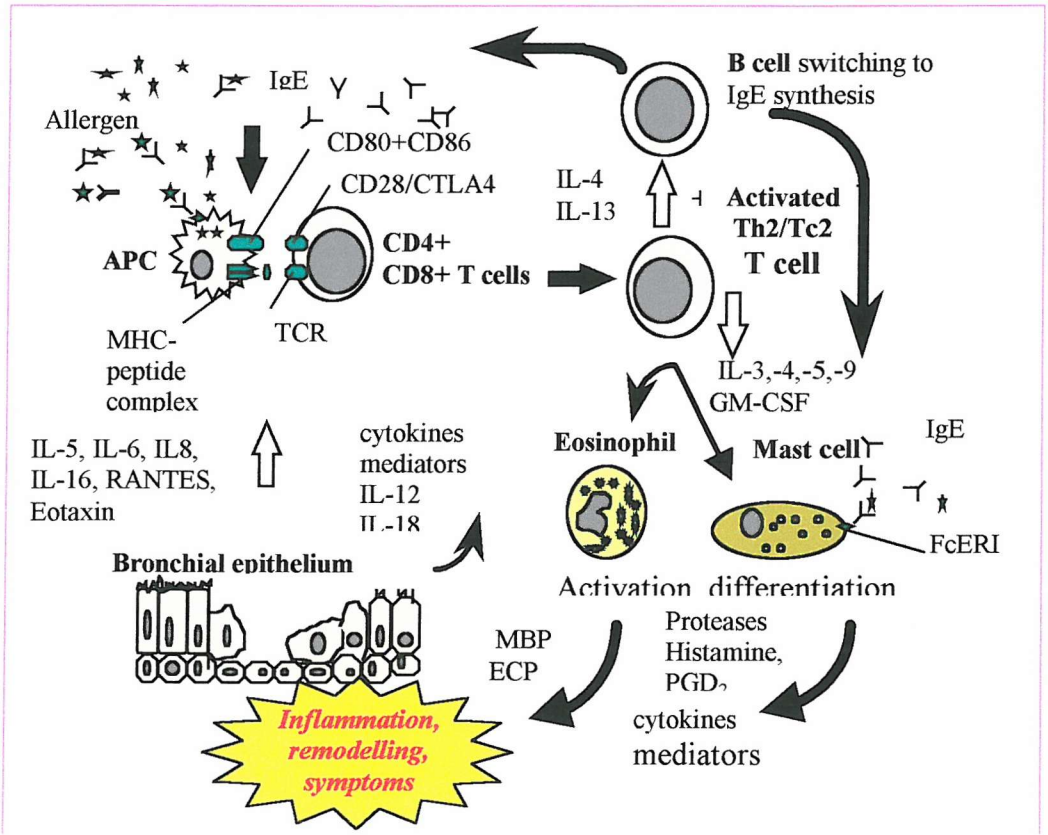
Other cells, such as macrophages or B cells, may also be involved in antigen presentation, but their role in asthma is poorly understood. Increased FcεR1 receptor expression has been confirmed on bronchial tissue macrophages in atopic and non-atopic asthmatics, suggesting an involvement in IgE mediated airway inflammation [88]. Macrophages have the potential to promote inflammation through the production of pro-inflammatory mediators, such as PGE_2, hydrogen peroxide, leukotrienes, and cytokines (IL-1, TNF-α, IL-6, IL-8, IL-10, IL-12, GM-CSF, IFN-γ, and TGF-β) [89], and it has been suggested that chronic activation of airway macrophages may be driving the inflammatory process in non-atopic asthma [90].

However, macrophages recovered by BAL are generally poor antigen presenting cells and have predominantly an immuno-suppressive role in the lungs [91, 92]. Alveolar macrophages (AM) have a reduced capacity to bind with T cells, which may be due to reduced expression of LFA-1 on their cell surface [93], and defective expression of B7-1 (CD80) and B7-2 (CD86) previously shown on AMs would reduce the ability to present antigen efficiently to T cells due to reduced costimulatory signalling via CD28 and CTLA-4 [94]. However, increased expression of CD86 [95] and CD80 has recently been shown on alveolar macrophages from atopic asthmatics compared with normal controls, suggesting that alveolar macrophages in asthma may be capable of antigen presentation.

1.10 Cellular involvement in asthma

Asthma has been traditionally viewed as a chronic inflammatory condition characterised by airway infiltration by activated mast cells and eosinophils, orchestrated by specific Th2 type T lymphocytes [96]. However, neutrophils have recently been implicated in more severe forms of asthma, and it is also increasingly evident that the structural elements of the airways, including the bronchial epithelium, endothelium, fibroblasts and the extracellular matrix, play a dynamic role in the airway inflammation and remodelling of asthma (Fig. 1.2).

Figure 1.2: Cellular involvement in airway inflammation in allergic asthma.



1.11 The Th-1 Th2 paradigm in Allergy and Asthma

$CD4^+$ T cells have been typically classified into functionally distinct Th-1 or Th-2 subsets on the basis of the restricted cytokine profiles expressed by *in vitro* murine T-cell clones. Th-1 cells produce IFN-γ, IL-2, and TNF-β (Tumor necrosis factor–β), promoting the development of cytotoxic T-cells and macrophages leading to cellular immunity, while Th-2 cells are noted to produce IL-4, IL-5, IL-9, IL-10, and IL-13 involved in promoting humoral immune responses and atopy. The situation is not so clear cut for human T-cell subsets, which can secrete a mixed pattern of cytokines.

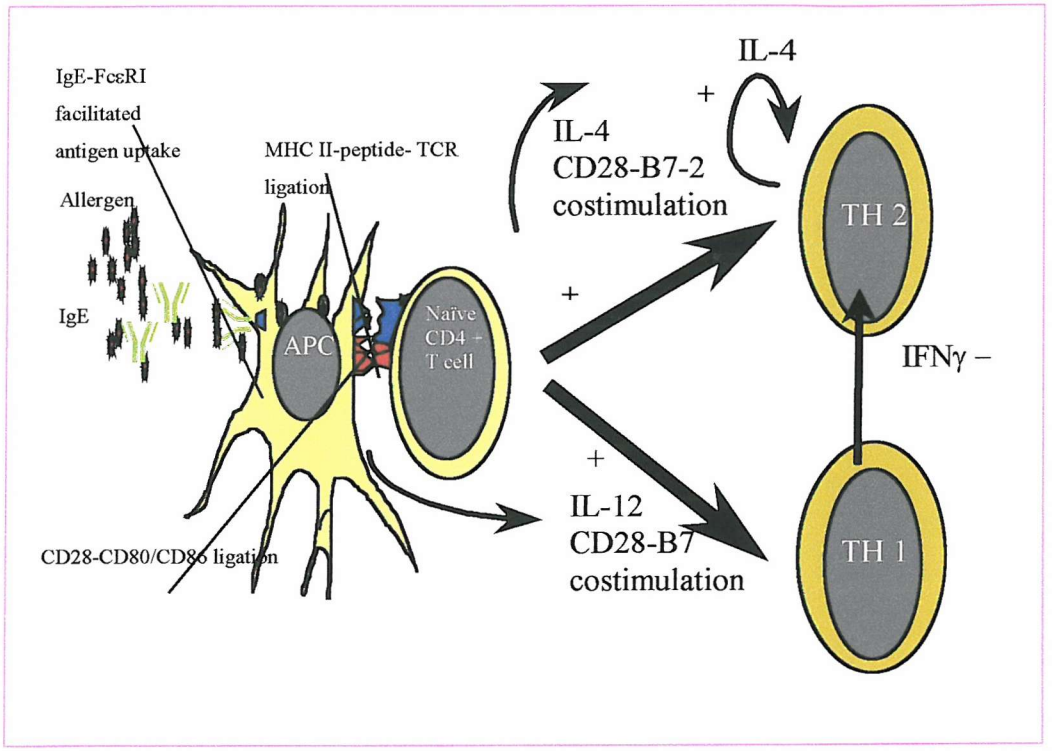
Recent findings show that human Th-2 T-cell clones can also produce IFN-γ, when cultured in the presence of IL-12, suggesting that the pattern of T-cell cytokine production in humans is determined by the local cytokine environment rather than by an irreversible primary differentiation step, as is found in murine T-cell clones.

A number of factors are involved in the development of $CD4^+$ Th2 cells in allergic processes, including the local cytokine environment, the level of antigen, the antigen presenting cell (APC), and the delivery of costimulatory signals from the APC via the CD28-B7-1 (CD80)/B7-2 (CD86) pathway (Figure 1.3). The paracrine release of IL-12 or IL-18 derived from APCs (e.g. dendritic cells), and IFN-γ, promotes the development of Th-1 responses, while the autocrine production of IL-4 by Th2 type T cells promotes the development of Th-2 type responses.

While a large number of studies support the hypothesis that Th2 type responses are involved in the pathogenesis of allergic diseases such as atopic asthma, allergic rhinitis and atopic dermatitis, some observations are difficult to rationalise with a simple Th-1 / Th-2 paradigm. Although bronchial biopsies from atopic asthmatics and allergen challenged skin test sites in atopic individuals have been shown to be infiltrated with $CD4^+$ and $CD8^+$ T-cells displaying a Th2 type cytokine repertoire, some studies in asthma and allergic rhinitis have also noted increased expression of the Th-1 cytokine IFN-γ. IFN-γ levels are elevated in serum during acute severe asthma and in BAL fluid of asthmatics after allergen challenge. Clinical trials of IFN-γ treatment to promote immune deviation towards a Th-1 environment have proved of little clinical benefit in the management of asthma. IFN-γ is known to induce activation markers CD69, Human Leukocyte Antigen DR (HLA-DR) and ICAM-1 on eosinophils, and increases their cytotoxicity and viability *in vitro*, which suggests that IFN-γ could contribute to eosinophil activation and may have pro-inflammatory actions in asthma. Although IL-12 promotes Th-1 type responses in primary immune responses, recent findings

show that IL-12 can amplify existing Th-2 responses. IL-10 has also been shown to inhibit pro-inflammatory cytokine production by both Th-1 and Th-2 type effector cells. These studies support the involvement of T-cells distinct from the classical Th-2 polarised subsets in allergic responses, and suggest that further studies of cytokine expression are required to fully understand the role of cytokines in immune regulation.

Figure 1.3: The role of cytokines and CD28-mediated co-stimulation in the differentiation of Th1 and Th2 type T lymphocytes.



1.12 The orchestrating role of T lymphocytes in airway inflammation

There is considerable evidence to support the view that the asthmatic airway inflammation is driven by the persistence of chronically activated T cells of a memory (CD 45 RO^+) phenotype, that are sensitised to specific allergenic, occupational, or viral antigens, and localise to the airways after appropriate antigen exposure [97], or viral infection. This hypothesis is supported by observations in studies using BAL and bronchial biopsies from asthmatic subjects, where elevated numbers of circulating activated $CD25^+$ $CD4^+$ T cells are seen in acute severe asthma, which are closely correlated with the level of airflow obstruction [98]. Analysis by immuno-histochemistry, *in situ* hybridisation (ISH), and flow cytometry of bronchial biopsies and BAL from symptomatic asthmatics has also confirmed increased numbers of activated T cells [7, 99]. Of particular interest is the correlation shown between activated $CD4^+$ lymphocytes, eosinophils, and cells positive for IL-5 mRNA in BAL, supporting the view that T cells regulate the accumulation and function of eosinophils in the airways in asthma by the release of interleukin-5 [100].

Studies have confirmed that $CD4^+$ (Th2) and cytotoxic $CD8^+$ (Tc) T cells are major sources of Th2 type cytokines in the airways in asthma [101-104]. Co-localisation studies of BAL and bronchial biopsy samples from both atopic and non-atopic asthmatics confirm that T cells are the predominant cells encoding mRNA for IL-3, IL-4, IL-5, IL-10, and GM-CSF. T cells are also considered to play an important role in regulating IgE synthesis in asthma. This is mediated by the increased T cell production of IL-4 and IL-13, and the accentuated expression of the accessory molecule CD40L on activated T cells in asthma, which binds to its ligand on B cells after B cell-T cell physical contact, delivering essential accessory signals required by B cells for clonal expansion, proliferation, and isotype class switching to the synthesis of IgE [8].

Therapeutic studies have demonstrated a role for T cells in asthma airway inflammation. Prednisolone treatment in corticosteroid sensitive asthmatics (CSA) results in improved lung function, reduced AHR, accompanied by a reduction in eosinophil counts, and a reduction in the numbers of $CD4^+$ T cells expressing mRNA encoding IL-3, IL-5 and GM-CSF but not IL-2, IL-4 or IFNγ in BAL [105-107]. However, a minority of subjects develops a severe form of asthma that is refractory to systemic corticosteroid treatment (Corticosteroid Resistant Asthmatic, CRA). Compared to CSAs these subjects have increased numbers of circulating $CD4^+$ $CD25^+$ T cells, and *in vitro* peripheral blood mononuclear cell (PBMC) cultures from CRAs show ongoing mitogen-induced proliferation when cultured in the

presence of dexamethasone [108]. Bronchial biopsies from CRAs also show increased expression of IFN-γ and persisting IL-4 and IL-5 mRNA expression, that is not reduced by steroid treatment [109].

1.13 The role of eosinophils in asthma

Elevated eosinophil counts have consistently been shown in the airways in asthma both at baseline and after segmental allergen challenge, and eosinophil influx has been closely related to the late phase bronchoconstrictor response [9]. Eosinophils have been closely associated with the degree of airway inflammation, epithelial damage, and disease severity in asthma as confirmed by eosinophil counts in the airway lumen, mucosal tissue, and induced sputum [110]. IL-3, IL-5 and GM-CSF release has been shown to prolong airway eosinophil survival by inhibiting apoptosis and to prime eosinophils for mediator release [111].

A recent study in atopic asthma has confirmed increased expression of high affinity IgE receptors (FcεR1) on eosinophils (80-91%) in BAL 24 hours after segmental allergen challenge compared to baseline (4%) [112], supporting the view that eosinophils may be involved in allergen presentation to T cells [113], and that cross-linkage of FcεRI receptors may result in eosinophil degranulation, and contribute to the bronchial epithelial damage characteristic of asthma.

A number of pro-inflammatory proteins (major basic protein, MBP; eosinophil cationic protein, ECP; eosinophil-derived neurotoxin; and eosinophil peroxidase, EPO) and lipid products (cysteinyl leukotrienes, LTC_4, LTD_4, LTE_4; prostaglandins, PGE_2; Thromboxane TxB_2; 15-HETE; and platelet activating factor, PAF) released by degranulation of activated eosinophils have been shown to promote smooth muscle contraction, tissue oedema, mucus secretion, and damage the epithelial integrity by disruption of epithelial desmosomes and tight junctions [114]. Eosinophils have also been shown to synthesise a number of pro-inflammatory cytokines including IL-1, IL-3, IL-4, IL-5, IL-6, IL-16, TNF-α, GM-CSF, IFN-γ, and transforming growth factors (TGF-α and TGF-β) involved in allergic inflammation and the remodelling processes in asthma [115].

The release of interleukin-5 and eotaxin in the airways in asthma has been closely associated with the bronchial infiltration and accumulation of eosinophils [116], by promoting the initial development of eosinophils from bone marrow progenitor cells that express $CD34^+$ and IL-5 receptor-α [100, 117-119]. IL-5 is highly effective at releasing eosinophils and their

precursors from the bone marrow. However, a recent study by Robinson *et al* has shown increased numbers of $CD34^+$/ IL-5 receptor-α mRNA positive cells in the bronchial tissue of asthmatic compared to non-asthmatic patients, suggesting that eosinophil differentiation may also occur *in situ* in the bronchial mucosa in asthma in response to local IL-5 production [120]. The administration of a single intravenous injection of a humanised blocking monoclonal anti-IL-5 antibody (SKB-240563) to allergic asthmatics resulted in the suppression of the circulating and sputum eosinophilia before and after allergen exposure [121]. Similar responses were also observed after the administration of recombinant IL-12 to asthmatic subjects. However, neither treatment resulted in a reduction of the early or late bronchoconstrictor response after allergen inhalation, improvements in BHR, or symptomatic benefit. It is possible that the numbers of eosinophils resident in the airway mucosa were not reduced by this therapeutic intervention, perhaps as a consequence of the effects of cytokines (e.g. GM-CSF) released by the airway epithelium and underlying myofibroblasts, which protects against programmed cell death or apoptosis of activated eosinophils in the airway tissue. However, this lack of efficacy of anti-IL-5 and rIL-12 does question the central role attributed to the eosinophil in the airway inflammation of asthma.

1.14 Mast cells

In normal and asthmatic subjects, the airways have been shown to contain numerous mast cells, which are originated from $CD34^+$ precursor cells, and are predominantly of the tryptase only phenotype (MC_T), as distinct from chymase positive tryptase positive mast cells (MC_{CT}) found in other tissues [122]. The polarisation of airway mast cells to the MC_T type is promoted by the presence of mast cell growth factors derived from infiltrating Th2 type lymphocytes (interleukin–3, IL-4, IL-6, and IL-9), and stem cell factor released by the bronchial epithelium and adjacent fibroblast/ myofibroblast layer [123]. Although some investigators have not noted any difference in mast cell numbers in endobronchial biopsies [122, 124] or BAL fluid [125] between asthmatics and normal subjects, other studies have reported increased numbers in BAL fluid of stable asthmatics [126], and endobronchial biopsy evidence of a further rise in mast cell numbers in parallel with the extent of the late allergic response to allergen challenge [127, 128].

Consistent with the hypothesis of increased activation of mast cells in asthma is the observation that mast cell mediator levels such as histamine, tryptase, and leukotrienes are increased in asthmatics after allergen challenge [129].

Mast cells have long been viewed as having a pivotal role in the development of the early allergic response (EAR) in asthma via the allergen-specific activation of IgE-bound FcεR1 receptors on the cell surface [130]. Receptor aggregation and signal transduction leads to the release of an array of pre-formed and newly synthesised pro-inflammatory mediators (histamine, heparin, tryptase; prostaglandins, PGD_2 and PGE_2; leukotriene C_4, LTC_4; and thromboxane A_2, TxA_2) with spasmogenic and vaso-active properties [131]. In asthma, a proportion of mast cells show ultra-structural evidence of degranulation, which is not found in normal subjects, and they become localised to the bronchial epithelium after allergen challenge, where they are more likely to encounter inhaled allergens or be activated by other noxious stimuli [132]. Flint *et al* showed an increased number of mast cells containing histamine in BAL fluid of asthmatics compared to normal subjects, with an inverse relationship between FEV_1, mast cell numbers, and the levels of histamine [132]. Increased levels of histamine have been found in the BAL fluid of subjects with active asthma, accompanied by increased levels of other mast cell derived products including PGD_2, LTC_4, and the protease, tryptase, but increased numbers of airway MC_T cells have been particularly associated with more severe forms of asthma. The mast cell has been confirmed as an important source of cytokine relevant to airway inflammation in asthma, (IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-16, RANTES, and tumor necrosis factor, TNF-α), both stored pre-formed in secretory granules, or newly generated by transcriptional mechanisms in response to cross-linkage of FcεRI receptors and other stimuli [133-136]. This contributes to the development of the EAR and the late allergic response (LAR) after allergen inhalation.

Increased numbers of $Fc\varepsilon R1^+$ cells have been shown in atopic and non-atopic asthmatics compared with control subjects, and are predominantly co-localised to mast cells, macrophages, and eosinophils [88]. The increased numbers of mast cells in bronchial biopsies and elevated mediator release in BAL from atopic asthmatics have been shown to correlate inversely with FEV_1 and AHR, respectively [96, 137].

Although primarily viewed as an effector cell, mast cells are also involved in regulating allergic responses. In allergic responses, mast cells are noted to express increased levels of FcεR1, CD40L, and produce IL-4 and IL-13, and can directly control the synthesis

of IgE by B cells [138]. *In vitro* studies have previously shown that mast cells can be induced to express CD40L and promote IgE synthesis by interaction with B cells in the presence of exogenous IL-4. Mast cells can also modulate T cell activation and Th2 type differentiation by cytokine release and the ability to present antigen to T cells in a MHC-II and co-stimulatory dependent fashion [139]. Mast cell proteases such as tryptase can activate matrix metallo-proteases that stimulate the growth of fibroblasts and smooth muscle cells, supporting the view that mast cells may be involved in the airway remodelling of asthma [140].

1.15 Basophils

Until recently, it has been difficult to distinguish between mast cells and basophils in tissues, as both cells have meta-chromatic granules and stain positively for FcεRI receptors. Similar to mast cells, airway and circulating basophils are derived from $CD34^+$ precursors. Upon activation by IgE-dependent or IgE-independent mechanisms, basophils release a number of potent mediators, including histamine, leukotrienes, proteoglycans, and cytokines including IL-4 and IL-13 [61, 141-144]. *In vitro* studies have confirmed the release of significant amounts of IL-4 early after allergen stimulation, and IL-13 release at a later stage, which may play a role in promoting the differentiation of $CD4^+$ T cells to a Th2 phenotype and the synthesis of IgE [61, 142].

Earlier studies have applied morphological criteria including cell surface expression of FcεRI receptors, the presence of metachromatic granules staining negatively for trypsin, and multi-lobulated nuclei to identify basophils in tissues. Using these indirect criteria, increased numbers of basophils have been described in the sputum of asthmatics during exacerbations. Guo *et al* also reported a 20 to 200 fold increase in the numbers of IgE-bearing, histamine containing cells in BAL fluid after sub-segmental allergen challenge with ragweed antigen in sensitised allergic asthmatics, of which 95% were considered to be basophils [145].

McEuen and colleagues have recently developed a monoclonal antibody (BB1) that stains a granule product called basogranulin (Molecular Weight $> 1 \times 10^6$) specific to basophils [146], and have applied this antibody to assess the role of basophils in the allergic airway responses of asthma. They have confirmed a trend of increased numbers of basophils in bronchial biopsies of atopic asthmatics compared to normal controls, atopic non-asthmatics, or indeed non-atopic asthmatics subjects. They noted a further increase in the

numbers of BB1 positive basophils at 24 hours post allergen challenge, co-incident with the late allergic response, with a proportion of these cells showing evidence of degranulation. However, the influx of basophils to the airways was significantly lower than the numbers of eosinophils recruited, suggesting that basophils may contribute to the development of the LAR.

1.16 Bronchial Epithelium

The bronchial epithelium has long been considered as a physical barrier between the external environment and the internal mileau of the lung parenchyma, and maintaining its integrity as an important component of airway defence. Epithelial damage in the airways of asthmatics was initially identified in post-mortem samples obtained from patients who died with severe asthma [147]. However, with the advent of fibreoptic bronchoscopy as a research tool, it was recognised that these changes of epithelial damage were also present in bronchial biopsy samples obtained from patients with mild asthma [114]. Of particular interest, a recent study by Pohunek *et al* has examined bronchial biopsies from children as young as two years of age. As early as four years prior to the onset of symptoms of asthma, the biopsies showed changes consistent with epithelial damage and airway remodelling with characteristic thickening of the *lamina reticularis* in the airways so typical of asthma [15]. Epithelial shedding has also been confirmed by the presence of epithelial cell clumps (Creola bodies) in the induced sputum of asthmatic subjects [148, 149]. There is a characteristic loss of differentiated ciliated and secretory cells from the epithelium, with exposure of the underlying basal cells that are more firmly attached to the underlying *lamina reticularis*. Although it has been suggested that this epithelial 'damage' is an artefact of biopsy [150], the increased expression of CD44 in areas of damage provides evidence that this has occurred prior to biopsy [151], as *in vitro* CD44 is expressed when epithelial cells lose cell-cell contact [152]. Increased expression of the epidermal growth factor receptor (EGFR, c-erbB1) is also obvious in areas of epithelial damage in the asthmatic bronchial epithelium [153, 154]. However, despite the increased numbers of damaged and repairing cells in the epithelium, there is no gross ultrastructural defect, with cells in undamaged areas forming normal cell-to-cell contact [155]. *In vitro* studies with epithelial cell cultures have confirmed the remarkable ability of the epithelium to repair itself [156]. It has been suggested that the bronchial epithelium is more

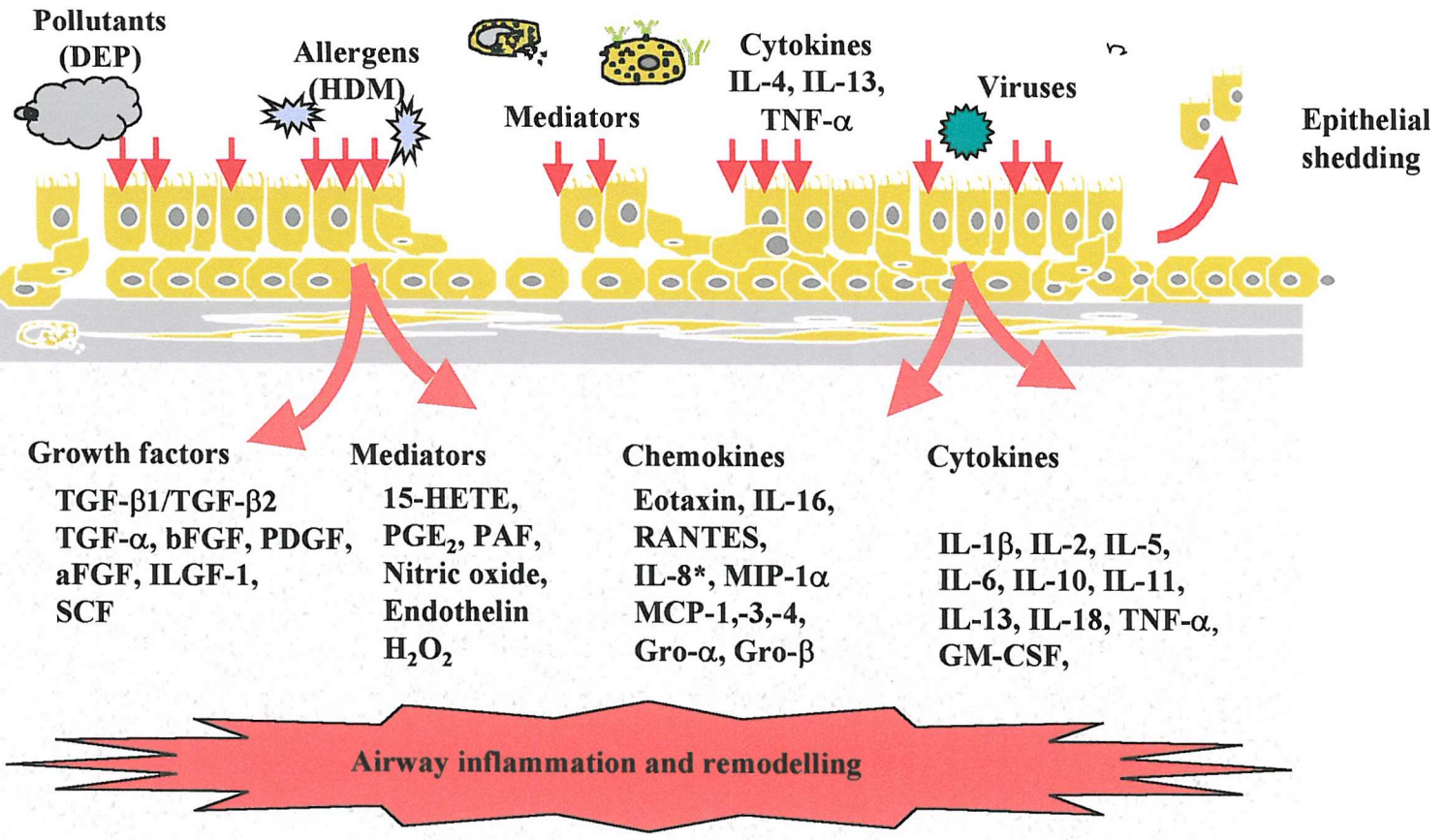
susceptible to environmental stress, or may have an altered ability to repair itself in response to injury [157].

1.17 Mediator release by the bronchial epithelium

Apart from epithelial cell damage, the stressed phenotype of the bronchial epithelium in asthma is reflected by the increased expression of heat shock protein 70 [158], EGFR (Epidermal Growth Factor Receptor) [153], and the transcription factors, Nuclear Factor kappa-B (NF-κB), activator protein (AP)-1 [159], and STAT-1 [160].

In vitro studies have shown that the bronchial epithelium can be activated by a number of stimuli including the enzymatic effects of allergen [54], proteases [161], oxidants, environmental pollutants [162, 163], viruses [164], bacteria [165], and physical distortion [166] (Figure 1.4). The activation of epithelial cells in response to these stimuli is mediated by specific protease activated receptors (PARs) [167], or by inducing a stress response with the activation of NF-κB and AP-1 [168, 169]. The 'stressed' phenotype of the bronchial epithelium in asthma is reflected by functional change with increased release of a number of autocoid mediators, growth factors, cytokines, and chemokines (Figure 1.4) [170, 171]. These include prostaglandin E_2 (PGE_2), 15-HETE, nitric oxide, endothelin-1, fibronectin, α and β-defensins, reactive oxygen (O^-), basic-Fibroblast Growth Factor (b-FGF), Transforming Growth Factors (TGF)-α, -β_1 and -β_2, Insulin like Growth Factor (IGF)-1, Platelet derived Growth Factor (PDGF), stem cell factor, Tumour Necrosis Factor (TNF)-α, IL-1β, IL-2, IL-6, oncostatin-M, IL-10, IL-11, IL-16, IL-18, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), IL-5, IL-8, Gro-α, Macrophage Chemotactic Factor (MCP)-1, RANTES, eotaxin-1, and eotaxin-2 [172]. Primary cultures of bronchial epithelial cells of atopic asthmatic subjects established from bronchial biopsies have been reported to show a higher basal release of IL-8, GM-CSF, and RANTES, with increased sensitivity to diesel exhaust particles and increased mediator release compared to non-atopic non-asthmatic subjects [173, 174]. The fact that these changes persist in passaged cells suggests an altered set point for mediator release in the epithelium of asthmatics.

Figure 1.4 : The bronchial epithelium as a source of cytokines, chemokines and mediators in asthma.



1.18 Role of EGFR in Epithelial repair

EGFR plays a central role as a regulator of epithelial function in response to internal and external stimuli [175], and can be activated independently of ligands by a number of pathways, including G-protein coupled 7-transmembrane receptors (eg Protease Activated Receptors; PARs), endothelin-1 (ET-1), bradykinin, by cytokine receptors, and by oxidant stress [157]. In biopsies from normal airways, there is increased expression of EGFR in areas of epithelial damage [153]. *In vitro* studies have shown that mechanical damage or oxidant stress results in increased expression of EGFR on bronchial epithelial cells, and indeed, *in vivo* exposure of subjects to ozone results in increased expression of EGFR in nasal epithelium [157]. The ability of EGF to promote epithelial repair is confirmed by studies showing that the addition of Epidermal Growth Factor (EGF) or heparin binding-epidermal growth factor (HB-EGF) to scrape wounded epithelial cell monolayers results in enhanced restitution of the epithelial barrier. This process can also be inhibited by the EGFR-specific tyrosine kinase inhibitor, tyrphostin AG1478 [153]. In subjects with asthma, there is a marked disease-related increased expression of EGFR, not only in areas of damage but also in morphologically intact areas, and this is seen to closely correlate with the degree of thickening of the sub-epithelial *lamina reticularis* [153]. This suggests that either the extent of epithelial damage is more widespread in asthma, or alternatively that there is a failure to down-regulate EGFR expression in asthma, such that the epithelium is held in a repair phenotype.

In epithelial diseases characterised by increased expression of EGFR, such as psoriasis, the increased expression of EGFR is associated with increased epithelial cell proliferation [176]. However, in the bronchial epithelium of asthmatics, the expression of proliferating cell nuclear antigen (PCNA) in basal epithelial cells is low despite obvious evidence of epithelial damage [177]. This is also associated with increased expression of the cell cycle inhibitor $p21^{waf}$ in the epithelium of asthmatics, which is related to disease severity [178]. A key candidate for the suppression of the proliferative response in asthma is transforming growth factor-beta (TGF-β). Studies have shown increased levels of TGF-β in the BAL of asthmatic subjects, which are related to the degree of sub-epithelial fibrosis [179]. TGF-β has also been shown to increase the expression of $p21^{waf}$ on primary epithelial cell cultures. The signalling pathways of EGFR and TGF-β converge on the Sma- and Mad-related (SMAD) proteins to determine the balance between cell proliferation and cell cycle arrest [180].

This is reminiscent of the regulation of branching morphogenesis during fetal development, which is also thought to involve a similar delicate balance of growth factors, including EGFR ligands and TGF-β. The epithelial mesenchymal trophic unit plays a critical role in regulating lung branching and growth during fetal lung development, by the release of soluble mediators by epithelial cells (TGF-α, IGF-1, b-FGF, Nerve Growth Factor (NGF), TGF-βs, and endothelin-1) and fibroblasts (Keratinocyte Growth Factor (KGF); acidic Fibroblast Growth Factor (FGF), amphiregulin, PDGF, and TGF-βs). These act to direct the deposition of extracellular matrix, which restricts epithelial growth at clefts, and directs the branching of the airways [181].

1.19 Features of airway re-modelling in asthma

A characteristic feature of asthma is the increased deposition of collagens type I, III, V, fibronectin, and tenascin in the *lamina reticularis* [182]. As noted earlier, these changes have been confirmed in a retrospective bronchial biopsy study in children of ages ranging from two to eleven years old, up to four years prior to the diagnosis of asthma. This thickening of the sub-epithelial basement membrane is a pathological feature of asthma, irrespective of the aetiology, either occupational [183], intrinsic asthma [33] or asthma superimposed on COPD [184]. In atopic non-asthmatic subjects, the degree of sub-epithelial basement membrane thickening is intermediate between that found in asthmatics and non-atopic non-asthmatics [185].

As early as 1990, Roche *et al* described an increased number of mesenchymal cells in the sub-epithelial basement membrane, with the characteristic features of myofibroblasts, which were closely related to the degree of thickening of the *lamina reticularis* [186]. These cells correspond to the attenuated fibroblast sheath described by Evans *et al* [187], that lies adjacent and beneath the epithelial layer to form an integrated unit, known as the epithelial mesenchymal trophic unit [188], capable of responding to endogenous and exogenous stimuli. The close spatial relationship between the epithelium and attenuated fibroblast sheath allows bi-directional signalling via soluble mediators and the release of neuropeptides [189]. The sophisticated nature of the sub-epithelial basement membrane is re-inforced by the presence of discrete pores at regular intervals, which may act as conduits for the migration of inflammatory cells into the epithelial layer [190].

1.20 Re-activation of the epithelial mesenchymal trophic unit in asthma

The extensive deposition of extra-cellular matrix and collagen in asthma is strong evidence for re-activation of the epithelial mesenchymal trophic unit in asthma. The increased production of TGF-β in the airways in asthma [179], the lack of epithelial proliferation in areas of damage [177], and the decrease in MMP/TIMP ratio in asthmatic airways [191, 192] leads to an enhanced deposition of matrix. The role of growth factors in the remodelling process has recently been investigated by Zhang *et al* using an *in vitro* co-culture model where epithelial cells were grown on a three-dimensional collagen gel seeded with fibroblasts [193]. They have shown that chemical or mechanical damage to the epithelium induces an increased proliferation of fibroblasts associated with increased release of growth factors (b-FGF, IGF-1, PDGF, TGF-β, and ET-1) in a temporal pattern with the epithelial repair process [193]. Inhibition of EGFR signalling during epithelial repair *in vitro* has been shown to release a number of growth factors that enhance collagen gene expression when applied to fibroblast cultures [153]. This provides a reciprocal relationship between the process of epithelial repair and the release of pro-fibrogenic growth factors that may promote the remodelling process in asthma. It is suggested that environmental signals may lead to initial activation of the susceptible epithelium that is amplified by the epithelial mesenchymal trophic unit and propagated through the airway wall, and the development of structural changes of remodelling.

1.21 Interaction of Th2 cytokines with the EMTU

Whereas the effects of IL-4 and IL-13 on immune cells are well recognised, studies in animal models have confirmed important effects of Th2 cytokines on the structural cells of the airways, which may contribute significantly to the remodelling process in asthma. IL-4 [194] and IL-13 [195] have been shown to induce goblet cell metaplasia and mucus hypersecretion in the airways of mice, as can EGFR [196, 197]. Of interest, deletion of the STAT-6 gene in mice prevents the allergen-induced development of airway hyperresponsiveness, Th2 differentiation, IgE synthesis, and goblet cell metaplasia [198].

1.22 The bronchial epithelium as a regulator of airway inflammation

The bronchial epithelium is increasingly being shown to play a dynamic role in regulating the inflammation, repair and remodelling process of asthma. It is well established

as an important source of mediators including arachidonic acid products (15-HETE, PGE_2), nitric oxide (NO) endothelins, cytokines (IL-1β, IL-5, IL-6, IL-11, GM-CSF, IL-16, IL-18) [172, 199-203], chemokines (IL-8, Gro-α, MCP-1, MCP-3, RANTES, (Macrophage Inflammatory Protein-1α) MIP-1α, MIP-2, eotaxin-1, and eotaxin-2), and molecules involved in airway inflammation and repair (Figure 1.4).

Activation of the bronchial epithelium can occur via a number of stimuli including mediators such as histamine [204], cytokines [205-207], leukotrienes, and environmental stimuli including allergens, occupational chemicals or pollutants [162, 163], viruses, and shifts in the epithelial lining fluid osmolarity that may occur in exercise induced asthma (Figure 1.4). Inflammatory cells recruited to the airways release a number of cytokines, tissue damaging proteases, and cationic proteins resulting in structural and functional damage to the epithelium. The mast cell derived serine protease tryptase, and matrix-metalloproteinase-9 (MMP-9) derived from eosinophils stimulate IL-8 release and increased ICAM-1 expression by epithelial cells [208]. Allergen derived proteases (*Der p* 1, a cysteinyl protease and *Der p* 6, a serine protease) have also been shown to promote IL-8 release and increase ICAM-1 expression by bronchial epithelial cells, possibly through PAR mechanisms, although this remains to be demonstrated [209]. The protease activity of allergens such as *Der p* are also capable of epithelial cell detachment and cytokine production most likely by interfering with the integrity of intercellular tight junctions on the epithelial barrier by cleavage of the tight junction adhesion protein occludin [52]. The disruption of the epithelial barrier by particular allergens would facilitate the delivery of antigens to APCs resident in the sub-mucosa and accentuate the activation of the immune system [49, 210]. The confirmation of FcεRI and FcεRII receptors on bronchial epithelial cells [211] also suggest that epithelial cells can be activated by IgE mediated mechanisms.

1.23.1 Cytokine networks in asthma

It is increasingly evident that an expanding array of cytokines plays a critical role in orchestrating the airway inflammation in asthma, by promoting the development, recruitment, activation, differentiation and survival of inflammatory cells. Individual cytokines have many overlapping cell regulatory actions, and function through complex cytokine networks. They interact with specific high affinity receptors on target cells, activating linked secondary intracellular cascades that regulate the specific transcription of genes and the ultimate cellular

response. Considerable progress has been made in characterising the cellular sources and actions of the numerous cytokines involved in asthma (Table 1.3).

Although *in situ* hybridisation studies of BAL and bronchial biopsies have confirmed T cells to be a major source of cytokines in asthma, it is clear that other cells including eosinophils, mast cells, bronchial epithelial cells, and the structural elements of the airways contribute to the cytokine milieu [170]. Cytokines derived from T cells and mast cells, such as IL-4, IL-5, and IL-13 play central roles in the pathogenesis of asthma by their ability to promote eosinophil recruitment, activation, terminal differentiation, and survival in the airways, and in the case of IL-4, promote B cell switching to IgE production [212]. IL-4 and IL-13 act in concert with TNF-α to stabilise vascular cell adhesion molecule (VCAM)-1 expression on endothelial cells, increasing the adherence of eosinophils to the endothelium, resulting in enhanced eosinophil recruitment to the airways [102, 213]. IL-3 and GM-CSF contribute to eosinophilic inflammation by increasing eosinophil survival, and mast cell and basophil development. A number of studies have confirmed increased expression of the Th2-type cytokines which promote the allergic response (IL-4 and IL-5), and reduced expression of the Th1-type cytokine IFNγ, in BAL and bronchial biopsies from asthmatics [214, 215]. Although T cells and mast cells are likely to initiate the production of eosinophilotactic cytokines, eosinophils are also capable of enhancing eosinophilic inflammation by producing IL-4, IL-5, GM-CSF and TNF-α [216].

Table 1.3: The cellular source and actions of cytokines in asthma.

Cytokine	Cell source	Molecular weight	Gene	Actions
IL-1(1α, β IL -1 Ra)	Monocytes, macrophages, smooth muscle & endothelium	17 kDa	2q13-21	Activation of T cells and airway epithelial cells. Promotes B-cell proliferation.
IL-2	T-cells Eosinophils	14-16 kDa	4q26-27	Promotes T-cell proliferation and clonal expansion. Eosinophil chemotaxis.
IL-3	T-cells Mast cells Eosinophils	14-28 kDa	5q 23-31	Stimulates development of mast cells and basophils, granulocyte differentiation and activation. Promotes eosinophil survival.
IL-4	T-cells Eosinophils Mast cells Basophils	20 kDa	5q23-31	Promotes T-cell activation and Th2 differentiation, B-cell differentiation, class switching to IgE production. Eosinophil recruitment VCAM-1 upregulation on endothelial cells.
IL-5	T-cells, Eosinophils, bronchial epithelium, mast cells	25-50 kDa	5q 23-31	Promotes growth and differentiation of eosinophil and basophils. Activates and prolongs survival of eosinophils.
IL-6	Macrophages, eosinophils, mast cells and fibroblasts	22-29 kDa	7p21	Activation of haemopoetic stem cells. Differentiation of T-cells and B-cells. Costimulatory factor for immune cells.
IL-8	Macrophages, eosinophils, T-cells, mast cells, endothelial cells, fibroblasts, neutrophils, airway epithelial cells	10 kDa	4q12-13	Neutrophil activation and differentiation. Chemotactic factor for primed eosinophils, basophils, and neutrophils.
IL-9	T-cells	40 kDa	5q31	Enhances mast cell growth. Mucus secretion. Activation of epithelial cells.
IL-10	T-cell B-cells Macrophages Monocytes	20 kDa	1q31-32	Inhibits T-cell proliferation and down-regulates pro-inflammatory cytokine production. Co-factor in mast cell growth and differentiation. Chemotactic for $CD8^+$ T-cells.
IL-12	T-cells, monocytes, macrophages, dendritic cells	70 kDa	5q31	Inhibits Th-2 development and cytokine expression. Supresses IgE production. Promotes Th-1 phenotype and IFNγ production.
IL-13	T-cells Basophils	-	5q31	B-cell proliferation, IgE class switching. Dendritic cell development. Eosinophil accumulation by increased expression of VCAM-1 on endothelial cells. Mucus hypersecretion and subepithelial fibrosis
IL-16	CD8+ T-cells, mast cells, eosinophils and airway epithelium	56kDA (Tetramer)	15q26.1	Activates monocytes and CD4+ T-cells. Recruitment of CD4+ T-cells and eosinophils.
GM-CSF	Macrophages, eosinophils, neutrophils, T-cells, mast cells, epithelial cells	-	5q23-31	Priming of neutrophils and eosinophils. Prolongs survival of eosinophils.
TNFα	Mast cells, T-cells, monocytes, neutrophils, epithelial cells.	26kDa	6	Enhances eosinophil and mast cell cytotoxicity. Chemoattractant for neutrophils and monocytes. Upregulates adhesion molecules.
IFNγ	T-cells NK cells Macrophages Eosinophils	20-25 kDa	12q	Suppression of Th-2 T-cells. Inhibits B-cell differentiation. Upregulates eosinophil activation markers CD69, HLA-DR. Increases ICAM-1 expression on endothelial and epithelial cells.

1.24 Chemokines involved in airway inflammation

Chemokines are a group of structurally related cytokine proteins of low molecular weight (8-10 kDa), expressed by a wide variety of cell types and tissues, that induce activation and directed migration of specific leucocyte subsets to sites of inflammation (Table 1.4) [221]. Chemokines have a number of functions apart from chemotaxis, including immuno-regulation, antiviral activity, and the control of haematopoiesis, angiogenesis, cell growth and metabolism. Chemokines including the eotaxins, monocyte chemotactic peptides (MCP-1,-2,-3), MIP-1α, and RANTES can promote histamine release by an IgE-independant mechanism. RANTES is a potent eosinophil chemoattractant, but also promotes eosinophil activation and release of mediators (eosinophilic cationic protein & superoxide).

A number of studies support the involvement of chemokines in allergic diseases. In sensitized animal models of allergic airway disease, allergen challenge results in increased production of MCP-1, MCP-3, MIP-1α, RANTES, and eotaxin. Pre-treatment with antibodies against MIP-1α, RANTES, and MCP-3 is noted to inhibit the allergen induced airway infiltration by eosinophils and lymphocytes. In patients with allergic rhinitis, MCP-1 levels are increased in nasal secretions during the allergy season, and increased levels of MCP-1, MIP-1α, RANTES and IL-8 are found in nasal fluid after local allergen challenge. Higher epithelial cell expression of MCP-1, but equivalent levels of RANTES are found in bronchial biopsies of asthmatic airways compared with normal subjects, and elevated concentrations of MCP-1, MIP-1α, RANTES and IL-8 are found in BAL fluid obtained from mild asthmatics. Segmental bronchial allergen challenge in asthmatics results in increased release of RANTES and IL-5 in BAL fluid. The addition of inhibiting antibodies for RANTES and MCP-3 is seen to abolish the *in vitro* chemotactic activity of BAL fluid obtained from asthmatics.

Table 1.4: Cellular source, actions, and chemokine receptors of chemokines in allergy and asthma.

CC chemokines	Target cells	Functions	Chemokine receptor	CXC chemokines
MCP-2,-3,-4; MIP-1α, RANTES MCP-3,-4; RANTES, Eotaxin, Eotaxin-1,Eotaxin-2	Eosinophil	Chemotaxis, activation	CCR-1 CCR-3	
MCP-1,-2,-3,-4,-5 RANTES, MCP-1,-2,-3,-4,-5 MIP-1α, -β, RANTES	T-cell	Chemotaxis Proliferation	CCR-1,-CCR-2 CCR-5	 SDF-1, IL-8, IP-10
MIP-1α, RANTES	Mast cell	Chemotaxis Metabolism	CCR-5	
MCP-3,-4, MIP-1α ,RANTES MCP-1,-2,-3,-4,-5 MIP-1α,-β;RANTES	Monocyte	Chemotaxis Activation	CCR-1 CCR-2 CCR-5 CXCR4	 SDF-1
MIP-1α,-β, RANTES	B-cell	Chemotaxis, IgE synthesis, Proliferation	CCR-5 CXCR4	 SDF-1
MIP-1α, I-309	Neutrophil	Chemotaxis Activation	CCR-1,CCR-8 CXCR-1, CXCR-2	IL-8, GCP-2 IL-8, GCP-2, Gro-α,-β,- γ, ENA-78, NAP-2
MCP-1,-2,-3,-4, RANTES, MIP-1α	Basophil	Chemotaxis Activation	CCR-2,-3	-
MCP-1,-2,-3,-4,-5 RANTES, MIP-1α,–β IP-10,	NK cells	Chemotaxis Activation	CCR-2 CCR-5 CXCR-3	-
MCP-3,-4, MIP-1α ,RANTES MCP-1,-2,-3,-4,-5 MCP-3,-4, eotaxin, RANTES MIP-1α,-β, RANTES	Dendritic cell		CCR-1 CCR-2 CCR-3 CCR-5	
I-309, MIP-1α, MCP-1	Smooth muscle cells	Chemotaxis Proliferation		
MIP-1β	Endothelial cell	Chemotaxis Proliferation	CXCR-2	IL-8, Gro, ENA-78, GCP-2, PBP, NAP-2
MIP-1α, HCC-1, MRP-2	Stem cell	Inhibition	CXCR-2	IL-8, Gro-α

1.23.2 Interleukin 16

Interleukin 16 (IL-16) is a newly characterised cytokine that is produced by $CD4^+$ and $CD8^+$ T cells, epithelial cells, mast cells and eosinophils [217-219]. It uses the surface molecule CD4 as its ligand and thus activates cells that are $CD4^+$, namely monocytes and $CD4^+$ T cells [220]. Bronchial biopsy studies using ISH have shown expression of IL-16 mRNA on epithelial cells from asthmatics but not normal or atopic controls. The epithelial and subepithelial IL-16 mRNA expression was significantly associated with airways hyperresponsiveness and $CD4^+$ T cell infiltration in the bronchial mucosa [200]. IL-16 has been detected early in BAL post endobronchial allergen challenge in asthmatics but not in control subjects [218]. This suggests a role for early IL-16 release in the selective recruitment of $CD4^+$ T cells and eosinophils to the inflamed bronchial mucosa in asthma.

1.25 Bronchial Explant models of asthma

The development of a novel bronchial explant culture system has been particularly useful to assess the involvement of particular cytokines in inflammatory responses in asthma. Unlike isolated cell culture systems, this is an integrated cell culture system using bronchial biopsies obtained by fibreoptic bronchoscopy from the lower airways of allergic asthmatics and normal subjects. The explanted bronchial tissue includes structural elements such as the airway epithelium and fibroblasts, and also the inflammatory cells resident in the airways, including resident T cells, mast cells, eosinophils, macrophages, and $CD1a^+$ dendritic cells, which makes observations in this system particularly relevant to asthmatic airway inflammation [222]. Jaffar *et al* has recently shown that the release of IL-5 and IL-13 by bronchial explants from mild allergic asthmatics is increased following *in vitro* exposure to *Der p* allergen [222]. The bronchial explant cultures are particularly useful for assessing the effects of novel therapeutic compounds on cytokine release by bronchial tissue, in particular for compounds not yet approved for use in clinical studies.

1.26 Involvement of CD28-B7 costimulation in airway inflammation in asthma

Recent interest has focussed on the requirement for co-stimulation in T cell-mediated inflammatory diseases such as asthma [223], in particular via the CD28-CD80/CD86 pathway [224]. The activation of naïve T cells in primary immune responses requires at least two distinct signals from APCs. The first signal is provided by the cognate interaction of the T cell

receptor (TCR) with MHC II complexes on APCs, and the second by CD80 (B7-1) or CD86 (B7-2) on APCs interacting with CD28 receptors on T cells (Figure 1.3) [225-227]. Co-stimulation via CD28 results in T cell activation, while ligation of its higher affinity homologue, Cytotoxic T Lymphocyte Antigen (CTLA)-4 suppresses immune responses by competing with CD28 for binding with CD80 or CD86 depriving the T cells of CD-28 mediated activation. There is also evidence to suggest that CTLA-4 may inhibit immune responses at a later stage, by affecting intracellular signal transduction pathways utilised by CD28 or the TCR [228]. Lack of co-stimulatory signalling via CD28 has been shown to result in T cell unresponsiveness or anergy [229]. Recent murine studies suggest a critical role for CD28-B7 co-stimulation in the production of Th2 cytokines, the development of airway hyperresponsiveness, and eosinophilic airway infiltration in asthma [230-233].

CTLA-4Ig is a chimeric fusion protein of the Fc component of human IgG_1 and the extra-cellular component of human CTLA-4, which inhibits signalling via the CD28-B7 pathway, and has been used to study the requirement for CD28-B7 co-stimulation in immune responses [234]. CTLA-4Ig has also been shown to inhibit allergen-induced proliferation and cytokine production by PBMCs obtained from atopic donors [235, 236]. Co-stimulatory signalling is also involved in cytokine production in asthmatic airways. It has recently been reported that allergen stimulation increases the production of IL-5 and IL-13 by explanted bronchial tissue of mild atopic asthmatics [222]. Addition of the fusion protein CTLA-4Ig, simultaneous with the allergen effectively inhibited the allergen-induced release of cytokines, chemokines, and related chemotactic activity supporting an obligatory requirement for CD28-B7 co-stimulation for the release of these pro-inflammatory mediators [237]. Whereas these earlier studies were performed in patients with mild disease, which is easily controlled with minimal medications, the responses in more severe persistent asthma have not been addressed. A significant proportion of asthmatics have persistent symptoms and evidence of ongoing airway inflammation, despite the use of adequate doses of inhaled corticosteroids [22, 238]. I propose to use the bronchial explant culture model to investigate the inflammatory responses in asthmatic subjects with moderate to severe disease.

1.27 Study objectives

1. To assess allergen-induced cytokine release by bronchial explant tissue cultures of moderately severe asthmatics, and to determine the requirement for CD28-B7 costimulation in cytokine release by bronchial tissue in severe asthma, bronchial explant culture studies were performed in subjects with moderately severe asthma.

2. To assess the production of cytokines by peripheral blood mononuclear cells and the requirement for CD28-B7 costimulation by circulating inflammatory cells compared to the bronchial tissue of moderately severe asthma, peripheral blood mononuclear cell cultures have been performed in parallel with the bronchial explant studies performed in moderately severe asthmatics.

3. To assess the effects of *Dermatophagoides pteronissinus* allergen extract or TNF-α on the production of pro-inflammatory cytokines (GM-CSF), chemokines (IL-8, RANTES, IL-16, and Eotaxin) by primary bronchial epithelial cell cultures of allergic asthmatics and normal subjects.

4. To assess the co-operative ability of *Der p* allergen and Th2 cytokines (IL-4 or IL-13) to promote the release of pro-inflammatory cytokines, chemokines, and growth factors by primary bronchial epithelial cell cultures of allergic asthmatics and normal control subjects.

5. To compare the ability of primary bronchial epithelial cells of allergic asthmatics and normal subjects to release growth factors (TGF-β2 and TGF-α) relevant to the remodelling process, so typical of chronic persistent asthma.

6. To assess the ability of corticosteroids to inhibit the production of cytokines, chemokines and growth factors in response to stimulation with *Der p* allergen, IL-4, IL-13, or TNF-α.

7. To assess the expression of receptor subunits for the IL-4 and IL-13 receptors by primary bronchial epithelial cells (including IL-4Rα, common-γ-c, IL-13Rα1, and the recently characterised IL-13Rα2 subunit) using RT-PCR and flow cytometry.

Overall, these studies will contribute to the current understanding of the patho-physiological mechanisms involved in the airway inflammation of asthma, particularly at the severe end of the spectrum. The primary bronchial epithelial cell culture studies will also provide an insight into the dynamic involvement of the bronchial epithelium in this process. In particular, this study will assess the ability of allergens and the Th2 cytokine environment to activate the airway epithelium and contribute to the airway inflammation and remodelling of asthma by the release of cytokines, chemokines and growth factors.

CHAPTER TWO

Materials and Methods

Methods

2.1 Subjects

Non-atopic non-asthmatic control subjects, mild and moderate persistent asthmatics were recruited from the Southampton area. Asthmatic subjects had a physician based diagnosis of asthma, and were characterised according to symptoms, pulmonary function, asthma medication requirements, and in particular, the ongoing requirements for β_2–agonists. Assessment of asthma severity was in accordance with the GINA guidelines on the diagnosis and management of asthma (Table 1.1). All subjects were non-smokers and were free from respiratory tract infections for a minimum of 4 weeks prior to inclusion to the study. Written informed consent was obtained from all volunteers prior to participation, and ethical approval was obtained from the Joint Ethics Committee of Southampton University and General Hospital.

Skin prick testing to a panel of common aero-allergens was performed, including house dust mite extract (*Dermatophagoides pteronissinus*), grass pollen, tree pollen, cat dander, dog dander, candida, aspergillus, as well as negative (saline) and positive (histamine) controls, using a standard lancet. Tests were considered positive if a wheal response of 3 mm greater than the negative control was observed. Subjects with positive responses to *Der p* were selected for inclusion in the study.

Serum IgE levels were measured by standard enzyme linked immuno-sorbent assay (ELISA) by the Regional Immunology Laboratory, Southampton General Hospital, UK.

BHR was assessed by either histamine or methacholine inhalation challenge, and expressed as PC_{20} (the cumulative dose of histamine or methacholine required to produce a fall in Forced Expiratory Volume in 1 second (FEV_1) by 20% from baseline).

2.2 Fibreoptic Bronchoscopy

Bronchoscopy was performed using a fibreoptic bronchoscope (Olympus FB-20D, Tokyo, Japan) in accordance with standard published guidelines (National Heart, Lung, and Blood Institute, 1992). Moderately severe asthmatic subjects stopped inhaled corticosteroids for a minimum of one week prior to bronchoscopy and bronchial biopsy. Subjects fasted for 5 hours prior to bronchoscopy, and were pre-medicated with salbutamol (2.5mg) and Ipratropium Bromide (0.5mg) nebulizer and intravenous atropine (0.6mg). Light sedation was achieved using intravenous midazelam (0-6 mg IV). Local anaesthesia was achieved by applying topical 10% lignocaine spray to the oro-pharynx and 1% lignocaine solution to the

lower airways via the bronchoscope (total maximum lignocaine dose < 300mg). Pulse oximetry was monitored throughout the procedure. Bronchial biopsies (6 to 8) were taken from sub-carinae of the 2nd and 3rd generation segmental bronchi of the right lower lobe using FB15 alligator forceps, and were placed in tissue culture medium for subsequent culture studies, or in ice cold acetone plus inhibitor for processing into glycomethacrylate resin for immuno-histochemistry analysis.

2.3 Primary Bronchial Epithelial Cell Brushings and Cell Culture

The endo-bronchial brush technique has traditionally been used to sample intra-bronchial cytological material for the diagnosis of lung malignancies, but has recently been identified as a method to obtain epithelial cells for the study of airway epithelial cell responses in inflammatory conditions such as atopic asthma. In the present study, this technique was used to assess the contribution of epithelial cells to airway inflammatory responses in mild and moderately severe asthma and normal control subjects (Figure 2.1). Briefly, fibreoptic bronchoscopy was performed as described above using the oral route to avoid contamination with nasal epithelial cells (section 2.2). A standard sterile single-sheathed nylon cytology brush (Olympus BC 9C-26101; Tokyo, Japan) was passed by direct vision via the bronchoscope channel into the lower airways, and six consecutive bronchial brushings were sampled from a three to four centimetre squared area of the lower airway epithelium. The brush material was dispersed in 5 mls sterile phosphate-buffered saline (PBS) after each brushing. 5mls RPMI with 10% fetal calf serum (FCS) was added and the sample was centrifuged at 1000 x g for five minutes to pellet the cell suspension. The cells were seeded onto culture plates in 5mls of serum-free hormonally-supplemented Bronchial Epithelium Growth Medium (BEGM; Clonetics, San Diego, CA) supplemented with 1% Penicillin and Streptomycin. The primary cell cultures were later passaged up to four times. Epithelial cell viability was assessed by the exclusion of trypan blue dye, and was consistently greater than 90%. Epithelial cell purity was assessed by initially performing differential cell counts on cytospins of the cell suspension, and also by immunohistochemical staining of primary epithelial cells cultured on culture chamber slides (Nunc, Labtek I I eight well chamber slides) with specific antibodies for pan-cytokeratin, cytokeratin 13 and cytokeratin 18.

The initial brushings were placed on petri dishes with BEGM plus 1% Penicillin/Streptomycin for 48 hours. A proportion of the epithelial cells adhered to the base of the plastic petri dish, but a significant number were non-adherent with visible ciliary

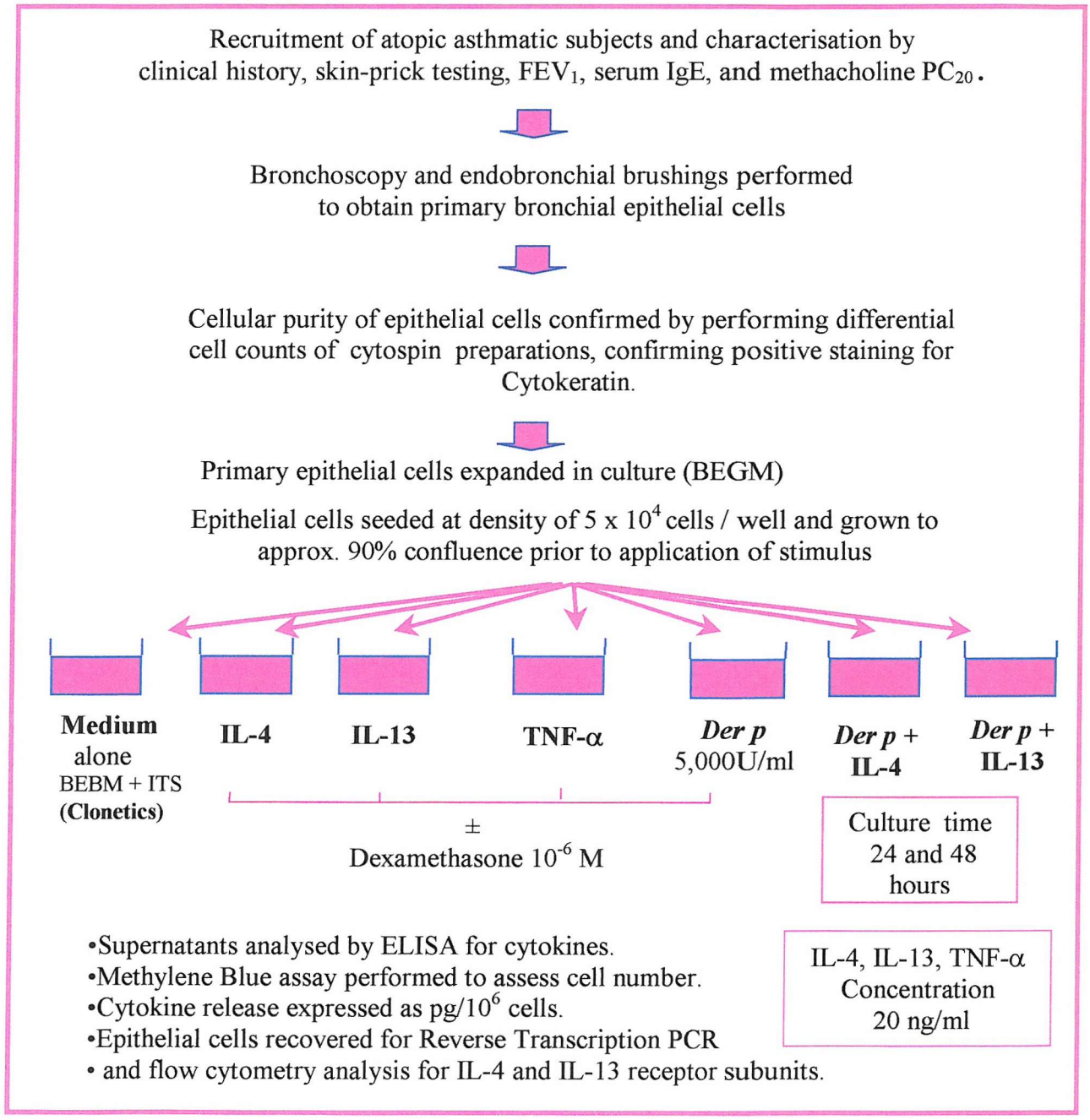
motility activity. The non-adherent cells were removed, centrifuged, and transferred to a second petri dish, and later to a third petri dish which facilitated further cell attachment and expansion of the cultures. By using this technique, sufficient primary epithelial cells were obtained from a single bronchoscopy and brushing. The epithelial cells could be expanded in culture for two to four passages, before becoming quiescent.

The cells that adhered and grew in culture had the phenotype of basal epithelial cells lacking visible cilia or other morphological features of differentiation. The cells were cultured in monolayers until the petri dish was 80-90 % confluent, the culture medium being replaced with fresh culture medium every 48 hours. The adherent cells were detached from the petri dish by the application of trypsin solution, washed with RPMI supplemented with 10-20 % fetal calf serum to neutralise the activity of the trypsin, and centrifuged. Cell counts were performed and viability assessed by trypan blue. The cells were then transferred to culture flasks at a density of 5×10^5 cells per flask, and grown in BEGM medium until ninety percent confluent.

Experiments were performed on epithelial cells at passage two and three. The adherent epithelial cells were detached by the application of trypsin, and were plated on twenty-four well culture plates (NUNC, USA) at a cellular density of 5×10^4 cells, in one millilitre of medium per well. The cells were cultured in BEGM, with the culture medium being changed every 48 hours until 80-100% confluence achieved. The medium was replaced with bronchial epithelial basal medium (BEBM) supplemented with insulin, transferrin, and sodium selenite solution (ITS, culture medium supplement volume added 1:100; Sigma, UK) for 24 hours. The culture medium in each well was then replaced with one millilitre of BEBM plus ITS. Cells were then cultured in either BEBM medium alone, *Der p* allergen extract (Aquagen ALK, Denmark, 2,500 and 5,000 SQ U/ml), interleukin-4 (10, 20, and 40 ng/ml), IL-13 (10, 20, and 40 ng/ml), TNF-α (10, 20, and 40 ng/ml). The allergen dose (Der p 5,000 SQ U/ml, 350 ng/ml) is representative of the estimated focal concentration of mite allergen in the airways[209] and relates to a level previously shown to induce a local inflammatory response (200 ng/ml) by Tonnel et al. [391]. In selected experiments, cells were cultured with a combination of stimuli including *Der p* (5,000 U/ml) plus IL-4 (20 ng/ml); *Der p* (5,000 U/ml) plus IL-13 (20 ng/ml); and *Der p* (5,000 U/ml) plus TNF-α (20 ng/ml) to assess the possibility of synergistic responses with combined stimuli. Stimuli were applied in the presence and absence of dexamethasone (10^{-6}M) to assess the ability of corticosteroids to suppress cytokine release by the epithelial cell cultures in response to the different stimuli.

The concentration of Dexamethasone (10^{-6}M) applied in this study has been considered to be equivalent with the estimated airway epithelial steroid concentrations achieved by the *in vivo* delivery of a 200 μg dose of beclomethasone by metered dose inhaler [392, 398, 399], and be capable of optimum activation of steroid responsive intracellular signalling pathways [393]. Cells were cultured for six, twenty-four, and forty-eight hours respectively at 37°C and 5 percent carbon dioxide. The supernatents were then removed, centrifuged (x g) (6,000 rpm) at 4°C and stored in aliquots for later analysis of cytokines. The adherent epithelial cells were suspended in 300μl of RNAzol and stored at –80°C for later analysis of cytokine mRNA expression by RT-PCR.

Figure 2.1: Study protocol for the recruitment and characterisation of subjects, fibreoptic bronchoscopy, bronchial brushings,and epithelial cell culture.



2.4.1 Methylene Blue Assay Principle

To adjust for any differences of plating efficiency or cellular density of bronchial epithelial cells cultures obtained from different individual subjects, a number of wells in each experiment were reserved for analysis by methylene blue, which gives an estimate of the amount of cellular protein in the cells cultured [394]. To determine if the application of allergen or cytokines affected epithelial cell detachment, the methylene blue assay was performed on cells exposed to either medium alone, house dust-mite (*Der p*) allergen, or allergen plus dexamethasone. In selected experiments, methylene blue analysis was performed on epithelial cell cultures exposed to cytokines. Using the methylene blue assay, the numbers of epithelial cells in individual cultures were quantified, cytokine release in individual culture supernatants were normalised, allowing a comparison between the cultures obtained from different asthmatic and normal subjects. The cytokine release was then expressed as picograms released by one million primary epithelial cells in culture.

2.4.2 Methylene Blue protocol

After completion of the epithelial cell cultures, supernatants were removed from the wells of the 24 well plate, and the epithelial cells fixed by the addition of 500 microlitres of formal saline for 60 minutes. The formal saline was then removed and the cells stained for 45 minutes by adding 300 microlitres of a filtered 1% methylene blue solution to each well. The methylene blue solution was then tipped off and washed repeatedly with water, and subsequently blotted dry on tissue paper. 400 microlitres of a 0.1M ethanol-hydrochloric acid (ETOH-HCl) solution was then added to each well, and gently agitated. 100 microlitre aliquots of this solution were then placed on a 96 well plate, and absorbance read using an ELISA reader at an absorbance of 630 nm. Sample values above the limit of detection of the ELISA reader were diluted (1:5) and readings repeated.

A standard curve for the methylene blue assay for the primary epithelial cells was performed by plating primary epithelial cells at incremental doubling cell densities ranging from 5×10^4 cells to 4×10^5 cells (Figure 1, Appendix). The epithelial cells were placed in culture overnight to allow time for the primary cells to adhere to the culture plates. These cells were then fixed, stained, and analysed using the methylene blue assay protocol and a standard curve for the methylene blue assay was generated. The methylene blue standard curve generated in this study has been validated by recent culture studies performed by Sarah Puddicombe in our laboratory. A good correlation was noted for the methylene blue standard

curves in both studies. Cell densities of subsequent cultures were then determined by reading off the cell densities corresponding to the absorbance reading by the methylene blue assay.

2.5 Bronchial explant culture protocol

The bronchial explant culture model of asthma has been developed in Southampton, and is a useful integrated cell system to study inflammatory immune responses in complex tissues using bronchial biopsies obtained from the lower airways of patients with asthma. Bronchial biopsies were cultured for 24 hours in serum free medium alone (500µl, AIM V, Life Technologies, Paisley, UK), *Der p* allergen (5,000 SQ U/ml, ALK, Horsholm, Denmark), and also in *Der p* allergen plus CTLA-4Ig (CTLA-4Ig 25µg/ml, generously provided by Dr.P Linsley, Bristol-Myers Squibb Institute, Seattle, USA) (Figure 2.2). Medium was supplemented with HEPES (10mM), glutamine (1mM), and 2-mercaptoethanol (2µM). Two biopsies were used in each culture condition to minimise effects due to tissue heterogeneity and to provide sufficient RNA for extraction and PCR analysis. After 24 hours, the cultures were stopped, the biopsies weighed and immediately stored in liquid nitrogen, and supernatents were stored at -80^0 C pending analysis. The allergen concentration and culture duration was optimised in preliminary studies performed in mild asthmatics by Jaffar *et al* [222, 239]. A time course experiment assessed the production of IL-5 by bronchial biopsies, stimulated *ex vivo* by *Der p* (5,000 U/ml). IL-5 production in explant supernatants commenced at 12 hours and peaked at 24 and 48 hours [239]. A 24-hour culture period was chosen for monitoring cytokine production by bronchial explants. Preliminary studies had been performed by Jaffar *et al* in mild asthmatic explants with particular reference to optimal concentration of CTLA-4Ig and control experiments using an isotype control antibody (IgG_1) [222, 239, 254]. Due to the small number of biopsies that could be taken in moderately severe asthmatic subjects for ethical reasons, it was not possible to repeat these control experiments in the moderately severe asthmatic subjects in this study.

2.6 Isolation and culture of peripheral blood mononuclear cells (PBMCs)

30mls of venous blood was collected into heparinised tubes at the time of bronchoscopy and was layered onto aliquots of Ficoll-Isopaque (20mls Lymphoprep, Nycomed, Oslo, Norway) in sterile tubes. After centrifugation at 1,000 x g for 20 minutes at 20^0 C, PBMCs were gently aspirated from the plasma / Ficoll interface, transferred to sterile universal tubes and washed twice (centrifugation at 250 x g for 10 minutes) with AIM V

medium. Cell counts were performed, and parallel PBMC cultures of 3 x 10^6 cells/ml were performed in medium alone (1 ml), medium plus *Der p* allergen (5,000 SQ U/ml), and also with addition of *Der p* and CTLA4Ig (25μg/ml). The PBMC cells were harvested for subsequent RT-PCR analysis for cytokine mRNA at 2 days, and the supernatants were recovered at 7 days for cytokine protein measurement. Studies by Larché *et al* support the use of a 7 day time point for assessing allergen-induced cytokine production by PBMC cultures and have also noted effective inhibition of cytokine production at a lower CTLA-4Ig fusion protein concentration of 10 μg/ml compared to the dose of 25μg/ml applied in this study [254].

2.7 Reverse Transcription-PCR analysis

2.7.1 Principles of RT-PCR

The application of Reverse Transcription-PCR is an ideal tool for the detection and amplification of a number of specific messenger RNA gene transcripts in small airway samples such as endo-bronchial biopsies, brushed epithelial cells, or indeed cell cultures.

2.7.1.1 RNA extraction

Messenger ribonucleic acid (mRNA) was extracted from bronchial biopsies or PBMCs using the RNAzol B (ams Biotechnology, Oxon, UK) technique. Trizol is a monophasic solution of phenol and guanidine isothiocyanate that disrupts cells and dissolves cellular components, while maintaining ribonucleic acid (RNA) intact. RNAse-free glycogen (molecular biology grade) is added to act as a carrier for RNA.

The addition of chloroform, homogenisation, and subsequent centrifugation separated the homogenate into an upper aqueous phase containing exclusively RNA, an intermediate phase containing DNA, and an organic phase containing protein. Total RNA was then transferred using disposable sterile filter-tipped micropipettes into sterile RNAse free Eppendorf tubes, and the RNA precipitated in isopropranolol (-20^{0}C). The addition of alcohol makes the solution more hydrophobic, reducing the solubility of the nucleic acids, and precipitates the RNA. Centrifugation of the sample pellets the RNA, which was then washed with ethanol to further improve RNA purity. The RNA pellet was then air-dried and resuspended in diethyl-pyrocarbonate (DEPC)-treated ultra-high quality water.

2.7.1.2 Principles of RNA measurement using Gene Quant

Nucleotides in solution absorb light in the ultra-violet region of the spectrum, with a maximum absorbance at a wavelength of 260 nm. Proteins also absorb ultraviolet light, but at a peak wavelength of 280 nm and to a lesser extent at 260 nm. Thus, nucleic acids can be quantified and the purity of an RNA preparation determined by measuring the ratio of UV absorbance at 260nm and 280 nm using a spectro-photometer, and comparison with results obtained from pure solutions. A spectrophotometer detects photoelectrically and compares electronically the amount of light transmitted through solutions containing different concentrations of an absorbing substance. Using this technique, the RNA concentration of samples was measured by inserting a 5μl sample of RNA in the spectrophotometer cuvette and measuring absorbance values at 260 nm. The purity of RNA extraction was then assessed by comparing the RNA and protein content of samples by measuring the absorbance at 260 nm (RNA) and at 280 nm (protein content) respectively, being expressed as a ratio. Samples with high absorbance readings were diluted to obtain a final RNA concentration of 200μg/ml. A five microlitre aliquot of this final RNA sample was then reverse transcribed (RT).

2.7.1.3 Reverse Transcription – Principle

Messenger RNA (mRNA) is the RNA that is further translated by the ribosome into protein. Messenger RNA gene transcripts possess a poly (A) tail (a string of Adenine (A) nucleotides that is added to the 3' end of mRNA after transcription). The bulk of RNA in total or cytoplasmic RNA preparations is composed of ribosomal or transfer RNA. Using AMV reverse transcriptase and oligo dT (a string of Thymine deoxyribonucleotides) as a primer, poly (A)-positive mRNA was reverse transcribed at 42 ^{o}C for one hour in the presence of 1 mM deoxynucleotides, 5 mM $MgCl_2$, 400 IU RNAse inhibitor, and a reverse transcription buffer, to obtain complementary DNA (cDNA).

2.7.1.4 Principles of polymerase chain reaction (PCR)

PCR involves the replication and amplification of complementary DNA sequences of a specific target genetic sequence by the action of specific oligonucleotide primer pairs that flank the DNA sequence to be amplified, and a thermostable DNA (Taq) polymerase enzyme. This involves repeated cycles of heat denaturation of the DNA strands, annealing of specific primers to their complementary sequences on the template DNA, and extension of annealed primers in the 5' to 3' direction with DNA polymerase in an automated thermocycler. The

primers hybridise to complementary strands of the target sequence, so that the DNA sequence of interest is specifically amplified. As the amplified product has a sequence identical to the initial DNA sequence of interest, the primers can hybridise to the complementary strands of the product, and successive cycles of the amplification process results in a doubling of the amount of target DNA synthesised in the previous cycles. This leads to an exponential accumulation of the specific target sequence. However, the rate of DNA synthesis reaches a plateau phase due to exhaustion of oligonucleotides, primers and denaturation of the polymerase. Large amounts of a particular DNA sequence can thus be specifically produced, to produce cDNA copies of 2^n, where n equals the numbers of cycles of PCR amplification.

2.7.1.5 Agarose gel electrophoresis

The amplified complementary DNA PCR product can usually be detected by electrophoresis of the DNA product on an agarose gel, staining with ethidium bromide, and visualised using ultra-violet illumination. To confirm the predicted size of the PCR product, a molecular base pair ladder was also run on the gel, which has bands of equal intensity every 100 base pairs from 100 to 1500 base pairs.

2.7.2 Methods of Reverse-Transcription-PCR

2.7.2.1 RNA extraction from bronchial biopsies

Bronchial biopsies stored in liquid nitrogen were initially suspended in sterile ground glass glass homogenizers containing 2μl of glycogen and 100μl of TRIZOL. Samples were left on ice for 15 minutes, and then thoroughly homogenized until the tissue was completely lysed. The homogenate was transferred to a 500 μl sterile Rnase-free Eppendorf tube. A further 100μl of TRIZOL was added to the glass homogenizer to recover the remaining RNA, and pooled in the Eppendorf tube. 40 μl (1/5 volume) of chloroform was added and the sample homogenised by vortex agitation, and kept on ice for 15 minutes. The sample tubes were then centrifuged at 13,000rpm at 4°C for 15 minutes to separate the homogenate into an upper aqueous phase containing exclusively RNA, an intermediate phase containing DNA, and an organic phase containing protein. Total RNA was then transferred using disposable sterile filter-tipped micropipettes into sterile RNase-free Eppendorf tubes. An equivalent volume (80μl) of isopropranolol was added to the eppendorf tube, vortexed thoroughly for 30 seconds, and the RNA precipitated overnight at -20^{0}C. The next day, the RNA pellet was then

recovered by centrifugation (13,000rpm) at 4^0C for 15 minutes, and the isopropranolol removed with a sterile mini-pastette. The RNA pellet was washed by twice adding 500µl of 80% ethanol and centrifugation at 13,000rpm for five minutes to further improve purity of RNA. The ethanol solution was aspirated, and the RNA pellet air-dried and re-suspended in 5 µl of diethyl-pyrocarbonate (DEPC) treated ultra-high quality (UHQ) water and dissolved by heating at 60°C for 10 minutes.

2.7.2.2 RNA extraction from PBMC or epithelial cell cultures

After culture the cell pellets were suspended in 300µl of TRIZOL and stored at –80°C until RT-PCR analysis. 2µl of glycogen was added to the Eppendorf tube, vortexed thoroughly, and the samples kept on ice for 15 minutes. The subsequent protocol was identical to that described in 2.7.2.1 above.

2.7.2.3 RNA measurement

The total RNA in the RNA samples was quantified using a Gene Quant to measure the optical density at 260nm, and the RNA concentration expressed as µg/ml. The purity of the RNA extraction from samples was assessed by measuring the ratio of the optical density obtained at 260nm and 280nm.

An equal volume of RNA (approximately 1µg) in paired samples was reverse-transcribed to ensure consistency and comparability between samples.

2.7.2.4 Reverse Transcription

Protocol:

- A master mix solution was made as follows (for one 20µl RT reaction volume)

Composition	Amount	Final concentration
$MgCl_2$ (25mM)	4µl	5mM
Reverse Transcriptase Buffer	2µl	(Promega)
dNTPs (10mM)	2µl	1mM each dNTP
Oligo dT[p(dT)$_{15}$]	1µl	0.5µg/µg RNA
RNase inhibitor	1µl	1 unit/µl
AMV Reverse Transcriptase	1µl	15u/µg
DEPC water	4µl	

15 µl of master mix solution was added to a 50µl PCR tube with 5µl of total RNA sample solution (approximately 1µg), and incubated at 60°C for 60 minutes in a Perkin-Elmer Thermal cycler.

- The Reverse Transcriptase enzyme was then inactivated by heating at 94°C for 3 minutes in the thermal cycler.
- The resultant 20µl of cDNA was diluted with 20 µl of ultra-high quality sterile water to obtain a total volume of 40 µl cDNA solution from each bronchial biopsy or PBMC cell culture and stored at –80°C until analysis by PCR.

2.7.2.5 PCR Protocol

For PCR amplification, specific primer pairs were used for the constitutively expressed adenine phosphoribosyl transferase (APRT), and for the cytokines IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF-α, GM-CSF, IFN-γ, IL-18, RANTES, and eotaxin (Table 2.1 of primer sequences and product size). For each PCR reaction, 2.5µl of cDNA was amplified using Taq DNA polymerase, in the presence of 15 pmol of each primer pair, 0.5µl of dNTPs, and magnesium free thermophilic PCR Buffer. $MgCl_2$ was added in a concentration as optimized for each primer pair (Table 2.1). Target cDNA was amplified for 30 cycles using annealing temperatures optimized for each cytokine primer.

Method:

- To ensure that the reaction was identical in all cases (with the exception of the cDNA under investigation) a stock master mix was prepared and then aliquoted into each tube. This contained the following components per reaction. The final volume of the master mix was then calculated using the number of PCR reactions plus one. The following master mix was prepared for each PCR reaction:

Component	**Volume**	**Final concentration**
UHQ water	15.5 µl	
$MgCl_2$ 25 mM	1 µl	1mM
Magnesium-free Buffer 2.5 µl		10mM TrisHCl (pH 9) 50mM KCl 0.1 % Triton ® X-100
dNTP*	0.5* µl	0.2 mM dATP, dCTP, dGTP, 0.19mM dTTP
Taq DNA polymerase	0.2 µl	1 Unit
Primer 1 †	1.5 µl	15 pmol
Primer 2 †	1.5 µl	15 pmol
Total volume	**22.5 µl**	

(*For PCR ELISA analysis (see section 2.7.2.6) 0.5 µl of dNTP was substituted by 2.5 µl digoxigenin-labelled dNTP mix, and the UHQ water volume was adjusted accordingly to make up a final master mix volume of 22.5 µl).

(† Some of the primers were added at a volume of 0.5 µl, and the UHQ volume was adjusted accordingly to make up a final volume of 22.5 µl).

2.5 µl of cDNA was added to 22.5 µl of master mix in 50µl PCR reaction tubes for each PCR reaction and placed in a thermal cycler programmed with the following temperature cycles:

1 Initial hot start to 95°C for one minute;

Then 35 to 40 cycles of: (steps 2 → 3 → 4)

2. 95°C for 20 seconds;

Ramp over 45 seconds to:

3. Specific primer annealing temperature for 30 seconds

(50°C / 54°C / 56°C / 58°C / 60°C, Table 2.1);

Ramp over 45 seconds to:

4 72°C for 1 minute

5 Hold at 72°C for 10 minutes (Primer elongation)

6 Hold at 10°C until stored at 4°C.

PCR products were then kept in the freezer at either 4 °C or at –20 °C for longer storage until analysis either by agarose gel electrophoresis or PCR ELISA.

Figure 2.2: Principle of ELISA-PCR.

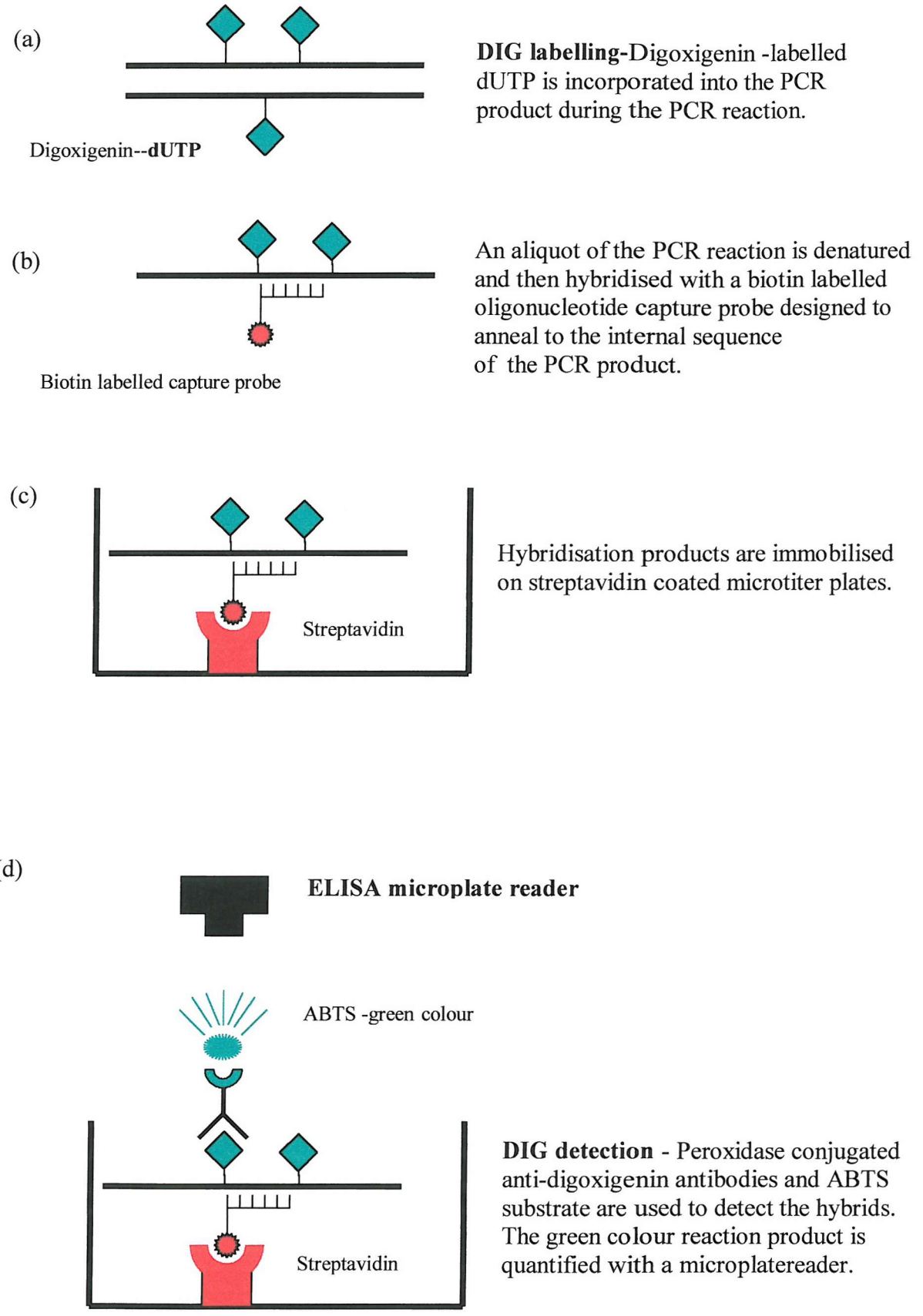


Table 2.1: Sequences of primer pairs used for RT-PCR analysis

Cytokine	Product size	PCR primer sequence
APRT	246	5' primer GCT GCG TGC TCA TCC GAA AG
		3' primer CCT TAA GCG AGG TCA GCT CC
IL-4	449	5' primer CTG CAA ATC GAC ACC TAT TA
		3' primer GAT CGT CTT TAG CCT TTC
IL-5	257	5' primer CTG AGG ATT CCT GTT CCT GT
		3' primer CAA CTT TCT ATT ATC CAC TC
IL-6	210	5' primer ATG AAC TCC TTC TCC ACA A
		3' primer CTC CTT TCT CAG GGC TGA GA
IL-8	190	5' primer GCA GCT CTG TGT GAA GGT GCA
		3' primer CAG ACA GAG GTC TCT TCC AT
IL-10	231	5' primer CTT GTC TGA GAT GAT CCA G
		3' primer CTC ATG GCT TTG TAG ATG CC
IL-12	300	5' primer CAT TCG CTC CTG CTG CTT CAC
		3' primer TAC TCC TTG TTG TTCCCT CTG
IL-13	500	5' primer CGG TCA TTG CTC TCA CTT GCC TT
		3' primer TTA CCC CTC CCT AAC CCT CCT T
IL-16	347	5' primer ATG CCC GAC CTC AAC TCC TC
		3' primer CTC CTG ATG ACA ATC GTG AC
IL-18	342	5' primer GCT TGA ATC TAA ATT ATC AGCT
		3' primer GAA GAT TCA AAT TGC ATC TTAT
GM-CSF	215	5' primer GCA TGT GAA TGC CAT CCA GG
		3' primer GCT TGT AGT GGC TGG CAA TC
IFN-γ	270	5' primer GGT CAT TCA GAT GTA GCG GA
		3' primer GCG TTG GAC ATT CAA GTC AG
TNF-α	324	5' primer CGA GTG ACA AGC CTG TAG CC
		3' primer CAT ACC AGG GCT TGG CCT CA
Eotaxin	320	5' primer GGA TCC AAC ATG AAG GTG TCCG
		3' primer GAA TTC TTA TGG CTT TGG AGT TGG AG
IL-4Rα	450	5' primer CTG ACC TGG AGC AAC CCG TAT
		3' primer CCG CTT CTC CCA CTG TGA CCC
Common-γ_c component	-	5' primer TAC CGG ACT GAC TGG GAC CAC
		3' primer TGG GGG AAT CTC ACT GAC GA
IL-13Rα1	459	5' primer TCA TGG TCC CTG GTG TTC
		3' primer AAC AAC TGG AGA ATG GGA AGA
IL-13Rα2	427	5' primer GGA GCA TAC CTT TGG GAC CT
		3' primer TTG GCC ATG ACT GGA AAC TG

2.7.2.6 Principle of PCR-ELISA (Figure 2.2)

Summary of Protocol

40µl of denaturation solution was added to 10 µl of the PCR product. A biotin labelled capture probe (50 ng/ml in 450 µl of hybridisation buffer provided with the kit), specific for the particular gene product, was then hybridised to each complementary digoxigenin (Dig)-labelled PCR product for 3 hours in an ELISA plate-shaker incubator at 37°C. During this incubation phase, the Dig-labelled PCR product-biotinylated probe hybrids were immobilised to streptavidin-coated microtitre plates. The plate was then washed four times to remove any remaining unbound antibody, and the bound PCR product detected using a peroxidase conjugated anti-digoxigenin antibody. The PCR products were then visualised using the peroxidase substrate ABTS development of a green colour within twenty minutes. The absorbancy at 405 nm was then measured on a microtitre plate ELISA reader.

Method:

The following solutions were prepared in advance from the kit according to the manufacturer's instructions (Boehringer Mannheim) including:

(a) Control PCR product:

The contents of vial 1 was resuspended in 100 µl of sterile UHQ water, briefly centrifuged and incubated at room temperature for 30 minutes to allow complete reconstitution of the DNA. The solution was then stored at –20°C.

(b) Control capture probe:

The contents of vial 2 were resuspended in 100µl of sterile UHQ water, briefly centrifuged, and incubated at room temperature for 30 minutes to allow complete reconstitution. The capture probe was stable for several weeks at 4°C.

(c) Anti-Dig-POD (Anti-Digoxigenin-peroxidase) conjugate:

The anti-Dig-POD was reconstituted by addition of 250 µl of sterile UHQ water and gently mixed for 15 minutes for full reconstitution. The solution was stable at 4°C for two months.

(d) Wash solution:

One washing tablet was dissolved in 2 litres of UHQ water. The solution was stored at 4°C for up to 6 weeks.

Assay protocol

- Preparation of anti-Dig-POD working solution:

One volume of anti-Dig-POD conjugate was diluted in 99 volumes of conjugate dilution buffer at least one hour prior to the start of the experiment, taking care to avoid foaming. This was allowed to reach room temperature prior to use, and stored away from light.

- 10 µl of PCR product was placed in 1 ml sterile Eppendorf tubes.
- 10 µl of control PCR product solution (vial 1) was placed in a sterile eppendorf.
- (a) 10 µl of biotin-labelled control capture probe was added to 500 µl of hybridisation buffer.

  (b) 11 µl of the cytokine-specific capture probes were added to 450 µl hybridisation buffer for each sample of the gene product.
- 40 µl of the denaturation solution was added to the Eppendorf tubes containing 10 µl of the sample PCR product. The tubes were vortexed and incubated at room temperature for at least 15 minutes. All samples were incubated for an identical duration of time.
- 450 µl of the cytokine specific capture probe / hybridisation solution was then added to each tube, and the tubes mixed well by vortexing.
- 200 µl of the solution from each sample, including negative and positive controls, was then transferred to duplicate wells of a Streptavidin-coated microtiter plate and the plates sealed with adhesive film.
- The plates were then incubated at 37°C on a microtiter plate shaker for 3 hours.
- The solution was discarded by inverting and washed 4 times by adding 250µl of wash solution to each well. After the last wash, the solution was discarded and the wells tapped dry on lint-free absorbent paper.
- 200µl of anti-DIG-POD working solution was added to each well. The plate was covered with an adhesive film, and incubated for 30 minutes at 37°C on a plate shaker.
- At least 15 minutes prior to use, the substrate solution was prepared by adding 1 ABTS tablet to 5 ml of substrate buffer, and stored at room temperature but protected from light.
- The solution was discarded from the plates and the plate wells washed 4 times with 250 µl of wash solution, and the wells tapped dry.
- 200 µl of ABTS substrate solution was added to each well. 200 µl was also added to empty wells to determine the intrinsic extinction of the ABTS solution.

- At 5 minute intervals during the colour development, the absorbance at 405 nm was read on an ELISA reader (Reference filter 492 nm). The background extinction of the ABTS substrate solution in the blank well was subtracted from the readings.
- To determine the relative expression of cytokine mRNA in the samples, the reading obtained for each cytokine PCR product was expressed as a percentage of APRT (house keeping gene).
  i.e. (Cytokine PCR product reading/ APRT reading) x 100.

2.8 Cytokine protein measurement by ELISA

The levels of cytokine protein in the supernatants of bronchial explant cultures, PBMC cultures, and primary epithelial cell cultures were determined using commercially available Enzyme Linked Immuno-sorbent Assay (ELISA) kits for the various cytokines in accordance with the manufacturers instructions (See Appendix for individual protocols). In the text of this thesis the terms cytokine release or production refer to cytokine protein measurements of culture supernatants.

- Interleukin-5 (Minimum detectable dose 4 pg/ml, Biosource, Cytoscreen).
- IL-13 (Minimum detectable dose < 1 pg/ml, Biosource, Cytoscreen).
- GM-CSF (Minimum detectable dose < 1.25 pg/ml, Biosource, cytoscreen).
- RANTES (Minimum detectable dose < 3 pg/ml, Biosource, cytoscreen).
- IL-16 (Minimum detectable dose < 5 pg/ml, Biosource, cytoscreen)
- Eotaxin (Minimum detectable dose < 2 pg/ml, Biosource Cytoscreen)
- IL-8 (Minimum detectable dose < 1 pg/ml, Eurogenetics, Pelikine)
- TGF-β2:E_{max}^{TM} ELISA (Minimum detectable dose 32 pg/ml, Promega, WI, USA).
- TGF-α ELISA (Minimum detectable dose 10 pg/ml. Oncogene Research Products, Boston, USA).

2.8.1 GM-CSF ELISA PROTOCOL:

All reagents were brought to room temperature. 50 µl of samples were diluted with assay diluent and added to a 96 well microtiter plate pre-coated with antibody for the appropriate cytokine. Standards were made by serial dilution in accordance with the manufacturer's instructions. 150 µl of secondary antibody was added to each well, the plate was sealed and incubated at room temperature for 1.5 hours. After a series of 4 washes, 100 µl

of streptavidin-horseradish peroxidase working solution was added to each well and incubated for an additional 30 minutes at room temperature, followed by 4 washes. Finally, 100 µl of chromogen was added to each well and colour development stopped after 30 minutes incubation by the addition of 100µl of stop solution. The colour reaction was quantified using an ELISA plate reader at 450 nm. A standard curve was plotted and cytokine concentrations (pg/ml) of the samples determined. Initial GM-CSF measurements were performed with the 'older format' ELISA assay by Biosource, cytoscreen in accordance with the manufacturer's instructions. The manufacturers have provided data to confirm equivalent quantitation by dividing results from their 'new format' ELISA assay by a factor of 0.559. Cytokine levels of biopsy supernatants were expressed in pg/mg wet weight of tissue. Cytokine release in primary epithelial cell cultures was corrected for the numbers of epithelial cells cultured and expressed as cytokine release per 10^6 cells cultured.

2.9 Immunohistochemistry Analysis

2.9.1 Processing of biopsies into glycolmethacrylate (GMA) resin

Tissue for immunohistochemical analysis was initially processed into glycol methacrylate resin prior to sectioning and immuno-staining. Bronchial biopsies obtained at bronchoscopy were placed in ice-cooled acetone plus protease inhibitors (phenylmethyl-sulfonyl fluoride [2nM] and iodoacetamide [2nM]), and stored overnight at -20^{0}C. The following day, the sample was placed in acetone at room temperature for 15 minutes and then in methylbenzoyl for 15 minutes. The biopsy was then immersed in glycol methacrylate (GMA) JB4 solution A (Polysciences, Northampton, UK) for 6 hours, the solution being changed every 2 hours. Finally the tissue was embedded in GMA resin (prepared by mixing GMA monomer, N.N-dimethylaniline PEG 400, and Benzoyl peroxide), and was polymerised overnight at 4^{0}C (Britten et al 1993). The resin blocks were stored at -20^{0}C in airtight containers until sectioning for immunostaining. 2µm thick sections were cut using a microtome (Supercut 2065, Leica, Germany), floated onto ammonia water (1:500), and picked up onto 0.01% poly-L-lysine coated glass slides, and air dried at room temperature for 1 to 3 hours. Biopsies were initially stained with Toluidine Blue to assess morphology of epithelium and submucosa, and biopsies with the best morphology from each subject were selected for sectioning and immunostaining for the various inflammatory biomarkers relevant to asthma.

2.9.2 Immunostaining

Endogenous peroxidases were blocked by applying 0.1% sodium azide and 0.3% hydrogen peroxidase to the sections, followed by 3 x 15 minute rinses with Tris buffered saline (TBS) adjusted to a pH of 6. Blocking medium was then applied for 30 minutes, subsequently drained, and primary antibodies (Table 2.2) applied and incubated overnight at room temperature. After 3 x 15 minute rinses with TBS, biotinylated rabbit anti-mouse IgG Fab(Dako Ltd, High Wycombe, UK) secondary antibodies were applied for 2 hours, followed by the streptavidin-horse radish peroxidase complex (Dako Ltd, High Wycombe, UK) for a further 2 hours. After rinsing with TBS, amino-ethyl carbazole (AEC) in acetate buffer (pH 5.2) and hydrogen peroxidase, was used as a substrate to develop a peroxidase-dependent red colour reaction. Sections were then counterstained with Meyer's haematoxylin, dried and mounted in DPX. As a negative control for each biopsy, 2 sections had TBS and isotype matched control (mouse IgGκ(MOPC 21), Sigma, Poole, UK) antibody applied at the appropriate concentrations to the primary monoclonal antibody.

2.9.3 Quantification of inflammatory cells

For each biopsy section, the numbers of positively staining cells in the sub-epithelial tissue, excluding glands and blood vessels, and in the airway epithelium, were counted using a Leitz Laborlux S microscope (Leica Ltd., Milton Keynes, UK) with the aid of an eyepiece graticule. A computer-assisted image analysis system (Apple Mackintosh Quadra 700 computer: Colourvision 1.6 software, Improvision, Coventry, UK) was used to measure the area of sub-epithelial tissue and the length of the epithelial basement membrane. Subepithelial and epithelial cell counts were then expressed per mm^2 tissue area and per mm length of epithelium, respectively.

The magnification factor of the GMA processed biopsy images (Figure 3.5) in this study can be determined using the following formula: (400 (Print magnification) x 20, 40, or 63 (objective lens at different magnifications) x 0.32 (Correction factor for microscope). Images were magnified by a factor of 2.56 x x 10^3, 5.12 x 10^3, or 8.06 x 10^3 when photomicrographs taken using objective lens of 20, 40, or 63.

Figure 2.3: Principle of streptavidin-biotin peroxidase enzyme complex immuno-histochemistry.

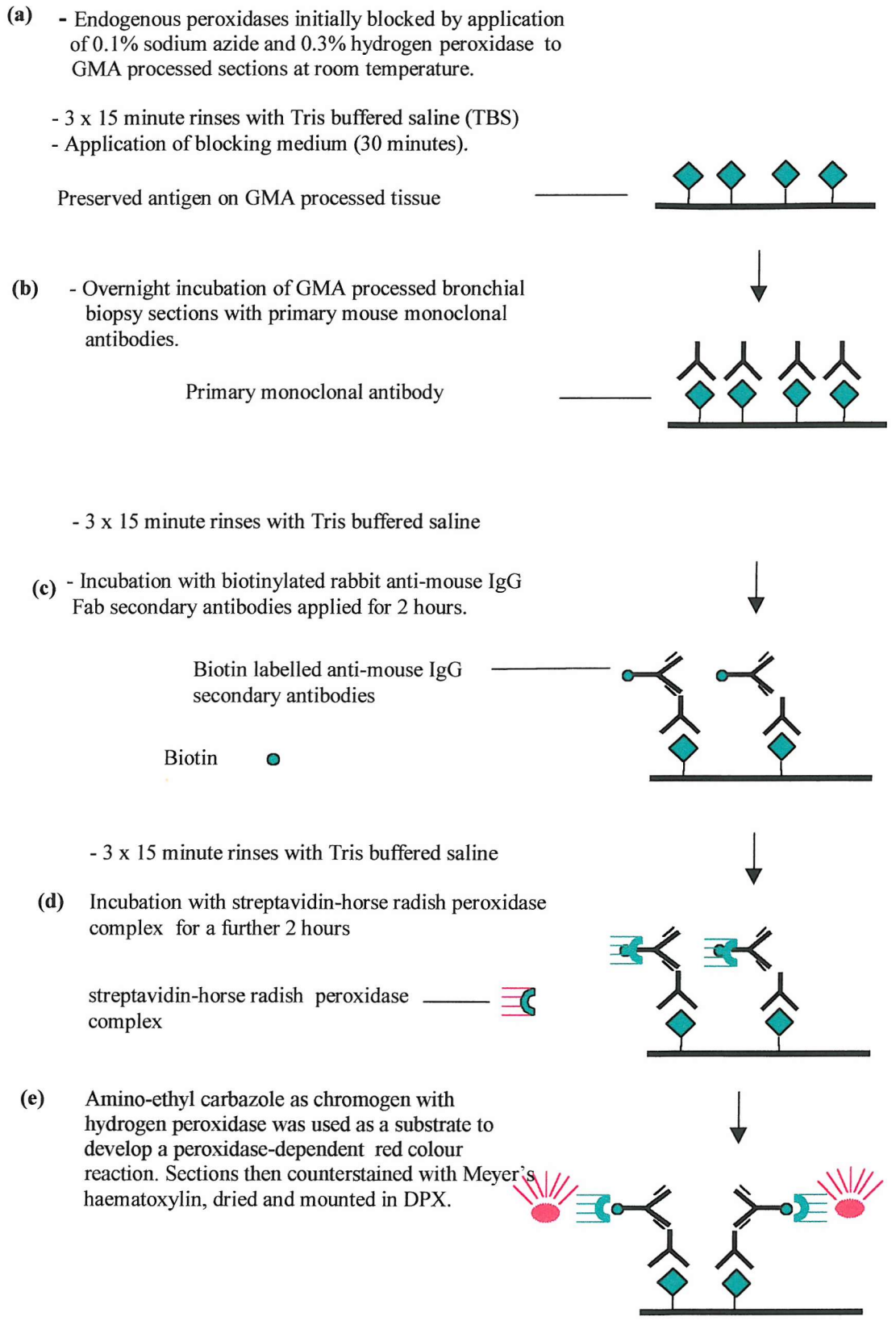


Table 2.2: Antibodies used for immunohistochemistry studies.

Name	Marker	Cells	Dilution	Source
CD3	CD3	T-cells	1 :100	DAKO, High Wycombe, UK
CD4	CD4	T-cells	1 : 10	Becton Dickinson, UK
CD8	CD8	T-cells	1 : 100	DAKO, High Wycombe, UK
CD25	Activated lymphocyte	T-cells	1 : 50	DAKO, High Wycombe, UK
EG2	ECP	Eosinophils	1 : 200	Pharmacia Upjohn, Milton Keynes, UK
NE	Elastase	Neutrophils	1 : 1,000	DAKO, High Wycombe, UK
AA1	Tryptase	Mast cells	1 :100	DAKO, High Wycombe, UK
Anti -CD80	CD80	Antigen presenting cells	1 : 5	Cymbus Biotechnology, Southampton, UK
Anti-CD86	CD86	Antigen presenting cells	1 : 10	Cymbus Biotechnology, Southampton, UK
Anti-CTLA4	CTLA4	T-cells	1 : 5	Autogen Bioclear, Calne, UK
Anti-CD28	CD28	T-cells	1 : 5	Cymbus Biotechnology, Southampton, UK
Biotinylated Anti – mouse	Secondary antibody	-	1 : 300	DAKO, High Wycombe, UK
Streptavidin biotin horse radish peroxidase	Third stage antibody	-	1 : 200	DAKO, High Wycombe, UK
IgG1	Isotype control	-	1 : 60	Sigma, UK

Figure 2.10 Flow Cytometry analysis

2.10.1 FACS protocol:

After completion of the epithelial cell cultures, the cells were detached from the culture plates by the addition of trypsin. A single cell suspension was then washed in staining buffer (PBS, 2% FCS) and resuspended at a concentration of $1x10^7$ cells/ml. 100µl of this cells suspension ($1x10^6$ cells) was then aliquoted into a 12 x 75mm polypropylene FACS tube. The primary antibody for the particular IL-4 / IL-13 receptor sub-units (Table 2.3) was then added, vortexed and incubated for 1 hour at 4 ^{0}C (on ice). The supernatant was discarded and the cells resuspended in 100 µl of blocking buffer (1% BSA in PBS) and then the secondary antibody (anti-mouse or anti-rabbit FITC conjugated) was added, vortexed and incubated for 30 min at 4 ^{0}C (on ice) in the dark. Cells were then washed with 2 ml of cold PBS and 0.5 ml of cold PBS were added for the FACS analysis. All the necessary control tubes (negative, positive and specificity controls) were prepared as required for immunofluorescence staining (See Appendix for individual protocols). See Appendix for detailed description of flow cytometry protocol for IL-4Rα detection using IL-4 fluorokine.

Table 2.3: Antibodies used for detection of IL-4 and IL-13 receptor sub-units by flow cytometry analysis.

Antibody	Manufacturer	Dilution
Common IL-2R γ_c	Pharmingen, 35335B	R-PE-conjugatedMouse IgG_1
IL-4Rα	R & D, Fluorokine, NF400	Biotinylated hIL-4
IL-13Rα2	Diaclone, B-D13	Mouse IgG_1
anti-mouse FITC conjugated	Pharmingen	
anti-rabbit FITC conjugated	Pharmingen	
Control IgG_1 antibody	Diaclone, B-Z1	Mouse IgG_1

2.11 Statistical analysis

Power calculations were performed using the sample size formula for a difference between two means to determine the number of variables required in each group to show a statistically significant difference between the comparisons of interest. This gives the number of subjects per group that you would require to have an eighty percent power (i.e. 80 % chance) of detecting a significant difference at the five percent level between the grouped variables of interest.

Formula: $n = \frac{14.978 \times (\text{common standard deviation, SD})^2}{(\text{Mean group difference})^2}$

The common standard deviation is the standard deviation of all variables in both groups. The mean group difference equals (mean group 1, e.g. mean TGF-β_2 production by atopic asthmatics) minus (mean group 2, e.g. mean TGF-β_2 production by normal control subjects).

(Reference: Medical Statistics, 3rd Edition by Martin Bland).

It was estimated that 30 asthmatics and 30 normal controls would be required to have an 80% power of showing a statistically significant difference (i.e. achieving a probability value of less than 0.05) between baseline TGF-β_2 production by epithelial cells of atopic asthmatics and normal control subjects). Although these power calculations were useful to estimate the numbers of subjects required to achieve a statistically significant result, it would not be possible to recruit such a high number of subjects for a study requiring bronchoscopy.

The data was assessed for normality using the Kolmogorov-Smirnoff test. This did not support a normal distribution of the data, so non-parametric tests of statistical significance were applied. Results are expressed as median (interquartile range). The Wilcoxon signed rank test for paired data was used for within-group comparisons of cytokine protein levels, using SPSS 7.5 for Windows. The Mann-Whitney U test was used for between-group comparisons. As the analysis of cytokine readouts were pre-planned in the individual groups, it was not considered necessary to perform Bonferoni correction for multiple comparisons. A probability value of less than 0.05 for the null hypothesis was accepted as indicating a statistically significant difference.

CHAPTER THREE

The role of CD28-B7 costimulation in allergen-induced cytokine release by the bronchial mucosa from patients with moderately severe asthma

3.1 INTRODUCTION

T cells play an important orchestrating role in asthmatic airway inflammation by the release of Th2 type cytokines (IL-4, IL-5, IL-9, IL-13, and GM-CSF) encoded on chromosome $5q_{31-33}$, which promote the development, survival, activation and recruitment of inflammatory cells to the asthmatic airways [214].

Recent interest has focussed on mechanisms of co-stimulation in T cell-mediated inflammatory diseases such as asthma [240], in particular via the CD28-CD80/CD86 pathway [224]. The activation of naïve T cells in primary immune responses requires at least two signals from APCs: the first provided by the cognate interaction of the T cell receptor (TCR) with MHC II complexes on APCs, and the second by CD80 (B7-1) and CD86 (B7-2) on APCs interacting with CD28 receptors on T cells [225-227]. Co-stimulation via CD28 results in T cell activation, while ligation of its higher affinity homologue, Cytotoxic T Lymphocyte Antigen (CTLA-4) limits immune responses by competing with CD28 for binding with CD80 or CD86 and thus depriving the T cells of CD-28 mediated activation. There is also evidence to suggest that CTLA-4 may inhibit immune responses at a later stage, by affecting intracellular signal transduction pathways utilised by CD28 or the TCR [228]. Lack of co-stimulatory signalling via CD28 has been shown to result in T cell unresponsiveness or anergy [229]. Recent murine studies suggest a critical role for CD28-B7 co-stimulation in the production of Th2 cytokines, the development of airway hyperresponsiveness, and eosinophilic airway infiltration in asthma [230-233].

CTLA-4Ig is a chimeric fusion protein of the Fc component of human IgG_1 and the extra-cellular component of human CTLA-4, which inhibits signalling via the CD28-B7 pathway, and has been used to study the requirement for CD28-B7 co-stimulation in immune responses (Figure 3.1) [233, 234]. CTLA-4Ig has also been shown to inhibit allergen-induced proliferation and cytokine production by PBMCs obtained from atopic donors [235, 236]. Co-stimulatory signalling is also involved in cytokine production in asthmatic airways. Using explanted bronchial tissue of mild atopic asthmatics stimulated with allergen, it has been reported that there is increased release of IL-5, IL-13, RANTES and IL-16 [222, 237]. Addition of the fusion protein, CTLA-4Ig, simultaneously with allergen effectively inhibited the allergen-induced release of all these cytokines, and the related chemotactic activity due to IL-16 and RANTES, supporting an obligatory requirement for CD28-B7 co-stimulation for the release of these pro-inflammatory mediators [237].

Whereas these earlier studies were performed in patients with mild disease, which is easily controlled with minimal controller and reliever medications, responses in more severe persistent asthma have not been addressed. Despite using high doses of inhaled corticosteroids, a significant proportion of asthmatics have persistent symptoms and evidence of ongoing airway inflammation [22, 238, 241]. To assess inflammatory responses in subjects with more severe disease, bronchial explant cultures were performed using bronchial biopsies obtained from the airways of patients with moderately severe asthma. In view of the possibility that co-stimulatory requirements may be specific for inflamed airway tissue, I have compared allergen-induced cytokine release and the requirement for CD28-B7 co-stimulation using CTLA4-Ig in explants with those obtained using peripheral blood mononuclear cell cultures from the same patients.

Objectives

To assess allergen-induced production of IL-5, IL-13, GM-CSF, and IL-16 by bronchial explant tissue cultures of moderately severe asthmatics, and to assess the effects of blocking CD28-mediated costimulation with the fusion protein CTLA-4Ig, on bronchial tissue cytokine production in patients with severe asthma.

To assess the parallel production of cytokines by peripheral blood mononuclear cells and the requirement for CD28-B7 costimulation by circulating inflammatory cells compared to the bronchial tissue of moderately severe asthma.

3.2 METHODS

3.2.1 Subjects

Table 3.1: Baseline characteristics of mild persistent to moderate persistent asthmatic subjects recruited for bronchial explant studies.

Subject	Age Years	Sex	FEV_1 Litres/ min	FEV_1 %Pred. Baseline	FEV_1 PC_{20} mg/ml	IgE IU/ml	$Beta_2$ Agonist Use/day	Inhaled steroid dose (µg/day)	Long acting Beta 2 agonists (Use per day)
EH	22	M	4.54	98	2.68	190	3	800	1
DB	36	F	2.1	81	0.73	1048	2	2,000	0
SB	28	F	3.2	100	7.89	66	2	800	2
AmC	41	F	1.75	61	0.13	40	2	0*	0
SO	35	M	2.9	61	0.60	2772	2	1,000	0
CE	40	F	2.25	66	0.29	59	2	800	0
NA	40	M	3.45	88	25.06	276	2	800	2
JC	34	F	2.41	77	0.15	208	-	400	0
TR	25	M	3.06	69	.07	188	3	1000	0
DS	32	M	2.41	88	.48	186	2	1000	0
TD	31	M	3.31	86	0.7	497	-	800	0
SK	55	M	2.26	68	0†	-	2	400	0
MK	21	M	4.9	100	1.8	102	-	400	0
RM	28	M	4.39	86	0.5	829	-	1000	2
VH	22	F	2.70	89	1.78	140	-	200	-
Mean	**32.7**	**9M**	**-**	**81**	**0.81‡**	**228‡**	**-**	**773**	**-**
SEM	**2.4**	**6 F**		**3.5**				**117**	

*Prescribed 1,000mg/day beclomethasone but poor compliance.

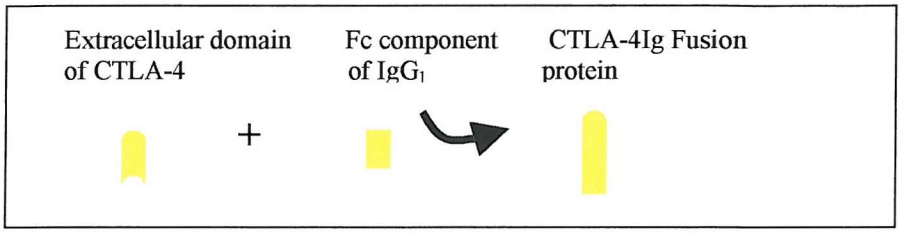
† 20% fall in FEV_1 with saline inhalation (Normal PC_{20} >16mg/ml).

‡ Geometric mean.

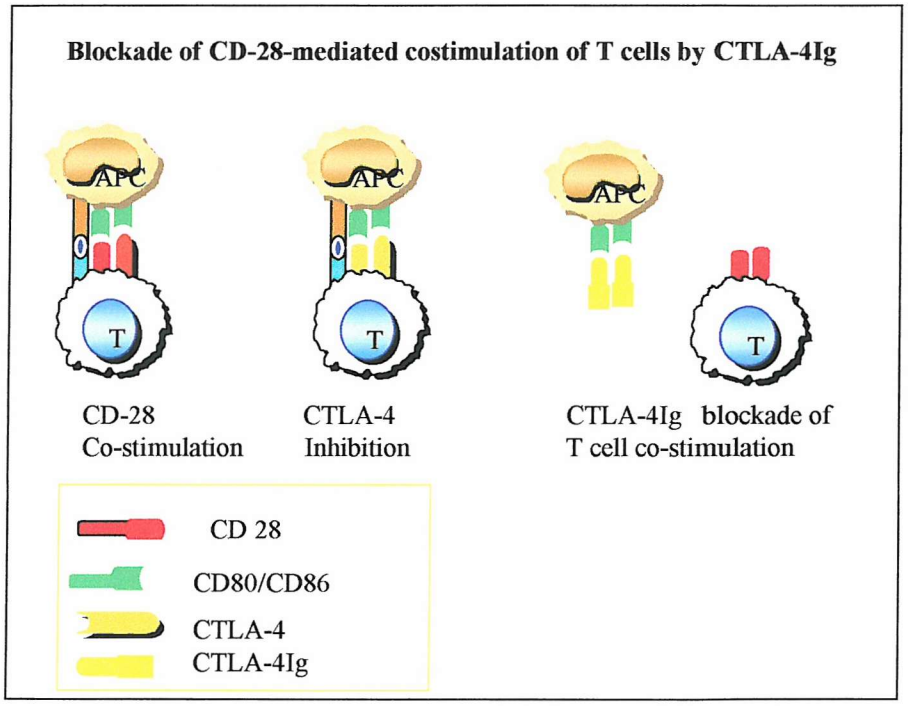
Figure 3.1: (a) CTLA-4Ig is a fusion protein composed of the extracellular component of CTLA-4 and the Fc component of IgG_1.

(b) Antigen presentation in context of MHC-II TCR interaction, associated with CD28 mediated co-stimulation leads to T cell activation. CTLA-4 ligation leads to a down-regulation in the immune response. CTLA-4Ig binds to CD80 and CD86 preventing CD28-mediated co-stimulation and inhibition of T cell activation.

(a)



(b)



3.2.1 Subjects

Fifteen persistently symptomatic asthmatic subjects (9 males) with a mean (± SEM) age of 33 ± 2.4 years were recruited for bronchoscopy and bronchial biopsy (Table 3.1). All had a history compatible with asthma, a mean FEV_1 of 81 ± 3.5 percent of predicted, with FEV_1 reversibility of 15%, geometric mean PC_{20} histamine of 0.81 mg/ml (normal > 16 mg/ml), and geometric mean serum IgE of 228 IU/ml. All the subjects were atopic as determined by positive skin prick testing to a panel of common aero-allergens (ALK, Horsholm, Denmark), and were sensitized to house dust mite (*Der p*). They were all receiving regular treatment with inhaled corticosteroids (mean ± SEM daily dose of 773 ± 117 μg per day of beclomethasone dipropionate), and short acting β_2-agonists as required for symptom relief. Three subjects were on maintenance therapy with inhaled long acting $beta_2$-agonists. Subjects withheld their inhaled corticosteroids for a minimum of one week prior to bronchoscopy and bronchial biopsy to optimise inflammatory responses in the bronchial tissue cultures. Written informed consent was obtained prior to inclusion in the study, and the study was approved by the combined Southampton University and Hospitals Ethics committee.

The methods section gives a detailed account of the bronchoscopy and endobronchial biopsy procedure (Section 2.2); the bronchial explant tissue culture (Section 2.5); PBMC isolation and culture (Section 2.6); RNA extraction, Reverse Transcription and PCR-ELISA protocol (Section 2.7); ELISA analysis for cytokine protein (Section 2.8); and immunohistochemistry (Section 2.9). Although explant studies were performed on all 15 subjects, there was only approximately 450 μl supernatant available for analysis by ELISA, and it was necessary to repeat the analysis in selected cases, which limited the number of assays that could be performed for each cytokine. Hence, there is a difference in *n* values for for the analysis of IL-5, IL-13, IL-16, and GM-CSF. Initially, RT-PCR was performed using gel electrophoresis, and the quantitative PCR only became available at a later stage in the study. This limited the number of samples that the PCR ELISA assay could be used for.

In the earlier explant studies in mild asthma [222, 237], control experiments were performed using CTLA-4Ig and isotype control antibodies, which showed no effect of the fusion protein alone on IL-5 mRNA or protein production. Due to the limited number of bronchial biopsies that could be taken from severe asthmatic subjects, for ethical and safety reasons, it was not possible to repeat these control experiments in the present study.

3.2.2 Statistical analysis

Data was analysed for statistical significance using non-parametric tests. Results are expressed as median (interquartile ranges). The Wilcoxon signed rank test for paired data was used for within-group comparisons of cytokine protein levels, using SPSS 7.5 for Windows. Values of $P<0.05$ were accepted as statistically significant.

3.3 RESULTS

3.3.1 Allergen-induced cytokine production in bronchial explant cultures of moderately severe asthmatics:

Consistent with previous explant studies using mild asthmatics [222, 239], the spontaneous release of IL-5 from bronchial explants of moderately severe asthmatics was extremely low (1.09 (0-12.5) pg/mg). Upon exposure to *Der p* extracts, IL-5 production was significantly increased (4.43 (2.2-46.5) pg/mg; P=0.01), although there was considerable variability between individual subjects (Figure 3.2 (b)). Where samples were available, parallel measurements of mRNA levels confirmed IL-5 gene expression upon exposure to *Der p* (Figure 3.2 (a)).

In selected experiments, CTLA-4Ig (25μg/ml) was added to the bronchial explant cultures simultaneously with the allergen extract. In previous experiments [222, 239] we have shown that CTLA-4Ig alone or an isotype control antibody had no effect on IL-5-mRNA expression or cytokine protein secretions. While the CTLA-4Ig fusion protein appeared to partially inhibit allergen-induced IL-5 mRNA and protein release in 3/6 and 5/12 explant cultures, respectively when the group was analysed as a whole, this failed to reach statistical significance (Fig. 3.3 (a, b). GM-CSF levels were not increased by allergen stimulation, and were also not significantly affected by CTLA-4Ig either at the protein or mRNA levels (Fig. 3.3 (e, f)).

IL-16 production was also noted in the supernatants of the bronchial explant cultures of moderately severe asthmatics (94.3 [26.8-126.8] pg/mg), which was significantly increased by stimulation with *Der p* allergen (157.5 [35.1-246.8] pg/mg; P=0.05). However, in similar fashion to IL-5 production, CTLA-4Ig did not significantly inhibit IL-16 production (115.9 [36.0-216.0] pg/mg (Figure 3.4).

Figure 3.2: Cytokine mRNA gene expression and protein production by bronchial explant cultures ofmoderately severe asthmatics after culture in medium alone, and after *ex vivo* stimulation with house dust-mite (*Der p*) allergen (5,000 SQ IU/ml) for 24 hours. (a) IL-5 mRNA gene expression (n=6), (b) IL-5 production (n=15), (c) IL-13 mRNA gene expression (n=6), (d) IL-13 production (n=8), (e) GM-CSF mRNA gene expression (n=6), (f) GM-CSF production (n=12).

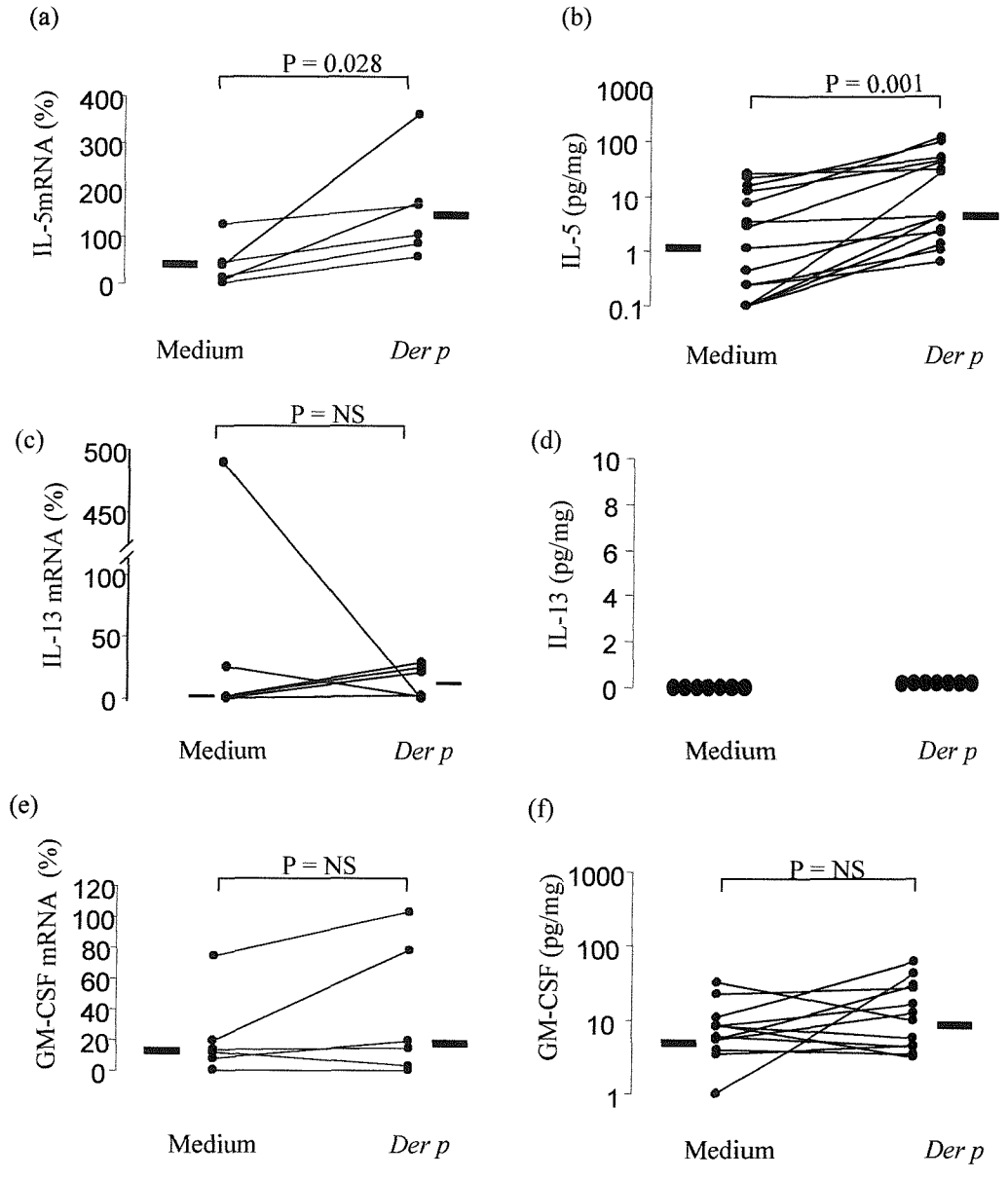


Figure 3.3: Cytokine mRNA gene expression and protein production by bronchial explants of moderately severe asthmatics after culture for 24 hours in the presence of *Der p* allergen (5,000 SQ IU/ml)in the absence or presence of CTLA-4Ig (25 μg/ml). (a) IL-5 mRNA transcripts (n=5);(b) IL-5 production (n=12); (c) IL-13 mRNA gene transcripts (n=6); (d) IL-13 production (n=8); (e) GM-CSF mRNA transcripts (n=6); (f) GM-CSF production (n=11).

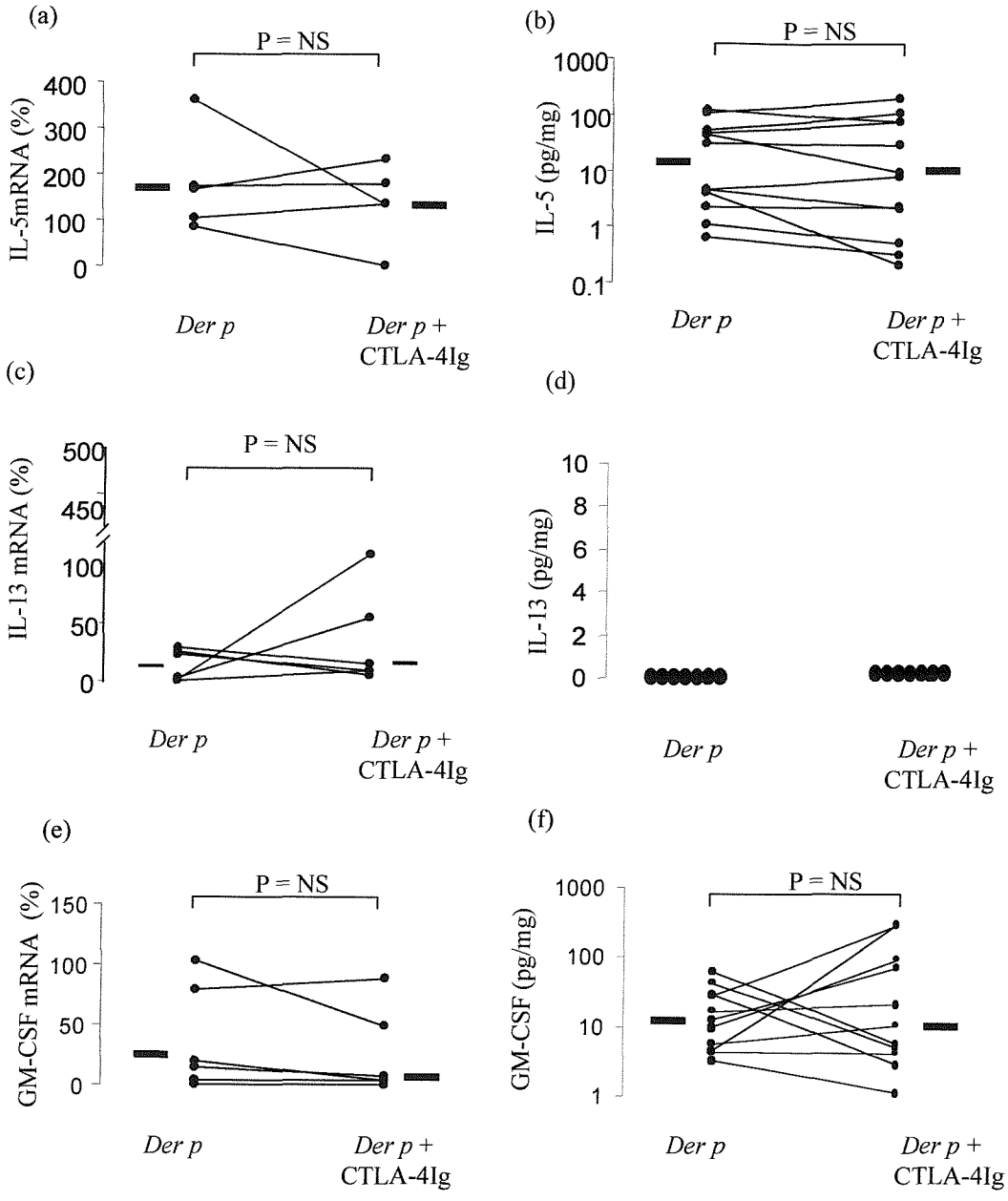
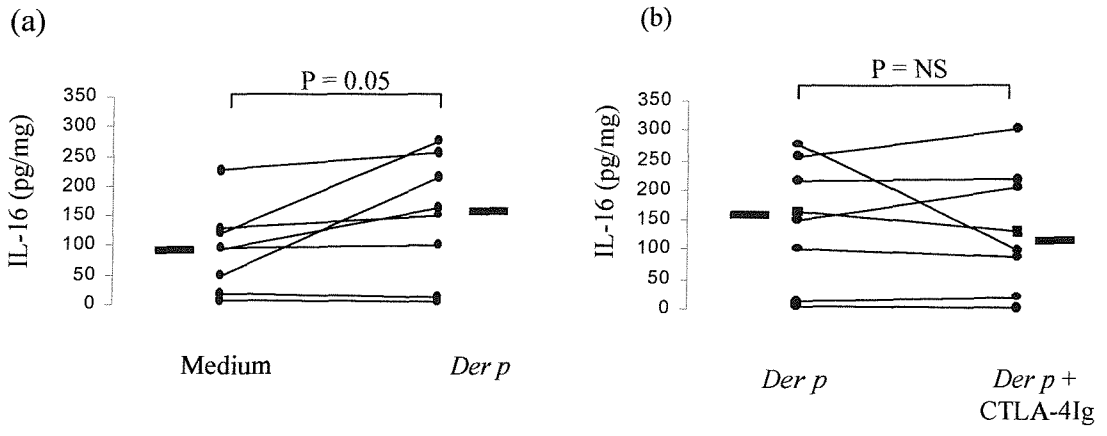
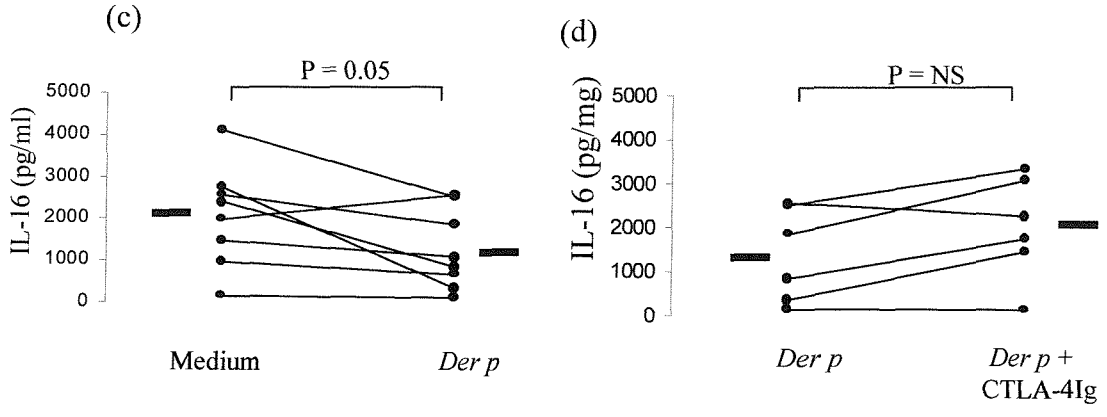


Figure 3.4: (a, b) Interleukin-16 production by bronchial explant cultures of moderately severe asthmatics after culture for 24 hours in either medium alone, *Der p* allergen (5,000 SQ U/ml), or *Der p* allergen plus CTLA-4Ig (25μg/ml), (n=8). (c, d) Interleukin-16 production by PBMC cultures of moderately severe asthmatics after culture for 24 hours in either medium alone, *Der p* allergen (5,000 SQ U/ml), or *Der p* allergen plus CTLA-4Ig (25μg/ml).

Bronchial Explants



PBMCs



3.3.2 Immunohistochemistry staining of bronchial biopsies

Immunohistochemical analysis of bronchial biopsies for infiltrating inflammatory cells

To characterise the numbers of inflammatory cells in the bronchial mucosa of the moderately severe asthmatics, a number of the asthmatic subjects had bronchial biopsies processed into GMA resin, and sections stained with immunohistochemical markers for resident inflammatory cells as described in the methods (section 2.9). These studies confirmed the presence of $CD3^+$ T lymphocytes (Figure 3.5), including $CD4^+$ and $CD8^+$ subsets; Neutrophil elastase (NE^+) neutrophils; ($EG2^+$) eosinophils; ($CD68^+$) macrophages; and ($CD19^+$) B lymphocytes, but only occasional $CD1a^+$ dendritic cells in the mucosa and submucosa of the moderately severe asthmatic subjects (Table 3.2). The numbers of eosinophils were low, but comparable with biopsies obtained from mild asthmatic subjects. It was not possible to obtain biopsies from all subjects due to the limitations on the total number of biopsies taken at each bronchoscopy for ethical reasons.

Table 3.2: Infiltrating inflammatory cell counts in submucosa and mucosa of bronchial biopsies from a proportion of moderately severe asthmatics. Results are expressed as the numbers of inflammatory cells per mm^2 of submucosa, and per mm length of epithelium, and expressed as medians (interquartile ranges).(n=6).

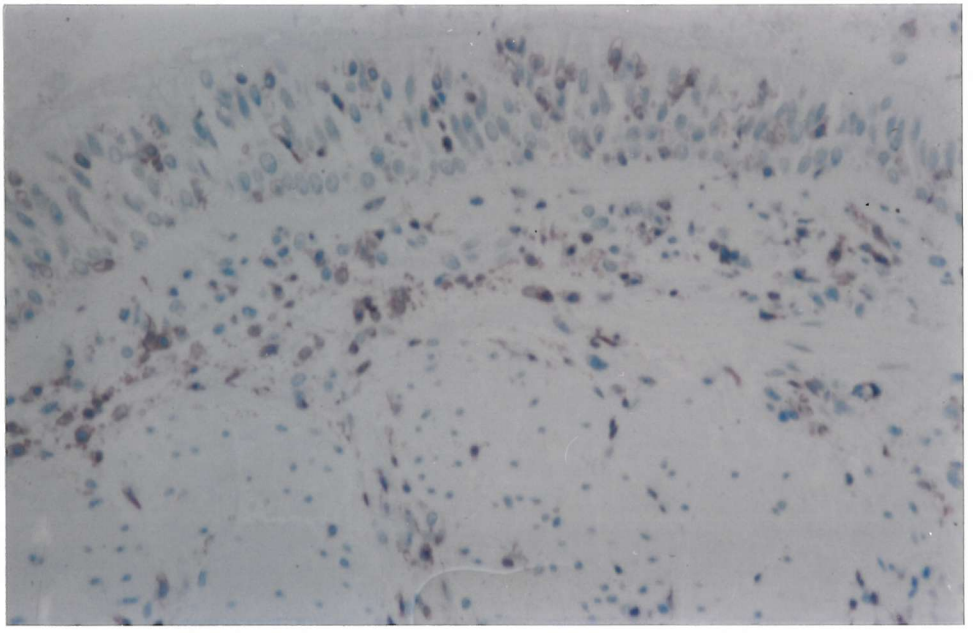
Inflammatory Cells (marker)	**Submucosa** (median (range))	**Bronchial Mucosa** (median (range))
Lymphocytes $CD3^+$	29.4 (15.2-69.5)	1.5 (0-8.7)
$CD4^+$	7.9 (0.3-16.3)	0 (0-0.3)
$CD8^+$	1.5 (0-4.4)	0 (0-0.6)
Eosinophils ($EG2^+$)	0.7 (0-7.2)	0 (0-0.6)
Mast cells ($AA1^+$)	8.7 (5.3-28.7)	0 (0-0.4)
Neutrophils (NE^+)	5.7 (0.3-9.6)	0
B lymphocytes ($CD20^+$)	0.96 (0.3-10.6)	0
Dendritic cells ($CD1a^+$)	0.7	0

Bronchial mucosal expression of CD80, CD86, CD28, and CTLA-4Ig

To assess the expression of co-stimulatory molecules in the bronchial mucosa of these asthmatic subjects, bronchial biopsy sections were stained with monoclonal antibodies for the co-stimulatory molecules CD28, CTLA-4, CD80 and CD86. The antibodies were initially titrated and optimised on human tonsil and nasal polyp. A characteristic staining pattern on the cell surface was noted for CD80 and CD86 on sections of nasal polyp, and an intracellular granular staining pattern was noted for CTLA-4 (Figure 3.5). CTLA-4 is stored in intracellular cytoplasmic vacuoles within T cells, and is transported to the cell surface upon cellular activation. CD28 staining was observed in human tonsil but not on sections of the bronchial tissue. However, there was no significant staining of CD28 or CTLA-4 cells in the bronchial biopsies obtained from our subjects, despite the presence of $CD3^+$ T lymphocytes.

Figure 3.5: (a) Immunostaining of bronchial biopsy sections from a moderately severe asthmatic for $CD3^+$ T lymphocytes (Magnification factor, 2.56 x x 10^3), 5.12 x 10^3, or 8.06 x 10^3). A typical bronchial biopsy includes the bronchial epithelium, subepithelial myofibroblast layer and the underlying submucosa with resident inflammatory cells. Smooth muscle tissue can also be identified in certain bronchial biopsies. Positive staining of nasal polyp tissue for: (b) CD80 (Magnification factor, 8.06 x 10^3); (c) CD86 (Magnification factor, 8.06 x 10^3); (d) CTLA-4 (Magnification factor, 8.06 x 10^3). (e) Immunostaining of human tonsil tissue for CD28 (Magnification factor, 8.06 x 10^3).

(a)



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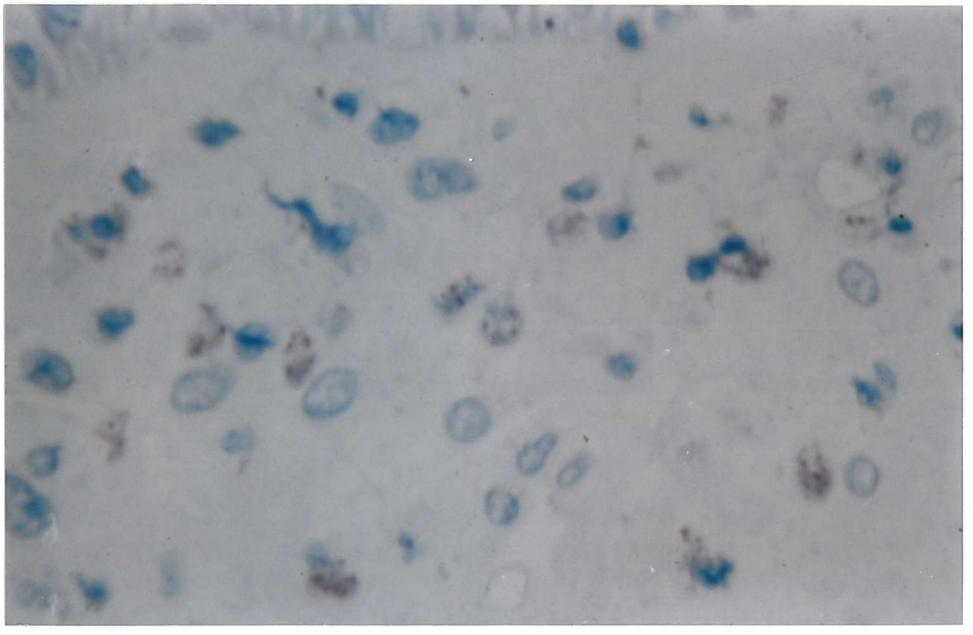

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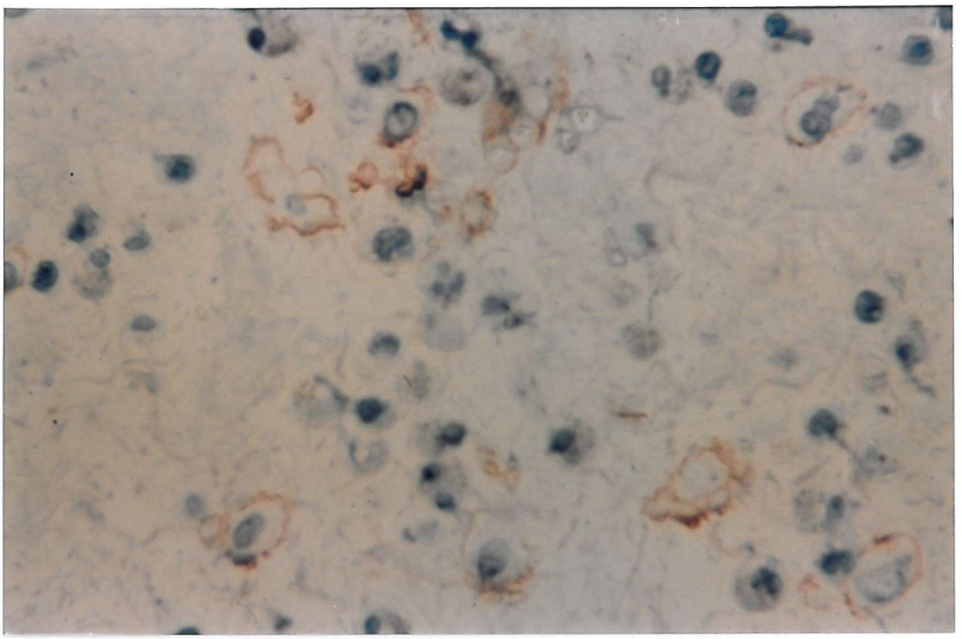
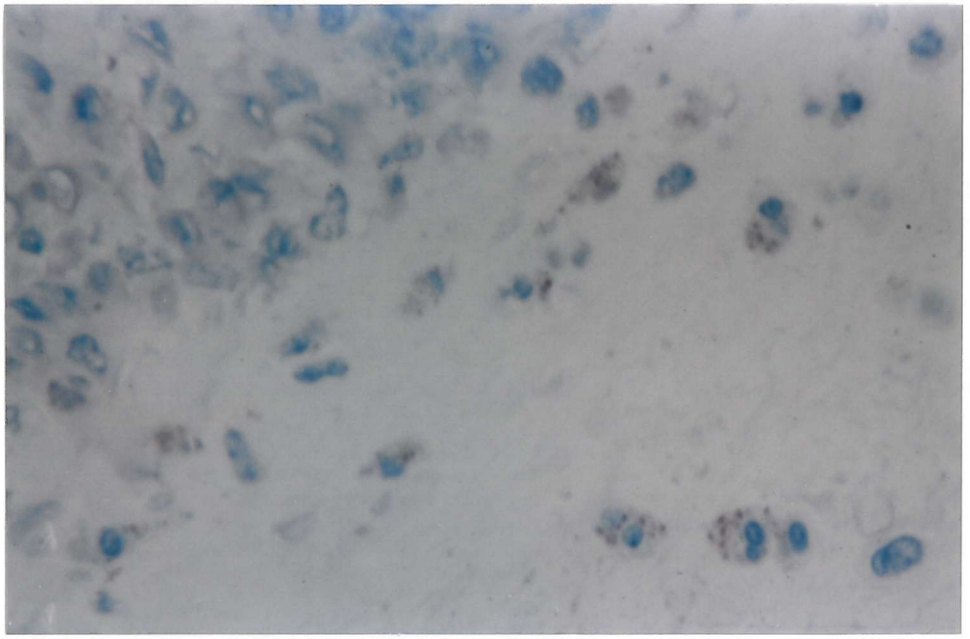
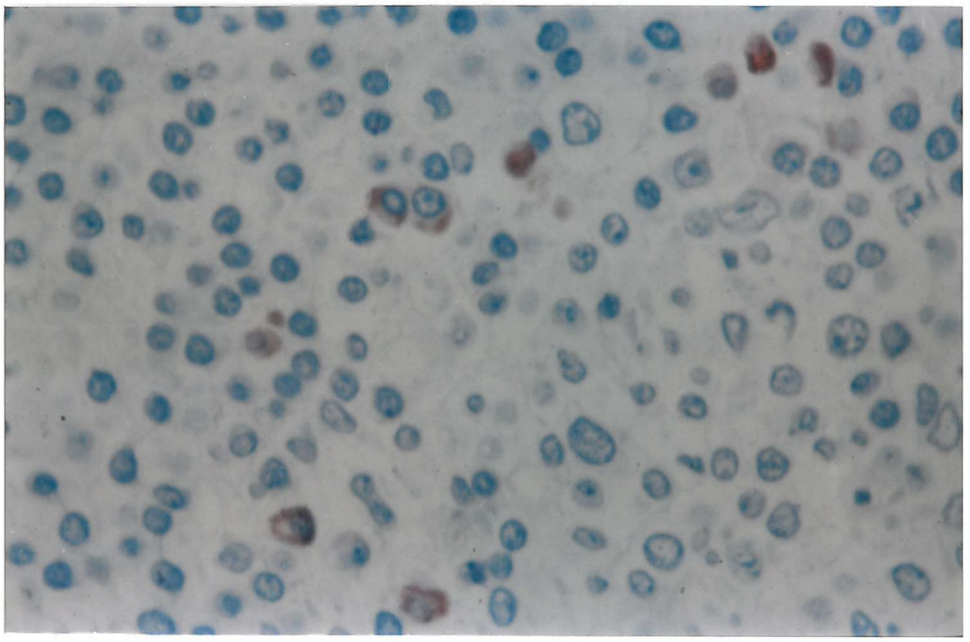


(d)



(e)



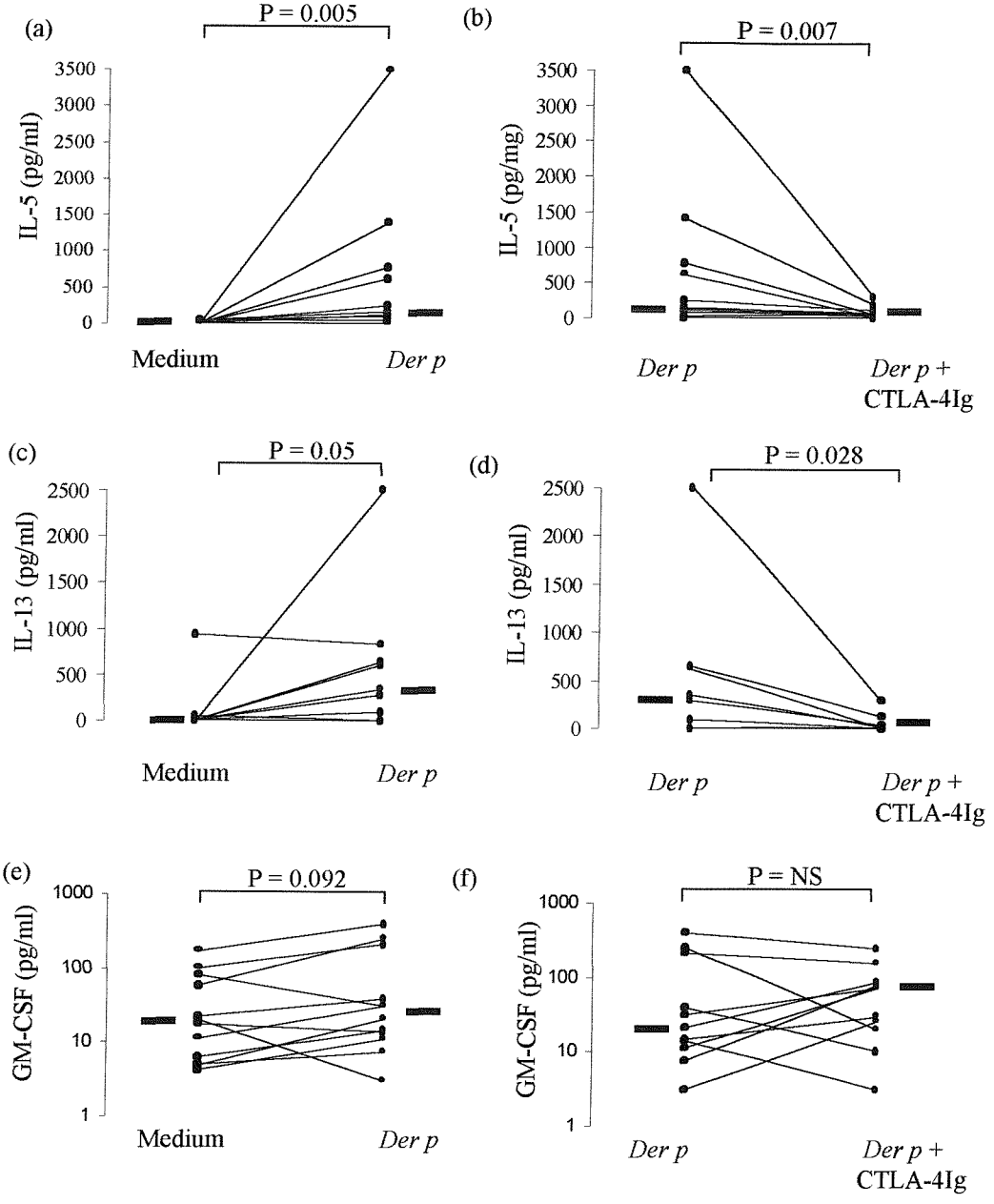
3.3.3 Allergen-induced cytokine release in PBMC cultures of moderately severe asthmatics:

Since there was no effect of CTLA-4Ig on allergen-induced cytokine release from the explants, control experiments were performed using PBMCs from the same volunteers. Using these cultures, spontaneous IL-5 production was low (21.7 (6.0-44.7) pg/ml), but increased significantly with allergen stimulation (106.6 (52.2-217.4) pg/ml; P = 0.005; Fig. 3.6a). Similarly, the baseline production of IL-13 was low (0 (0-22.6) pg/ml) but increased significantly with allergen stimulation (345 (51.1-727.0) pg/ml; *P* = 0.05; Fig. 3.6c). GM-CSF demonstrated a trend for increased production with allergen stimulation (18.5 (5.3-74.3) pg/ml versus 25.7 (11.8-168.8) pg/ml), however, this did not reach statistical significance (*P*=0.092, n=12; Fig. 3.6e).

In contrast to the bronchial explant cultures, the allergen-induced release of IL-5 (150 (91-770) pg/ml) was effectively suppressed by CTLA-4Ig (46.1 (26.7-95.0) pg/ml; *P*=0.007; Fig. 3.6b). In similar fashion to IL-5 production, the allergen-induced release of IL-13 in the PBMC cultures of moderate asthmatics was significantly inhibited by the addition of CTLA-4Ig (11.4 (0-100.1) pg/ml; *P* = 0.028), (Fig. 3.6d). Allergen stimulation augmented the release of GM-CSF from 17 (5-57) pg/ml to 20.4 (11.1-212) pg/ml (*P* = 0.029, n=11), but the addition of CTLA-4Ig had no effect.

Basal IL-16 production was also noted in the PBMC cultures of moderately severe asthmatics (2474 [1532-3080] pg/ml), which was lower in cells exposed to allergen (1353 [310-2523] pg/ml; *Der p*; n=6; *P*=0.05) (Figure 3.4). IL-16 production was not significantly affected by the addition of CTLA-4Ig to the PBMC cultures (1994 [1109-3137] pg/ml).

Figure 3.6: Cytokine release by PBMC cultures from moderately severe asthmatics after culture in medium alone or House dust-mite allergen (*Der p* 5,000 SQ IU/ml) in the absence or presence of the fusion protein CTLA-4Ig. (a,b) IL-5 production (n=11); (c,d) IL-13 production (n=9); (e,f) GM-CSF production (n=12).



3.4 DISCUSSION

In this study, every attempt was made to recruit patients with well characterised chronic severe asthma within clear specifications of their symptoms, baseline spirometry and bronchial hyperresponsiveness. Despite this, some heterogeneity was apparent in measures of lung function. This is a characteristic feature of moderate-severe asthma where there is little relationship between baseline spirometry or bronchial hyperresponsiveness and clinical manifestations of asthma, and considerable independent variations in these indices over time [388, 389]. It is likely that different subphenotypes exist as asthma becomes more severe and chronic, which may also explain heterogeneity of responses to various therapeutic modalities such as leukotriene receptor antagonists or antiIgE.

The bronchial explant culture model developed is a useful integrated cell system for assessing allergic responses in bronchial tissue using biopsies obtained by fibreoptic bronchoscopy [222]. Unlike isolated cell culture systems, the explanted bronchial tissue includes structural elements such as the airway epithelium, and also the inflammatory cells resident in the airways, including resident T cells, mast cells, eosinophils, macrophages, and $CD1a^+$ dendritic cells. This makes observations in this complex cell system particularly relevant to asthmatic airway inflammation. As two bronchial biopsies are required for each culture condition of the explant study to optimise measurable cytokine production and reduce variability, this places a limit to the number of simultaneous comparisons that can be made in the bronchial explant system due to the limitation on the number of bronchial biopsies that can be safely obtained at bronchoscopy from an individual subject. It is also possible that differences in the composition of individual bronchial biopsies (e.g size of biopsy, length of epithelium, or depth of submucosa in biopsy) may limit the widespread application of this system to study the contribution of individual cells such as the bronchial epithelium in an integrated system like the bronchial explant.

In this study, the bronchial tissue of moderately severe asthmatics was infiltrated by inflammatory cells including $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes, eosinophils, neutrophils, macrophages, but only occasional $CD1a^+$ dendritic cells were noted. However, there was no significant cell surface expression noted for the CD28-B7 co-stimulatory molecule ligands (CD28, CTLA-4, CD80 and CD86) using the current antibodies in the bronchial mucosa of the moderately severe asthmatic subjects assessed. The activity of these antibodies had been initially titrated and optimised on human tonsil and nasal polyp, with a characteristic cell surface staining pattern being noted for CD80 and CD86. The absence of significant

expression of co-stimulatory molecules in the airways of moderate asthmatic subjects may be related to prior treatment with inhaled corticosteroids, which has been shown to reduce the number and activation of dendritic cells in the bronchial mucosa of asthmatic subjects [83]. The lack of expression of CD80 and CD86 in the biopsies may also explain the lack of effect of the fusion protein, CTLA-4Ig in the severe asthmatics.

The involvement of CD28-B7 co-stimulatory molecules in the airway inflammatory process has been assessed by a number of studies. Hofer *et al* [242] has noted increased expression of CD86 on circulating B cells from patients with asthma after exposure to specific allergen. Studies performed by Jaffar *et al* in mild asthma using FACS analysis of enzymatically digested bronchial biopsies from mild asthmatics noted a low number of $CD1a^+$ dendritic cells, and only occasional cells staining positive for CD80 and CD86 [239]. However, Agea *et al* [95] has documented increased expression of CD86 on alveolar macrophages in the BAL of mild asthmatics compared to non-asthmatic controls. Balbo *et al* also noted increased basal expression of CD80 but not CD86 on alveolar macrophages of asthmatic subjects compared to healthy subjects, and the expression of CD86 but not CD80 was further increased following allergen challenge [243]. Burastero *et al* confirmed higher expression of CD80 on alveolar macrophages from asthmatic subjects, with an increased ability to stimulate T cell proliferation and cytokine production compared to alveolar macrophages from healthy controls [244]. This suggests that alveolar macrophages from mild asthmatics may have an increased potential for antigen presentation compared to non-asthmatic subjects. However, there was no significant expression of co-stimulatory molecules in the bronchial mucosa or submucosa of moderately severe asthmatics in this study.

A number of studies support the role of IL-5 in promoting the development, recruitment, and activation of eosinophils in inflamed asthmatic airways [245-247]. In the present study, we have demonstrated IL-5 production by bronchial explant cultures and PBMC cultures of moderately severe asthmatics, with a significant increase in IL-5 upon allergen stimulation. Overall, the levels of IL-5 in the explant cultures from these moderately severe asthma subjects were comparable to those found in mild asthma, despite the fact that these patients had been receiving prolonged inhaled corticosteroid therapy until one week before the biopsies were taken [222, 239]. However, in some cases, the production of IL-5 was low, even upon exposure to allergen. We also demonstrated mRNA expression for IL-13 in the explanted bronchial tissue of moderately severe asthmatics, but in contrast to the explant

studies performed in mild disease, we did not detect IL-13 protein production in the explants [222, 239]. It is possible that the low levels of IL-5 in some explant cultures and inability to detect IL-13 in the bronchial explant cultures of severe asthmatics in this study may be related to the persistent action of inhaled corticosteroids in these particular patients. Studies have shown that withdrawal of inhaled corticosteroids, even for one week, results in the return of asthma symptoms [248, 249], a reduction in peak flow readings, increases in airway hyperresponsiveness, and an associated increase in airway inflammation, as reflected by increased eosinophil progenitors in blood, and increased eosinophils and mediators in sputum [249, 250]. Whether withholding corticosteroids for one week is sufficient to completely restore the increased production of IL-5 and IL-13 is unknown. Given the risk of unacceptable exacerbation of asthma symptoms it was felt ethically unacceptable to withhold corticosteroids for more than one week.

GM-CSF plays an important role in promoting airway inflammation by promoting the maturation of dendritic cells, and prolonging the survival of neutrophils and eosinophils in the airways [251]. Constitutive release of GM-CSF was noted in the explant cultures of moderately severe asthmatics, although production was not significantly augmented by allergen. This differs from the explant studies in mild asthma, where under identical *in vitro* culture conditions, no significant levels of GM-CSF were noted, either spontaneously or following allergen exposure. This difference suggests a potential role for GM-CSF in perpetuating the airway inflammation at the severe end of the disease spectrum [251]. Since bronchial epithelial cells, mast cells, myofibroblasts [252], and macrophages [170] generate plentiful amounts of GM-CSF, it is likely that its release in the airways of asthmatics is derived at least partially from these cells, which would not be expected to require CD28-mediated co-stimulation for cytokine release.

In contrast to the relative effectiveness of CTLA-4Ig to suppress Th2 cytokine responses in mild asthma [222, 237], we have found that this fusion protein did not significantly inhibit IL-5, GM-CSF or IL-16 release in the bronchial explant studies of moderately severe asthmatics. However, the effectiveness of CTLA-4Ig at suppressing IL-5 and IL-13 in the PBMC cultures suggests that the activation of circulating T cells of these subjects still requires CD28-mediated co-stimulation as also is found in patients with mild allergic asthma [239]. These data support the idea that allergic responses in the bronchial mucosa in moderately severe asthma are less dependent on CD28-mediated co-stimulation

when compared to circulating mononuclear cells from the same subjects. While it is possible that relatively higher doses of the fusion protein may be required to inhibit co-stimulation in the bronchial tissue in more severe asthma this seems unlikely in view of its efficacy in the PBMC cultures and in the explant studies of mild asthmatics [222].

There was substantial basal production of IL-16 by the bronchial tissue cultures of moderately severe asthmatics, which is comparable to the levels noted by Dent *et al* using a similar bronchial explant model [253]. However, only in the present study was the production of IL-16 significantly increased following allergen exposure, which may be related to the higher dose of allergen utilised in the present study (5,000 IU/ml versus 2,000 U/ml). However, the allergen-induced production of IL-16 was not inhibited by the addition of CTLA-4Ig, suggesting that this process was independent of CD28-B7 co-stimulation. The role of co-stimulatory signalling in the production of IL-16 and the T lymphocyte chemotactic activity (TLCA) of bronchial explant supernatents of mild and moderate asthmatics has recently been investigated by Dent *et al.* Exposure of bronchial explants to allergen led to a significant increase in the production of IL-16 in the explant culture supernatants of mild but not steroid-dependent asthmatics. The TLCA of mild asthmatics was inhibited by antibodies to the counter-ligands of CD28, CD80 (94%) and CD86 (62%), but IL-16 production was only inhibited by anti-CD86 (77%). Of particular interest, CTLA-4Ig did not inhibit the production of IL-16 or the TLCA in the supernatents of bronchial explant cultures of a similar group of moderately severe asthmatics [253]. These data suggest that CD28-B7 co-stimulation is not as critical for the generation of cytokines and chemokines in the bronchial tissue of more severe forms of asthma.

Differences in the requirement for CD80 and CD86 co-stimulation have been shown in the circulation compared to the bronchial mucosa of mild asthmatics. Larchè *et al* [254] noted that the allergen-induced proliferation of T cells isolated from broncho-alveolar lavage (BAL) fluid was inhibited by a selective anti-CD86 antibody, but not by a selective anti-CD80 antibody. In the earlier explant studies in mild asthma, it was observed that allergen-induced IL-5 and IL-13 production in bronchial explant cultures was inhibited by an anti-CD80 antibody, and to a lesser extent by an anti-CD86 monoclonal antibody [239]. However, it was also noted that cytokine production by PBMCs of mild asthmatics was not affected by anti-CD80, but was reduced by anti-CD86, and was strongly attenuated with a combination of both antibodies [239].

The requirement for CD-28 mediated co-stimulation in circulating inflammatory cells has previously been demonstrated. Both Van Neervan *et al* [255] and Larché *et al* [254] have shown that allergen-induced cytokine production and cell proliferation in PBMC cultures from mild asthmatics are inhibited by either CTLA-4Ig or anti-CD86, but not by anti-CD80. These observations have been confirmed in both $CD45RA^+$ and $CD45RO^+$ T cells, suggesting that CTLA4-Ig can inhibit immune responses in T cells of both naïve and memory phenotype. However, when the responses of allergen-specific peripheral blood $CD4^+$ T cell lines were assessed, cell proliferation and cytokine production were found to be independent of CD80 or CD86 costimulation. This supports data from *in vitro* studies by Gause *et al* [256] showing that ongoing, memory-type T cell-dependent immune responses are less dependant on CD28-B7 costimulation than are primary immune responses involving naïve T cells. There is recent evidence to suggest the existence of other co-stimulatory pathways [257], including ligation of Ox40-Ox40L [258], ICOS (Inducible co-stimulator) signalling [259], or T1/ST2 (member of the IL-1 receptor family) [260], that may play a more important role in Th2-mediated immune responses in the lung mucosa. ICOS is of particular interest in this respect, because in ovalbumin sensitised mice, ICOS mRNA is markedly reduced in the lungs after allergen challenge, and systemic administration of an ICOS-Ig fusion protein markeedly inhibited antigen-induced IL-5 production in parallel with an 80% reduction in eosinophil infiltration, while it failed to influence a neutrophilic Th1 response involving IFN-γ . Effective antagonists of these pathways were not available for use while this study was being carried out. It would be important to test these agents, in particular the recently availabe human ICOS-Ig, in the integrated human tissue system of the severe bronchial asthmatic explant system.

It is important that appropriate blocking reagents against these ligands and their receptors are developed to investigate whether they can substitute for B7/CD28 as the second signal in the airway mucosa of more severe asthma. While the earlier studies in mild asthma support a role of CD28 co-stimulation in regulating airway T cell cytokine production in asthma, the responses appear to differ in more severe and chronic disease. Taken together, these results suggest that CD28-B7 co-stimulation may not be as critical for cytokine production in the bronchial mucosa in more severe forms of asthma. T cells resident in the airways of severe asthmatics may behave more like T cell lines, being capable of activation and cytokine production independent of CD28-B7 co-stimulatory signals from APCs. Alternatively, pro-inflammatory cytokine production in the airway tissue of more severe asthmatics may also be derived from cells distinct from T cells, which may not require co-

stimulation for cytokine production. While T cells are considered to be the principal source of IL-5 in asthma, other cells have the capacity to generate and release this eosinophilopoietic cytokine, including mast cells, eosinophils, and more recently, the bronchial epithelium [134, 172].

In conclusion, the current study has confirmed ongoing allergen responsiveness and pro-inflammatory cytokine production by inflammatory cells in the airways and circulation of moderately severe asthmatics. CTLA-4Ig was found to effectively suppress allergen-induced IL-5 production in PBMC cultures but not in bronchial tissue cultures. It is possible that airway cytokine production in more severe asthma is less amenable to modulation by antagonists of co-stimulation than is found for mild asthmatics. A recent clinical trial has reported the use of CTLA-4Ig in the treatment of patients with severe psoriasis, and the investigators noted a reduction in parameters of T-cell mediated inflammatory skin lesions and clinical parameters of disease severity [261, 262]. Further studies are required to clarify the precise role of CD28-B7 co-stimulation in asthmatic airway inflammation and to assess the importance of the tissue microenvironment in pulmonary inflammatory responses in severe asthma.

CHAPTER FOUR

Cytokine release by epithelial cell cultures of atopic asthmatics and normal control subjects after exposure to *Dermatophagoides pteronissinus* allergen or Tumor Necrosis Factor-α

4.1 INTRODUCTION

It is well recognised that environmental exposure to allergens plays a major role in the initial development of asthma, and exacerbation of pre-existing asthma. *Der p* is one of the most important environmental aero-allergens worldwide [46]. Consistent with this, the bronchial epithelium is the primary interface between the external and internal mileau of the airways, and is likely to be exposed to the highest concentrations of inhaled aero-allergens, such as house dust mite allergens, in the airways of asthmatic subjects. It is increasingly being recognised that the interaction of allergens with the bronchial epithelium may play an important role in the initiation and maintenance of airway inflammation in asthma [53,49]. In addition to its function as a physical barrier, the bronchial epithelium has also been identified as a major source of pro-inflammatory mediators, cytokines, chemokines, and growth factors. *In vitro* and *in vivo* studies have noted the production of cytokines and growth factors, including IL-8, GM-CSF, and RANTES, by airway epithelial cells in response to a variety of stimuli [53]. Although a number of studies have been performed using epithelial cell lines, there is limited information available regarding the effects of house dust mite allergen extracts on the release of cytokines and chemokines from epithelial cells derived from the airways of patients with allergic asthma.

The studies presented in chapter three have shown an increased release of cytokines and chemokines relevant to asthmatic inflammation by bronchial explant tissue cultures of patients with allergic asthma after exposure to *Der p* allergen extract. To assess the ability of the bronchial epithelium to contribute to cytokine release in the airways of asthmatics, epithelial cell cultures were established from primary bronchial epithelial cells taken by bronchial brushings from the airways of allergic asthmatics, and cytokine release assessed after exposure to *Der p* allergen, and TNF-α was used as a control stimulus. In parallel the effects of corticosteroids were analysed.

4.2 Objectives

- To establish the contribution of the bronchial epithelium to the allergen-induced release of cytokines and chemokines, primary bronchial epithelial cell cultures were established from bronchial brushings of the airways of mild and moderate asthmatic subjects, and normal subjects, and the production of GM-CSF, IL-8, and RANTES measured after *in vitro* stimulation with *Der p* allergen or TNF-α.

- To determine the ability of corticosteroids to inhibit the epithelial production of cytokines and chemokines, primary bronchial epithelial cell cultures of normal and allergic asthmatic subjects were pre-incubated with dexamethasone prior to stimulation with *Der p* allergen or TNF-α, and cytokine release measured after culture in the absence or presence of dexamethasone.

4.2 METHODS

4.2.1 Subjects

Mild and moderately severe asthmatics and non-atopic non-asthmatic control subjects were recruited and were characterised according to symptoms, pulmonary function, asthma medication requirements (Table 4.1) as described in the methods section (Chapter 2).

4.2.2 Fibreoptic bronchoscopy and bronchial brushings

Fibreoptic bronchoscopy was performed and epithelial cells obtained by bronchial brushings in accordance with standard published guidelines [16] as described in chapter 2 (section 2.2).

4.2.3 Primary bronchial epithelial cell cultures

The epithelial cells were expanded in culture, and grown to confluence on 24 well plates as described in detail in chapter 2 (section 2.3). Prior to allergen exposure, the cells were rendered quiescent by replacing the culture medium in each well with one millilitre of basal medium (BEBM), lacking EGF or bovine pituary extract, but supplemented with ITS (Insulin, transferin, and sodium selenite). After 24 hours, the epithelial cells were then cultured for a further 24 hours in either BEBM alone, or *Der p* allergen extract (Aquagen ALK, Denmark) at a concentration of 2,500 and 5,000 SQ IU/ml or TNF-α (20 ng/ml). To assess the ability of corticosteroids to suppress allergen-induced cytokine release, parallel cultures were performed in *Der p* allergen (5,000 SQ IU/ml) plus dexamethasone (10^{-6}M), or TNF-α (20ng/ml) plus dexamethasone (10^{-6}M). The supernatants were then removed, centrifuged at 4°C and stored in aliquots, at –80°C for later analysis for cytokines by ELISA as described in detail in section 2.8 of chapter 2.

4.2.4 Methylene Blue Assay

The numbers of epithelial cells in individual cultures were quantified using a methylene blue assay and cytokine release in individual cultures corrected for cell number, and expressed as picograms (or nanograms) per million of epithelial cells cultured as described in detail in section 2.4 of chapter 2.

4.2.5 Cytokine measurement by ELISA

The levels of cytokine released into the supernatants of primary epithelial cell cultures were determined using commercially available ELISA kits as described in section 2.8 in chapter 2.

4.2.6 Statistical analysis

Cytokine values were analysed by non-parametric statistical tests. The Wilcoxon signed rank test for paired data was used for within-group comparison of cytokine protein levels, using SPSS 7.5 for Windows. The Mann Whitney U test was used for between-group comparison. Values of $P < 0.05$ were accepted as statistically significant.

Table 4.1: (a) Clinical characteristics of atopic asthmatic subjects.

Subject	Age	Sex	Atopy	Asthma severity	FEV_1	FEV_1 %Pred	Histamine PC_{20}
B.O.	26	M	Y	Mild	3.78	89	<8*
E.L.	29	F	Y	Mild	3.07	-	-
C.R.	19	M	Y	Mild	-	91	<8*
M.J.	20	M	Y	Mild	-	-	4
A.B.	23	M	Y	Mild	4.2	-	-
A.C.	21	M	Y	Mild	2.83	68	0
N.Z.	19	M	Y	Mild	5.15	100	15.78
K.S.	37	M	Y	Mild	3.95	99.7	0.9
E.W.	27	M	Y	Mild	4.1	103	0.32
H.Y.	19	F	Y	Mild	3.05	94	>32
T.R.	25	M	Y	Mod. Severe	3.06	69	.07
M.K.	21	M	Y	Mod. Severe	4.9	100	1.8
S.K.	55	M	Y	Mod. Severe	2.26	68	0
R.M.	28	M	Y	Mod. Severe	3.81	74.3	0.5
C.L.	39	M	Y	Mod. severe	2.06	78	0.76

* Screening histamine PC20 was performed by Dr D. Buchanan and the calculated PC20 was not recorded but noted as less than 8. In selected induviduals, the FEV1 as a percentage of predicted was not recorded in the screening records.

Table 4.1: (b) Clinical characteristics of healthy control subjects.

Subject	Age	Sex	Atopy	Asthma	FEV_1	FEV_1 %Pred	Histamine PC_{20}
DD	40	F	N	N	4.7	100	>16
GJ	20	M	N	N	5.45	104	>8
RC	21	M	N	N	5.05	100	>8
SL	20	M	N	N	5.55	110	>8
FB	25	M	N	N	3.90	100	>8
JL	33	M	N	N	-	100	>16
FW	29	F	N	N	3.5	100	>8
AL	21	M	N	N	4.64	100	>8
MN	22	M	N	N	4.7	100	>8
WP	19	M	N	N	5.2	100	>32
AH	21	F	N	N	-	100	>32
KI	19	F	N	N	3.54	100	>16
SP	20	F	N	N	3.0	78	>16

4.3 RESULTS

4.3.1 Bronchial epithelial cell cultures

Primary cultures of bronchial epithelial cell cultures were established from both atopic asthmatics and normal control subjects (Figure 4.1), with no significant differences being noted in growth characteristics between normal or asthmatic subjects.

4.3.2 Characterisation of epithelial cells obtained by bronchial brushings and epithelial cells in culture

Differential cell counts were performed on cytospin preparations of the cellular suspension obtained by bronchial brushings. The yield of epithelial cells obtained by brushings of either normal or asthmatics was greater than 90 percent, with the remaining cells being composed of neutrophils or eosinophils. Immunohistochemistry was used to show that the cells were predominantly columnar-type epithelial cells, and stained positive for cytokeratin (CK-18) and negative for cytokeratin-13 (CK-13). However, in each case, some of the epithelial cells were of a basal cell phenotype co-expressing both CK-13 and CK-18 (Figure 4.3).

Primary epithelial cell cultures were established in culture in BEGM, with a frequency of 9/10 and 11/16 cultures for normal and asthmatic epithelial cells, respectively. The failure to establish cultures invariably resulted from overgrowth of fungal or bacterial infection rather than an inability of the cells to proliferate. The risk of infection was increased in bronchial brushings heavily contaminated with blood, so extreme caution was taken at bronchoscopy and brushings to minimise the trauma to the airway mucosal surface. There was no morphological differences noted between bronchial epithelial cell cultures derived from normal or asthmatic subjects. There was no difference in viability of the epithelial cell cultures from normal or asthmatic cultures, being 98% and 95%, respectively. There was no difference in the mean generation time of the cultures (Figure 4.2), with cell yields from 5-6 brushings being in the range (1.5-4.0 x 10^6) and (2.0-4.5 x 10^6) at the end of Passage 1. Cultures usually expanded through passage 2 and passage 3, but thereafter became quiescent. For large scale cultures, up to a maximum of 12-14 brushings have been collected, and was well tolerated by most patients; the increased numbers of brushings resulting in a corresponding increase in cell yield. Immuno-histochemical analysis of the culture monolayers confirmed staining for CK-13 and CK-18, suggesting these cells were of a basal cell phenotype.

Figure 4.1 : Photo-micrograph of primary bronchial epithelial cell cultures obtained from atopic asthmatic subject.

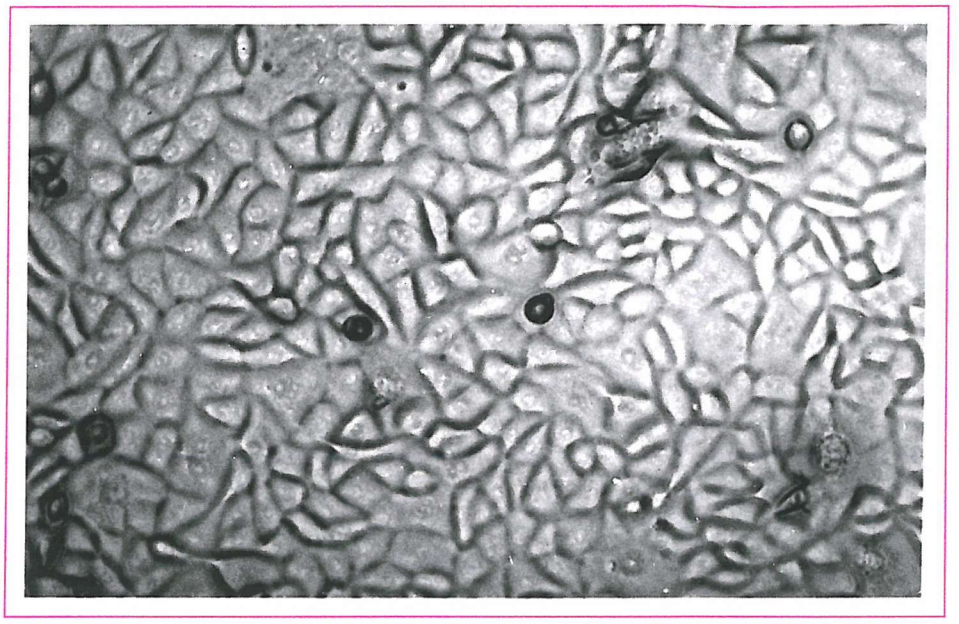


Figure 4.2 : Growth characteristics of primary epithelial cell cultures of normal and atopic asthmatic subjects represented as doubling index.

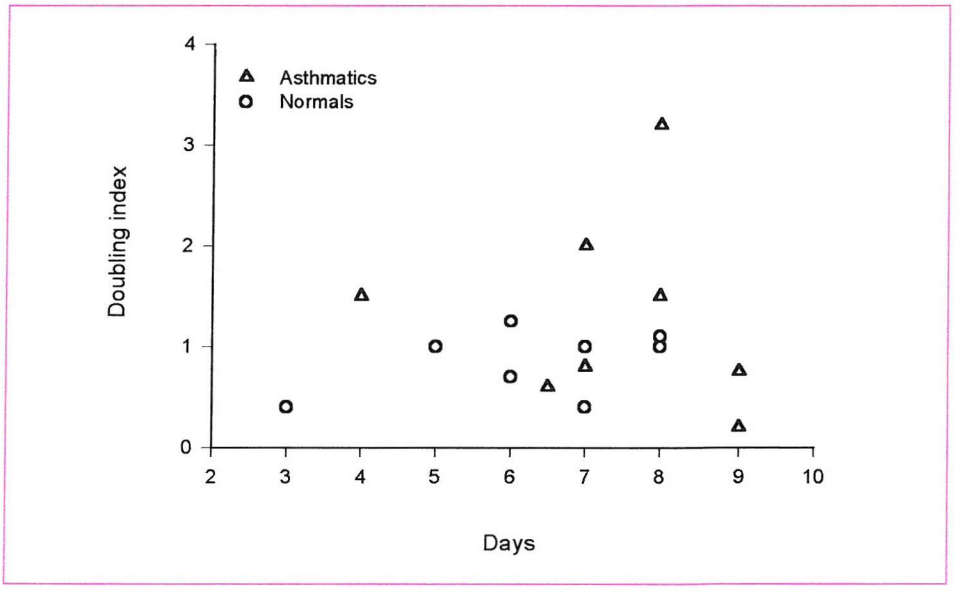
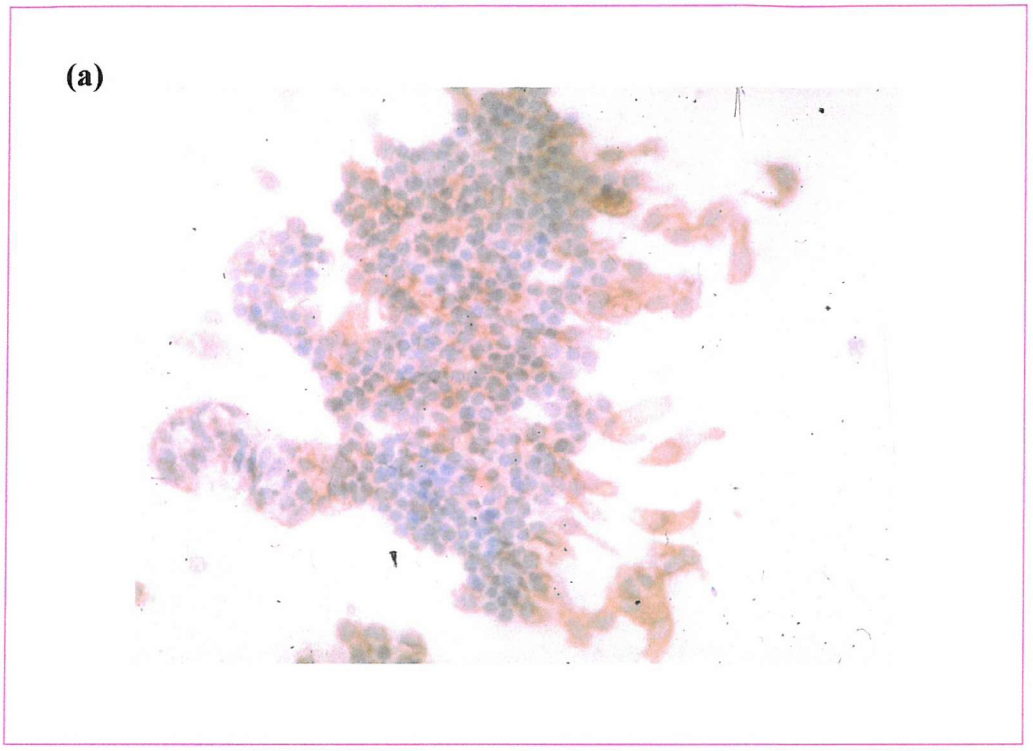
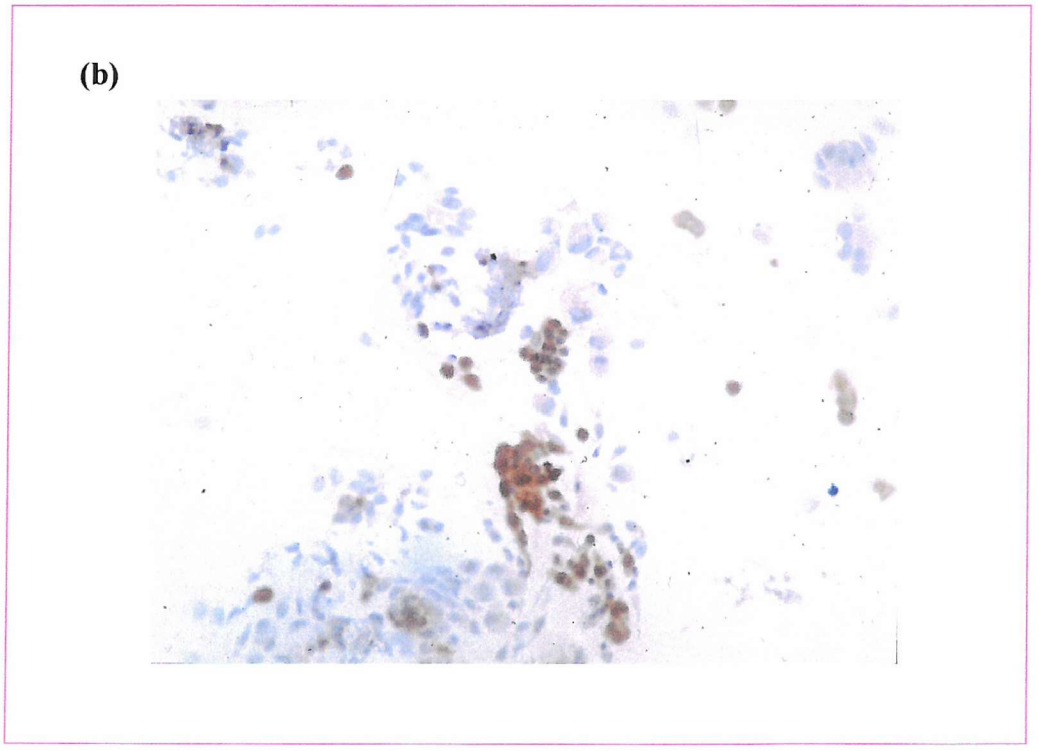


Figure 4.3 : Immunostaining of primary bronchial epithelial cells for cytokeratin (a) cytokeratin-13, and (b) cytokeratin-18.





4.3.3 Interleukin-8 release by epithelial cell cultures of both atopic asthmatics and normal control subjects is stimulated by *Der p* allergen, and TNF-α

To determine the effects of allergen and *TNF-α* on epithelial cells, bronchial epithelial cell cultures were exposed to either *Der p* allergen or TNF-α for 24 hours. There was constitutive release of IL-8 by epithelial cells from both normal and asthmatic subjects, with no significant differences noted between normal or asthmatic subjects. There was considerable heterogeneity in the amount of IL-8 release in individual cultures. The release of IL-8 by epithelial cells of both normal and atopic asthmatic subjects was significantly increased by exposure to *Der p* allergen at allergen concentrations of 2,500 IU/ml or 5,000 IU/ml (Figure 4.4). TNF-α was a potent stimulus for the release of IL-8 by epithelial cell cultures of both normal and asthmatic subjects, although there was no significant differences noted for IL-8 production between cultures derived from normal or asthmatic subjects (Figure 4.5).

4.3.4 GM-CSF release by epithelial cell cultures of both atopic asthmatics and normal control subjects is stimulated by *Der p* allergen, and TNF-α

In similar fashion to IL-8, there was a low basal production of GM-CSF by epithelial cell cultures of both normal and atopic asthmatics, with no significant difference being noted between the two groups. The release of GM-CSF was significantly increased after exposure of the epithelial cell cultures of normal and atopic asthmatic subjects to *Der p* allergen at concentrations of 2,500 IU/ml or 5,000 IU/ml (Figure 4.6). TNF-α also stimulated increased production of GM-CSF by epithelial cell cultures of both normal and asthmatic subjects (Figure 4.7). However, there was no significant difference noted in GM-CSF production by epithelial cell cultures from normal or asthmatic subjects.

4.3.5 RANTES release by epithelial cells of allergic asthmatics after exposure to *Der p* allergen or TNF-α

RANTES was released in a low basal level by epithelial cells of asthmatic and normal subjects, in the absence of stimulation. RANTES production was significantly increased by stimulation of epithelial cell cultures of asthmatic subjects with *Der p* 5,000 U/ml (Figure 4.8) and also after stimulation with TNF-α (Figure 4.9). However, there was no significant increase in the release of RANTES by epithelial cell cultures derived from normal subjects.

4.3.6 Dexamethasone reduces the release of GM-CSF, IL-8, and RANTES by epithelial cell cultures

The release of GM-CSF by epithelial cell cultures of normal or asthmatics after stimulation with allergen or TNF-α was significantly reduced by pre-treatment of the epithelial cell cultures with dexamethasone, although GM-CSF release still remained higher than that of unstimulated cultures suggesting a certain degree of steroid-insensitivity (Figure 4.6, 4.7).

IL-8 production after stimulation of epithelial cell cultures of both normal or asthmatic cell cultures with *Der p* or TNF-α was also reduced by dexamethasone, but again not to basal levels (Figure 4.4, 4.5).

In similar fashion to IL-8 and GM-CSF production, the release of RANTES by epithelial cells of asthmatic subjects was also significantly inhibited by pre-treatment with dexamethasone, although the production of RANTES was not completely suppressed to that of un-stimulated cultures (Figure 4.8, 4.9).

4.3.7 Absence of detectable production of IL-16 or Eotaxin by epithelial cells of normal or asthmatic subjects

Whereas there was significant increased production of GM-CSF, IL-8, and RANTES by the primary epithelial cell cultures, there was no detectable release of IL-16 or eotaxin by epithelial cells from normal or asthmatic subjects cultured in medium alone, or after stimulation with either allergen or TNF-α. However, the application of RT-PCR did confirm ongoing mRNA gene transcription for IL-16 by epithelial cells, but not for for eotaxin (Figure 4.10).

Figure 4.4: (a) IL-8 production by primary bronchial epithelial cells of atopic asthmatics (n = 14) after culture in medium or *Der p* allergen (2,500 or 5,000 SQ IU/ml) in the absence or presence of dexamethasone (1μM).

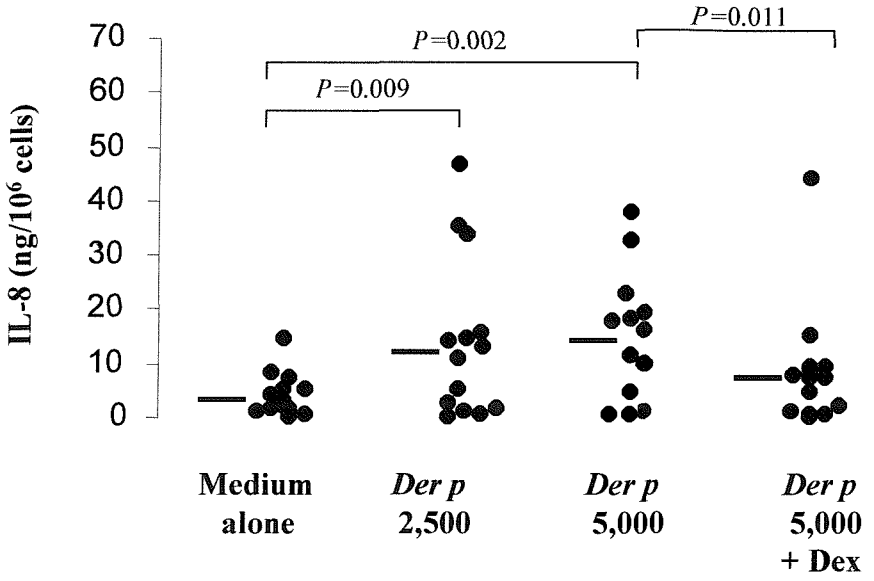


Figure 4.4: (b) IL-8 production by primary bronchial epithelial cells of normal controls (n = 12) after culture in medium alone, or *Der p* allergen (2,500 or 5,000 SQ IU/ml) in the absence or presence of dexamethasone (1μM).

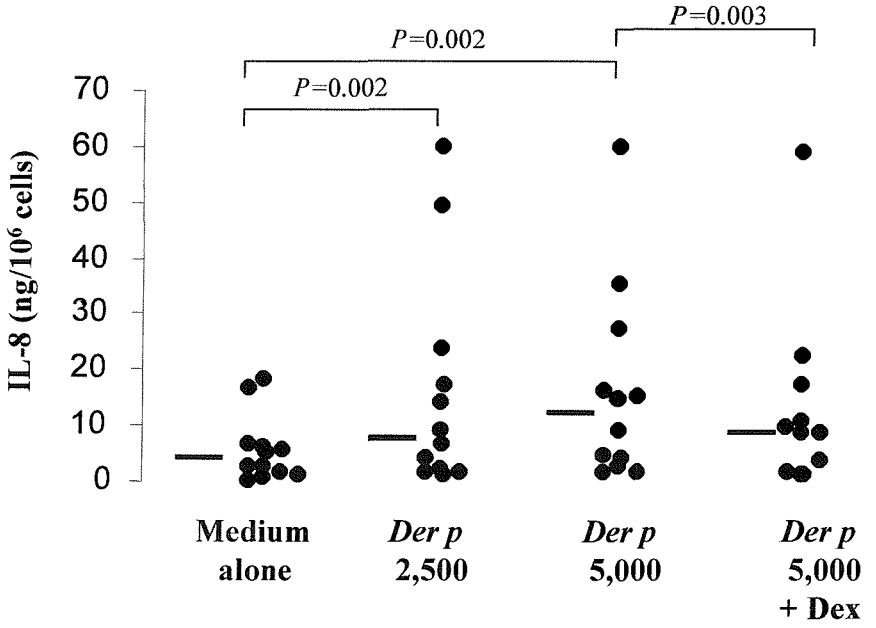


Figure 4.5: (a) IL-8 production by primary bronchial epithelial cells of atopic asthmatics (n = 14) after culture in medium alone, or after exposure to TNF-α (conc. 20 ng/ml) in the absence or presence of dexamethasone (10^{-6} M).

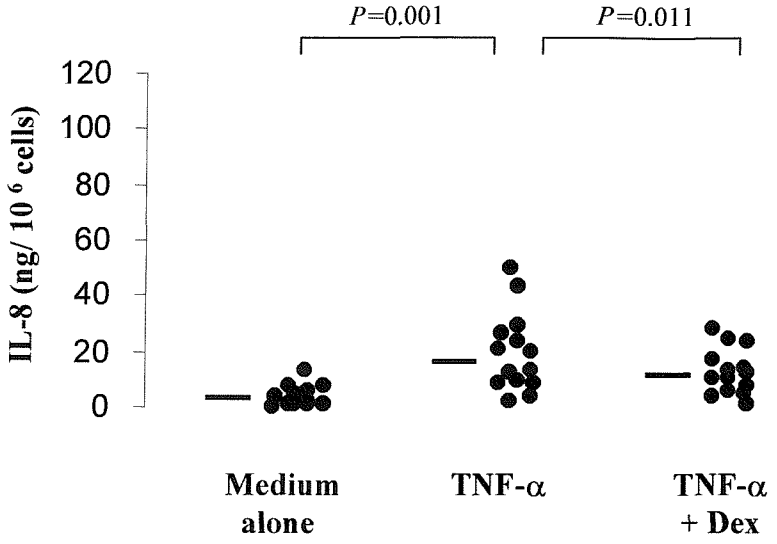


Figure 4.5: (b) IL-8 production by primary bronchial epithelial cells of normal controls (n = 12) after culture in medium alone or TNF-α (conc. 20 ng/ml) in the absence or presence of dexamethasone (10^{-6} M).

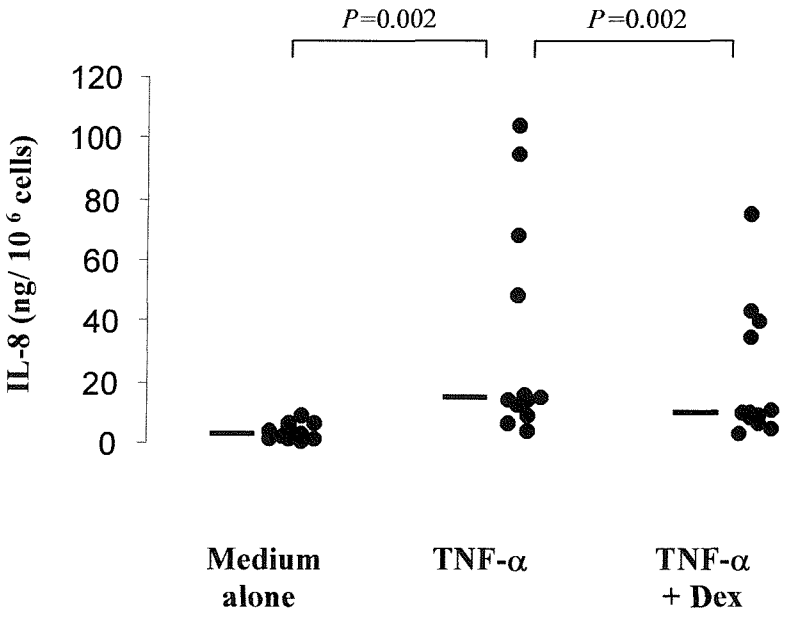


Figure 4.6: (a) GM-CSF production by primary bronchial epithelial cells of atopic asthmatics (n = 14) after culture in medium,or *Der p* allergen (2,500 or 5,000 SQ IU/ml) in the absence or presence of dexamethasone (1μM).

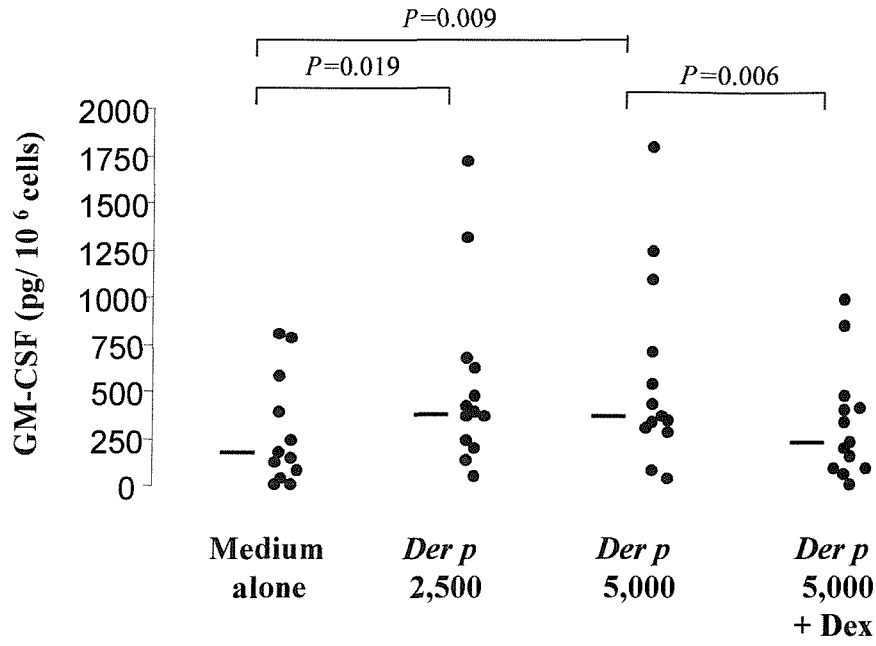


Figure 4.6: (b) GM-CSF production by primary bronchial epithelial cells of normal controls (n =12) after culture in medium, or *Der p* allergen (2,500 or 5,000 SQ IU/ml) in the absence or presence of dexamethasone (1μM).

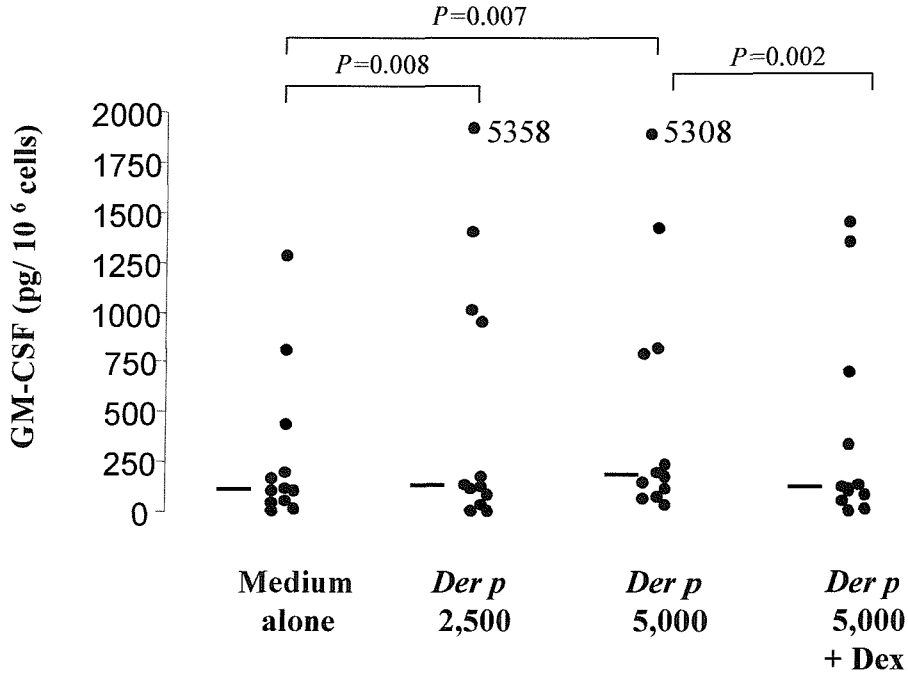


Figure 4.7: (a) GM-CSF release by primary bronchial epithelial cells of atopic asthmatics (n = 15) after culture in medium or TNF-α (20 ng/ml) in the absence or presence of dexamethasone (10^{-6}M).

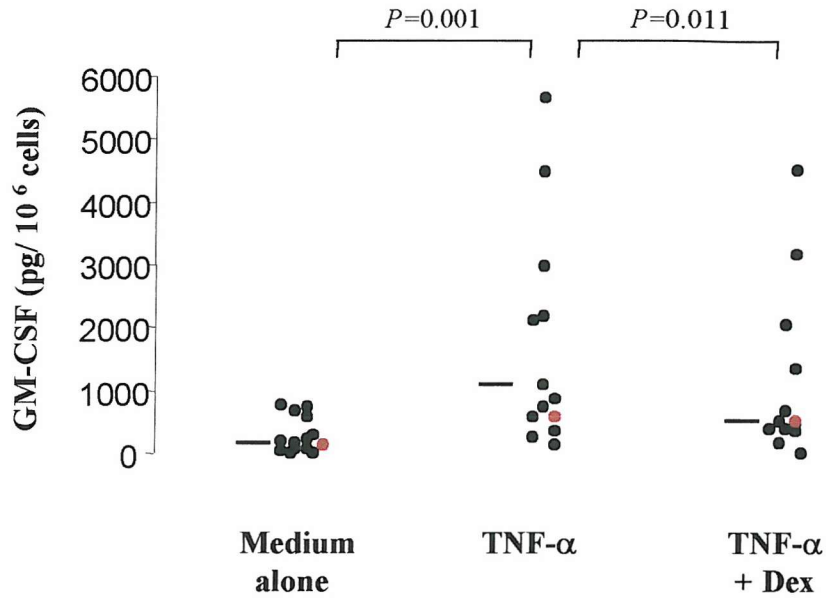


Figure 4.7: (b) GM-CSF production by primary bronchial epithelial cells of normal controls (n = 11) after culture in medium or TNF-α (20 ng/ml) in the absence or presence of dexamethasone (10^{-6}M).

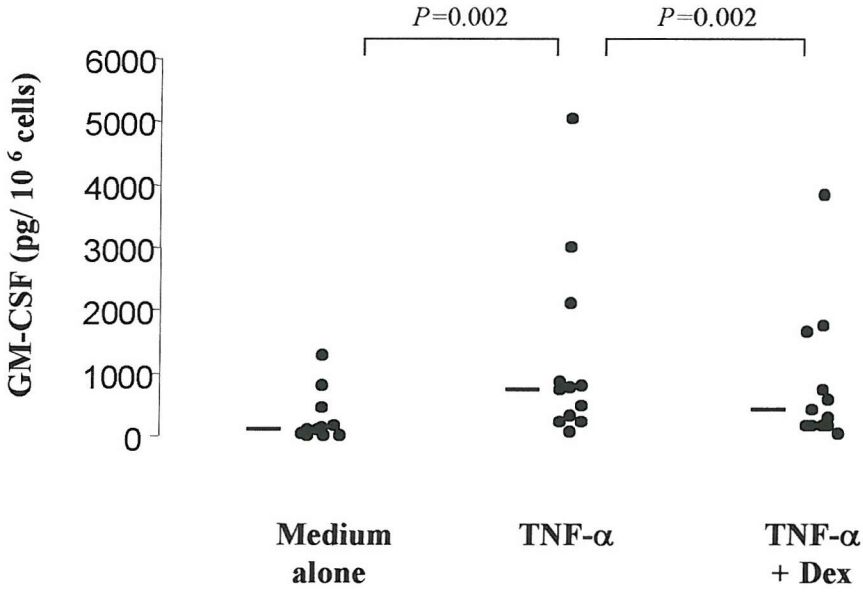


Figure 4.8: (a) RANTES production by primary bronchial epithelial cell cultures of atopic asthmatics (n = 13) after culture in medium or *Der p* allergen (5,000 SQ IU/ml)in the absence or presence of dexamethasone (10^{-6}M).

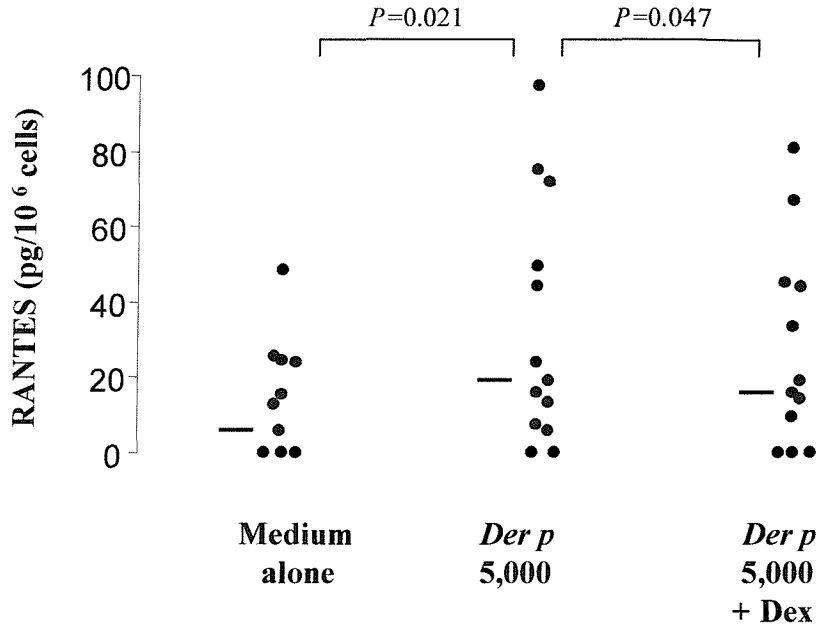


Figure 4.8: (b) RANTES production by primary bronchial epithelial cell cultures of normal controls (n = 6) after culture in medium or *Der p* allergen (5,000 SQ IU/ml) in the absence or presence of dexamethasone (10^{-6}M).

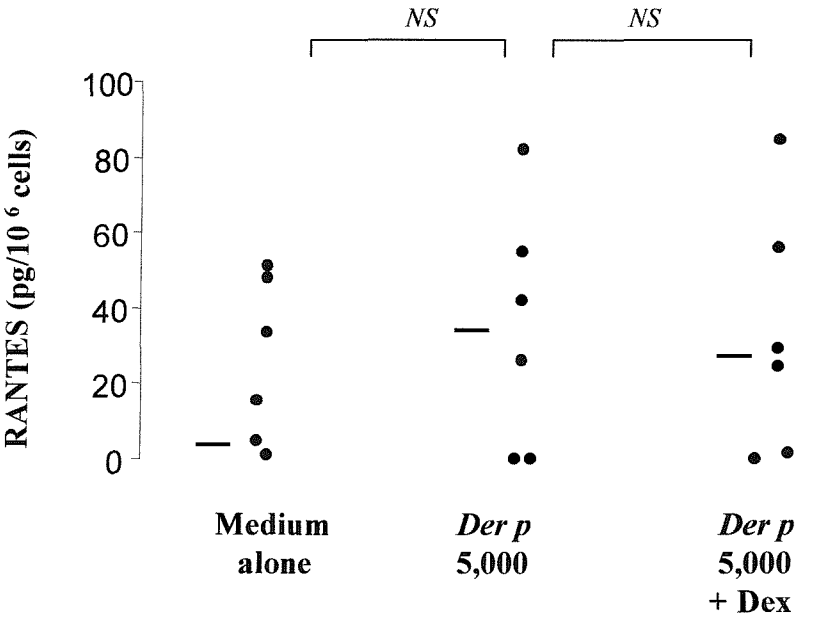


Figure 4.9: (a) RANTES production by primary bronchial epithelial cells of atopic asthmatics (n = 12) after culture in medium or TNF-α (20 ng/ml) in the absence or presence of dexamethasone (10^{-6} M).

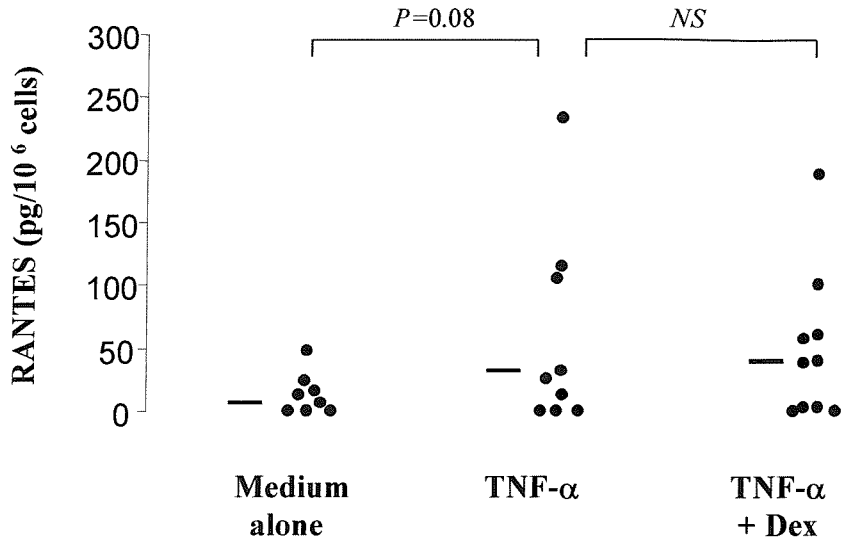
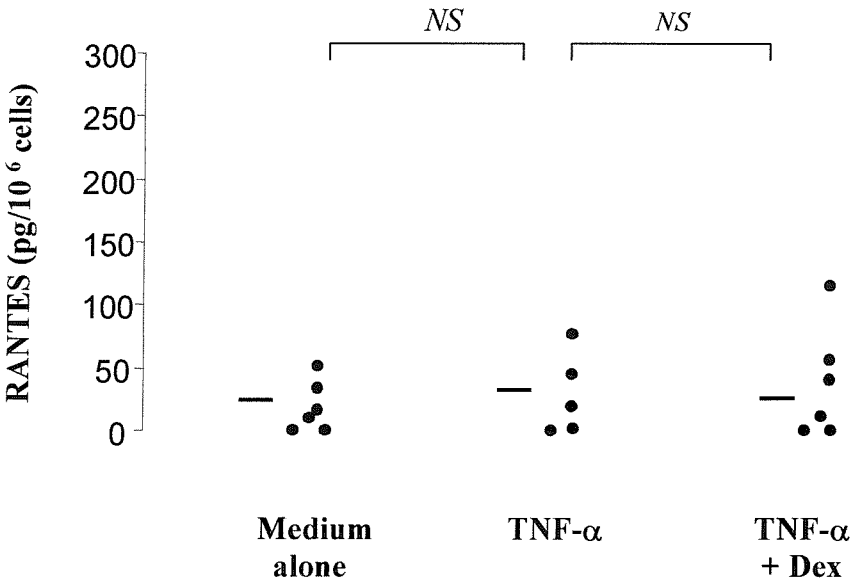


Figure 4.9: (b) RANTES production by primary bronchial epithelial cells of normal controls (n = 6) after culture in medium, or TNF-α (20 ng/ml) in the absence or presence of dexamethasone (10^{-6} M).



4.4 DISCUSSION

In this study, it has been demonstrated that bronchial epithelial cells can safely be obtained by bronchial brushings of the lower airways of healthy control subjects and mild or moderately severe asthmatics at fibreoptic bronchoscopy. This is the first time that primary bronchial epithelial cell cultures have been established from bronchial brushings in our department. It is also the first time that bronchial epithelial cell cultures have been succesfully established from the airways of allergic asthmatics in our department, which paves the way for assessing disease-specific epithelial responses using cells derived from patients with asthma of variable severity. These epithelial cells were expanded in culture to study dynamic airway inflammatory responses in asthma, using cells derived from patients with real disease. It was possible to obtain sufficient primary bronchial epithelial cells for culture from a small amount of brushed cells to perform a number of representative experiments. This novel approach has allowed a comparative study of inflammatory responses using cells obtained from the airways of healthy control subjects and mild or moderately severe asthmatics with real disease. This unique approach has extended and complemented observations from previous studies that have been performed using immortalised airway epithelial cell lines, or cells derived from resection specimens of patients with co-existent lung disease.

Epithelial cell cultures of both healthy subjects and allergic asthmatics were noted to release increased amounts of GM-CSF and IL-8 after exposure to allergen extract or TNF-α, although there was no significant difference in the levels of cytokine production between epithelial cell cultures from normal or allergic asthmatic subjects. There was constitutive production of RANTES by epithelial cell cultures from normal and asthmatic subjects in the absence of stimulation. However, the production of RANTES was further increased after exposure to either *Der p* allergen or TNF-α in epithelial cells of allergic asthmatics, but not healthy controls. Dexamethasone significantly reduced the release of GM-CSF, IL-8, and RANTES after stimulation with allergen, and although statistically significant, the suppression of cytokine release was incomplete.

Increased GM-CSF mRNA expression has been demonstrated in the mucosa of asthmatic subjects [263, 264], and elevated GM-CSF has also been found in the BAL of asthmatics [264]. The ability of *Der p* allergen to stimulate increased production of GM-CSF by bronchial epithelial cells may contribute significantly to the airway inflammatory process in asthma. GM-CSF induces the expression of adhesion molecules on endothelial and epithelial cells [265], and primes granulocytes to release increased amounts of mediators on

stimulation [266]. By this means, GM-CSF plays an important role in promoting the recruitment, survival, and activation of eosinophils and the final differentiation and maturation of monocytes to dendritic antigen presenting cells in the airways of asthmatics [267].

IL-8 is a potent CXC chemokine with a number of biological effects on various cells, with a predominant effect on neutrophils [268]. IL-8 induces trans-endothelial migration of leucocytes, stimulates degranulation and release of proteolytic enzymes from neutrophil intracellular storage granules, enhances the production of LTB_4 and 5-HETE (Hydroxyeicosotetranoic acid) from exogenous arachidonate, and induces respiratory burst with the formation of O_2^- and hydrogen peroxide [269-271]. Eosinophils and T lymphocytes have also been reported to migrate in response to IL-8 [272-278]. IL-8 induces the release of eosinophil peroxidase [279], and is a potent activator of eosinophils, when associated with secretory IgA [280]. IL-8 is also chemotactic for human basophils and induces the release of histamine [281] after pre-treatment of basophils with IL-3, IL-5 or GM-CSF [282]. IL-8 is extremely stable, even in the presence of proteolytic enzymes or fluctuations in pH, capable of exerting a prolonged biological activity [283].

Increased IL-8 levels have been reported in the BAL of mild asthmatics after allergen challenge [284, 285], and in the sputum of severe asthmatics [286, 287] compared to normal subjects. Devalia *et al* have previously reported that bronchial epithelial cells of allergic asthmatics constitutively release greater amounts of IL-8, GM-CSF, and RANTES when compared to epithelial cell cultures of normal subjects [174]. The studies presented here confirm that IL-8 release by primary epithelial cells can be increased by exposure to *Der p* allergen. Tomee *et al* has previously reported increased IL-8 release by the alveolar epithelial cell line (A549) after exposure to *Der p* allergen [53]. More recently, it has been demonstrated that purified *Der p* 1 and *Der p* 9 allergen induce the release of IL-1β, IL-6, IL-8 and GM-CSF from the epithelial cell line, BEAS-2B, or primary epithelial cells derived from lung resection specimens [54, 288]. *Der p* allergen has also been shown to induce the up-regulation of expression of the transcription factor nuclear-factor kappa B (NF-κB) on airway epithelial cells [289].

RANTES is a member of a large supergene family of pro-inflammatory cytokines called C-C chemokines, and is expressed by T lymphocytes, fibroblasts, endothelial cells,

platelets, and epithelial cells [290]. RANTES is a potent chemo-attractant for eosinophils [291] [292] [293], $CD45RO^+$ memory T lymphocytes, and monocytes/macrophages [294], and induces the release of eosinophil cationic protein and superoxide anions from eosinophils [292].

Some investigators have reported identical expression of RANTES in normal and asthmatic airways [295], whereas others have documented higher mRNA expression and protein for RANTES in the submucosa of asthmatics using immunohistochemistry [291], and *in situ* hybridisation [296]. An increased quantity of RANTES protein has also been noted in the BAL of asthmatic subjects compared to normal subjects [297]. There have been variable reports of increased release of RANTES [284] [298] after segmental endo-bronchial allergen challenge in asthmatic subjects. In this study, there was a low basal release of RANTES by epithelial cells of both normal and asthmatic subjects. However, the release of RANTES was increased by stimulation with *Der p* allergen in epithelial cultures derived from asthmatic subjects, but not from normal subjects. This suggests that epithelial derived release of RANTES may contribute to the regulation of cell trafficking in asthma following allergen exposure [284]. TNF-α was also noted to be a potent stimulus for the release of RANTES by epithelial cell cultures derived from asthmatic airways.

Eotaxin has been identified as a major chemo-attractant involved in the recruitment of eosinophils to the respiratory tract after allergen challenge in animal studies [299]. Increased expression of eotaxin protein and mRNA has been demonstrated in the airways of asthmatic patients at baseline [119], and has been co-localised predominantly to epithelial cells [119]. Allergen challenge has been shown to increase the numbers of eotaxin-positive cells in BAL [300], [301] and induced sputum of asthmatics [302]. *In vitro* studies have also shown that stimulation of airway epithelial cell lines (A549 and BEAS-2B) with TNF-α induces the expression of eotaxin mRNA in a dose-dependent fashion, particularly when combined with IL-4 stimulation, associated with activation of NF-kB and STAT-6 expression [202, 303, 304]. In this study, eotaxin protein was not detectable after stimulation of primary epithelial cell cultures with either *Der p* allergen or TNF-α. It is possible that the generation of eotaxin in the airways of asthmatics may be derived from more differentiated ciliated epithelial cells, fibroblasts, or indeed inflammatory cells. It has recently been shown that human lung fibroblasts release increased amounts of eotaxin after stimulation [305].

The recently characterised cytokine IL-16, a natural ligand for the CD4 molecule, is a selective inducer of the migration of $CD4^+$ T lymphocytes and eosinophils, and is considered to play an important role in asthma [219]. A variety of cells including mast cells, eosinophils, epithelial cells, and T lymphocytes are able to produce IL-16 [136, 306]. Increased expression of IL-16 has been noted in the mucosa and submucosa of asthmatics compared to normal subjects [200], and increased levels of IL-16 are found in BAL of asthmatic subjects after allergen challenge associated with increased influx of eosinophils and T lymphocytes [306]. However, in the present study, IL-16 was not detectable after allergen-stimulation of primary bronchial epithelial cells from normal or asthmatic subjects. This suggests that stimuli other than allergen or TNF-α may be involved in the release of IL-16, or alternatively, cells distinct from epithelial cells play a more prominent role in the production of IL-16 in the airways of asthmatic subjects [136].

The precise mechanisms responsible for the allergen-induced generation of cytokines by epithelial cells are unknown, although there are studies to support the involvement of protease-dependent and protease-independent mechanisms [53]. It is note-worthy that several important allergens of the house dust-mite [49] also have intrinsic protease activity. The group 1 allergens from HDM (*Der p* 1) have cysteinyl protease activity, whereas allergens from Group 3 (*Der p* 3), group 6 (*Der p* 6), and group 9 (*Der p* 9) all have serine protease activity, and probably also act as digestive enzymes [49]. *Der p* 1 has been shown to cleave the low affinity IgE receptor (CD23) on B lymphocytes, and inactivates a major component of the anti-protease defenses of the respiratory tract, along with its striking effects on airway epithelial cells [210, 307].

In vitro experiments have shown that exposure to allergen proteases causes epithelial cell detachment resulting in increased permeability of epithelial sheets and cultured monolayers of airway epithelium [308], associated with disruption of tight-junction associated proteins [52]. The injury to airway epithelial cells by this mechanism may facilitate the trans-epithelial passage of allergenic proteins, increasing the access of allergens to the sub-epithelial dendritic-type antigen presenting cells, and result in increased activation of the immune response.

In this study, TNF-α was noted to be a potent stimulus for cytokine production by epithelial cells of normal and asthmatic subjects. However, there was no significant difference in the relative amounts produced between epithelial cells from normal or asthmatic subjects.

TNF-α is considered to play an important amplifying role in airway inflammation in asthma [309], and increased TNF-α expression has been reported in asthmatic airways. Increased TNF-α mRNA has been reported in bronchial biopsy specimens of asthmatics, and TNF-α has also been noted in BAL fluid of asthmatics [264]. TNF-α is produced by many cells, including macrophages, T lymphocytes, mast cells, eosinophils, and epithelial cells. TNF-α is expressed as a type II membrane protein, and is released from cells by proteolytic cleavage of the membrane-bound form by a metalloproteinase, TNF-α converting enzyme (TACE). The release of TNF-α is enhanced by IL-1, GM-CSF, and IFN-γ. TNF-α has been shown to stimulate increased production of GM-CSF, IL-8, and RANTES by bronchial epithelial cells [199, 310, 311]. TNF-α interacts with two cell surface receptors TNF-R55 and TNF-R75, members of the nerve growth factor receptor super-family. Activation of TNF-receptor associated family (TRAF) of adaptor proteins, such as TRAF-2, may have a role in the signal transduction pathway from the TNF receptor to the activation of mitogen activated protein (MAP) kinase cascades with subsequent activation of NF-κB and AP-1 [170, 303].

Inhaled corticosteroids are currently considered as the mainstay of treatment for chronic asthma [312]. The airway epithelium is considered as one of the most important targets for inhaled corticosteroids in asthma. These data confirm that the production of GM-CSF, IL-8 and RANTES by epithelial cell cultures of normal and asthmatic subjects after exposure to *Der p* allergen or TNF-α was significantly reduced by the potent corticosteroid, dexamethasone. The doses of corticosteroid applied in this study are comparable to the estimated beclomethasone concentrations in the airways from a 200μg metered dose inhaler, as determined by regional lung deposition studies of 99mTc-labelled beclomethasone [395]. However, corticosteroids did not completely suppress cytokine production, suggesting that the pathways involved in the release of cytokines by bronchial epithelial cells are at least partially unresponsive to the effects of corticosteroids. This is consistent with previous studies that showed that corticosteroids inhibited the transcription of IL-8 [310] and RANTES [201] by airway epithelial cell lines. Inhaled corticosteroids have also been shown to inhibit the increased expression of GM-CSF, MIP-1α, and RANTES in the epithelium of patients with asthma [313].

Corticosteroids are known to exert their effects by binding to glucocorticoid receptors localized to the cytoplasm of target cells, leading to direct or indirect regulation of the

transcription of target genes [312]. It has been suggested that corticosteroids may act by direct interactions between the transcription factors, activator protein-1, NF-kB, and the glucocorticoid receptor leading to mutual repression [168]. Allergens and TNF-α have been shown to increase the expression of NF-κB by epithelial cell cultures. It is likely that the anti-inflammatory effects of corticosteroids on cytokine release by epithelial cells in this study is mediated, at least partially, by inhibition of NF-kB signalling pathways, possibly downstream of NF-κB binding to DNA [168].

As stated, inhaled corticosteroids are known to suppress airway inflammation by inhibiting the transcription of several inflammatory genes [393, 395, 312]. The double helix of DNA is tightly wound around core histone proteins under resting conditions, excluding the binding of RNA polymerase II, the key enzyme that results in gene transcription and the formation of mRNA. The binding of pro-inflammatory transcription factors, such as NF-κB or AP-1 to co-activator molecules such as CREB-binding protein (CBP) that act as molecular gene switches results in acetylation of core histone proteins by histone acetyltransferase (HAT). Acetylation by HAT of lysine residues on histones opens up the chromatin structure, allowing polymerase II and transcription factors to bind, and switching on gene transcription. Recent studies suggest that corticosteroids act by reversing the histone acetylation that leads to to increased transcription of pro-inflammatory genes. This is achieved by inhibition of HAT activity of CBP, and more importantly by the recruitment of histone deacetylases (HDACs) to the transcription start site. This has been shown to occur *in vitro* at dexamethasone concentrations of 10^{-6} M, equivalent to those applied in the current study. By this process, corticosteroids are able to switch off multiple inflammatory genes.

4.5 CONCLUSIONS

This study has confirmed that primary bronchial epithelial cells can be safely obtained by bronchial brushings from the airways of asthmatic or normal subjects. These primary bronchial epithelial cells can be effectively expanded in culture to provide a sufficient number of cells to assess functional responses in the bronchial epithelium of asthmatic subjects. In this chapter, it has been demonstrated that exposure of bronchial epithelial cells of normal and atopic asthmatics to either *Der p* allergen or TNF-α stimulate an increased production of GM-CSF, IL-8, and RANTES in the case of asthmatic subjects. The ability of allergens and pro-inflammatory cytokines to activate the bronchial epithelium with increased release of cytokines may act to perpetuate airway inflammation in asthma. Although there was no

significant difference in the absolute amount of cytokine production between epithelial cells derived from normal or asthmatic subjects, it is possible that other soluble factors, such as Th2 cytokines, may act in concert with allergen to augment cytokine release in the bronchial epithelium in asthma.

CHAPTER FIVE

The expression of IL-4 and IL-13 receptor subunits on primary bronchial epithelial cells of atopic asthmatics and normal control subjects

5.1 INTRODUCTION

IL-4 and IL-13 have several common functional properties determined by the common expression of IL-4 and IL-13 receptor components on certain target cells [65, 314]. However, they also have distinct actions on other cell types (eg IL-4, but not IL-13, promotes the Th2 differentiation and survival of T cells, which lack functional IL-13 receptors) [314, 315].

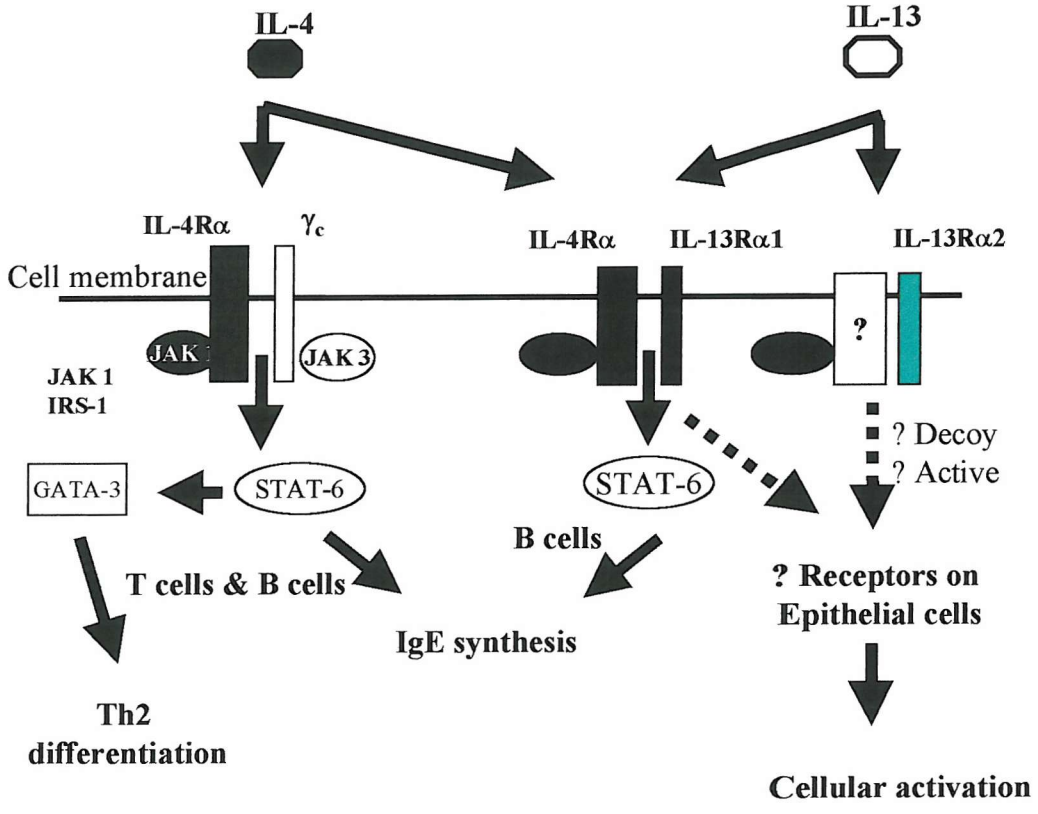
The classical IL-4 receptor consists of a heterodimeric complex of the 140-kd IL-4Rα chain, which binds IL-4 with relatively high affinity, and the 70-kd common γ-chain (γc), a shared component of the receptors for IL-2, IL-4, IL-5, IL-7, and IL-15 (Figure 5.1). However, the IL-4Rα also interacts with the IL-13 receptor alpha (IL-13Rα), and this complex can bind both IL-4 and IL-13. IL-4 receptors are widely expressed on T lymphocytes, B-lymphocytes, mast cells, basophils, macrophages, endothelial cells, fibroblasts, and have also been recently reported on airway epithelial cells [77].

IL-13 receptors are expressed on B cells, monocytes, macrophages, basophils, eosinophils, mast cells, endothelial cells, and airway and skin fibroblasts. mRNA transcripts for IL-13Rα1 have recently been identified intra-cellularly in T cells, but whether this results in the surface expression of functional IL-13Rα chains remains to be established [315]. The high affinity IL-13 receptor complex is composed of the 140-kd IL-4Rα chain, which binds IL-4 but not IL-13, and an IL-13 binding component. Two different cDNAs encoding IL-13-binding proteins have recently been cloned and designated IL-13Rα1 and IL-13Rα2. These proteins are 27% homologous and are expressed as 65 to 70 kD glycosylated molecules [316 317]. The genes encoding IL-13 receptors are located on chromosome Xq13. IL-13Rα1 is a protein of 427 amino acids and selectively binds IL-13 with low affinity (Kd-4nmol/L), whereas IL-13Rα2 is a 380 amino acid protein, which binds IL-13 with high affinity (Kd-50 pmol/L) in the absence of the IL-4Rα chain. The functional role of IL-13Rα2, either decoy or active, is not yet known [318, 319]. Soluble IL-13Rα2 has been used as an antagonist of IL-13 in animal studies of airway inflammation.

Studies with animal models have recently revealed common effects of IL-4 [194] or IL-13 [195] on the structural elements of the airways. Of particular interest, the targeted over-expression of IL-13 in the airways of mice leads to sub-epithelial fibrosis, smooth muscle proliferation, and bronchial hyperresponsiveness, suggesting that IL-13 may play a key role in sustaining the asthma phenotype [195].

Recent studies using immortalised bronchial epithelial cell lines (BEAS-2B) suggest that bronchial epithelial cells can be activated by either IL-4 or IL-13, resulting in increased production of cytokines and chemokines relevant to airway inflammation [320]. Th2 cytokines have also been shown to stimulate mucus hypersecretion and goblet cell metaplasia of the bronchial epithelium. Considering the effects of Th2 cytokines on bronchial epithelial cell function, it is important that the magnitude of these responses be compared using cells derived from the airways of normal and asthmatic subjects. As responsiveness to the Th2 cytokines depends on the type of surface receptor expressed, the objective of this chapter was to characterise the expression of IL-4Rα, IL-13Rα1, IL-13Rα2, and γ_c on bronchial epithelial cells.

Figure 5.1 : Signalling pathways and cell surface receptors for IL-4 and IL-13. This involves the ligation of IL-4 with IL-4Rα on the classical or alternative IL-4 receptor and the subsequent intracellular activation of the JAK-STAT signalling pathway. IL-13 binding with either the low affinity IL-13 receptor sub-unit IL-13Rα1 or the high affinity IL-13Rα2 sub-unit results in activation of the JAK-STAT6 pathway and cellular activation.



5.2 METHODS

5.2.1 Subjects

Mild and moderately severe asthmatics and non-atopic non-asthmatic control subjects were recruited and characterised as described in chapter 2 (Table 4.1). Bronchoscopy was performed in all individuals, and brushings of the lower airway epithelium were obtained and epithelial cell cultures established and characterised, as described in chapter 2 (Methods section 2.2).

5.2.2 Primary bronchial epithelial cell cultures

The epithelial cell cultures were maintained as described in the methods (section 2.3). Confluent epithelial cells were cultured for 24 hours in either basal medium alone, *Der p* allergen extract (Aquagen ALK, Denmark; 5,000 SQ U/ml), interleukin-4 (20 ng/ml), IL-13 (20 ng/ml), TNF-α (20ng/ml)(Peprotech EC, London, UK). Upon completion of individual cultures, epithelial cells were retained for RT-PCR analysis (section 2.7) or FACS analysis (section 2.10) respectively as described in detail in chapter 2.

5.2.3 Detection of mRNA gene transcription for IL-4Rα, Common γ_c, IL-13Rα1, and IL-13Rα2 by human bronchial epithelial cells using RT-PCR

RNA extraction from the cultured epithelial cells, Reverse Transcription (Quiagen Omnioscript Reverse Transcriptase), and PCR Amplification was performed as described in chapter 2 (section 2.7). Oligonucleotide primers (Table 2.1) were designed and PCR conditions optimised (Department of Human Genetics, Southampton General Hospital) as described in detail in chapter 2 (section 2.7). PCR product was displayed on agarose gel electrophoresis and the expression of mRNA gene transcription for IL-4Rα, IL-13Rα1, IL-13Rα2, and γ_c was quantified by densitometry and expressed as a percentage of APRT gene transcription. The results are expressed as median (interquartile range).

5.2.4 Flow cytometry Analysis

With the assistance of Fabio Buccheri, flow cytometry was performed using antibodies for IL-4Rα, IL-13Rα1, IL-13Rα2, and common-γ receptor as described in chapter 2 (section 2.10).

Study protocol for the recruitment and characterisation of subjects, fibreoptic bronchoscopy, bronchial brushings,and epithelial cell culture.

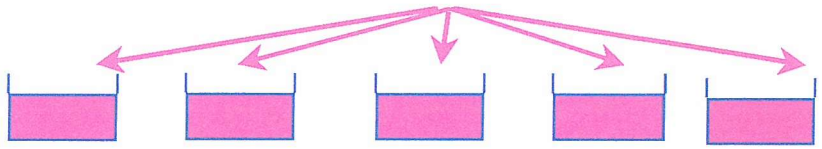
Recruitment of atopic asthmatic subjects and characterisation by clinical history, skin-prick testing, FEV_1, serum IgE, and methacholine PC_{20}.

Bronchoscopy and endobronchial brushings performed to obtain primary bronchial epithelial cells

Cellular purity of epithelial cells confirmed by performing differential cell counts of cytospin preparations, confirming positive staining for Cytokeratin 13 and Cytokeratin 18.

Primary epithelial cells expanded in culture (BEGM)

Epithelial cells seeded at density of 5 x 10^4 cells / well and grown to approx. 90% confluence prior to application of stimulus



Medium | ***Der p*** **5,000 U/ml** | **IL-4** | **IL-13** | **TNF-α**

Culture time 24 hours

IL-4, IL-13, TNF-α Concentration 20 ng/ml

- Supernatents analysed by ELISA for cytokines.
- Methylene Blue assay performed to assess cell number.
- Cytokine release expressed as pg/10^6 cells.
- Epithelial cells recovered for Reverse Transcription PCR and flow cytometry analysis for IL-4 and IL-13 receptor subunits.

5.3 RESULTS

5.3.1 mRNA expression of IL-4Rα, common γ_c , IL-13Rα1, and IL-13Rα2

RT-PCR has been used to detect mRNA transcription of genes for IL-4Rα, γ_c, IL-13Rα1, and IL-13Rα2 in bronchial epithelial cells of normal and asthmatic subjects (Figure 5.2). However, by comparison mRNA transcription of genes for IL-13Rα1 and γ_c, but not IL-13Rα2 was noted in PBMCs. A low intensity signal was noted for mRNA transcription of genes for IL-13Rα2 and γ_c by primary lung fibroblasts.

The expression of mRNA transcripts by epithelial cells exposed to medium alone, *Der p* allergen, IL-4, IL-13 or TNF-α were compared using densitometry of PCR product on gel electrophoresis. The expression of mRNA transcripts for γ_c and IL-13Rα1 were equivalent in bronchial epithelial cells of either atopic asthmatics (n=6) or normal controls (n=6), following exposure to either medium alone, *Der p* allergen, IL-4, IL-13 or TNF-α. mRNA transcription of genes for IL-4Rα by bronchial epithelial cells of atopic asthmatics (n=6), but not normal control subjects (n=6), was significantly increased by exposure to IL-13 (101(70-237); *P*=0.046) or TNF-α (132(73-503); *P*=0.046) compared to bronchial epithelial cells maintained in medium alone (54(30-80)), but this did not reach significance for IL-4 (90(77-127); *NS*) or *Der p* (82(63-136); *NS*). Of particular interest, mRNA gene transcripts for IL-13Rα2 were also higher in bronchial epithelial cells of asthmatics (n=6) exposed to *Der p* allergen (54.9(27.9-63.3); *P*=0.028), IL-4 (61.0(51.0-73.5); *P*=0.028), IL-13 (53.3(43.7-63.3); *P*=0.028), but not TNF-α (45.5(34.1-55.3); *NS*) compared to epithelial cells maintained in medium alone (28.9(13.1-41.2)). The expression of mRNA gene transcripts for IL-13Rα2 were lower in bronchial epithelial cells of normal subjects after culture in medium alone(1.8(0-15.3)). This was increased by exposure to IL-4 (41.3(10.3-53.6)), but was not significantly altered by *Der p*, IL-13, or TNF-α.

5.3.2 The expression of IL-4Rα, common γ_c , and IL-13Rα2 receptors on the surface of epithelial cells of normal and asthmatic subjects

FACS analysis was performed to confirm the presence of IL-4 and IL-13 receptor sub-units on the cell surface of cultured epithelial cells of asthmatics (n=3) and normal subjects (n=3), using selective antibodies for IL-4Rα, IL-13Rα2, and the common γ_c receptor. This confirmed the presence of IL-4Rα on epithelial cells of both normal and asthmatic subjects (Figure 5.3). Of particular interest, FACS analysis confirmed the presence of the recently

described IL-13Rα2 component on the surface of primary bronchial epithelial cells of healthy control subjects and atopic asthmatics (Figure 5.5). This is the first time that epithelial cells have been shown to express IL-13Rα2, which is considered to either act as a decoy receptor, or be involved in down-regulating IL-13 induced responses on target cells. Both IL-4Rα and IL-13Rα1 were readily detectable in every case, but in the case of IL-13Rα2, the levels of expression were found to be variable (Table 5.1). In contrast to IL-4Rα and IL-13Rα2, only low levels of γ_c were detectable on the epithelial cells (Figure 5.4). This differs to a previous study performed by Van der Velden *et al*, who suggested that the expression of γ_c on bronchial epithelial cells using FACS analysis were equivalent to that of IL-4Rα [205]. In contrast to the effects of *Der p* allergen and Th2 cytokines on IL-13Rα2 mRNA gene expression, a preliminary assessment did not confirm an effect of *Der p* allergen or Th2 cytokines on the cell surface expression of IL-4Rα or IL-13Rα2.

Unfortunately, it was not possible to evaluate the presence of IL-13Rα1 on primary bronchial epithelial cells in this study due to the poor performance of a commercially available antibody applied, and the absence of an alternative antibody for use by FACS analysis.

Table 5.1: Level of expression of IL-13Rα2 and IL-4Rα receptor subunits by primary bronchial epithelial cells of atopic asthmatics (A1-3) and normal controls (n1-3) as determined by percentage immunoflourescence using FACS analysis.

Subject	IL-13Rα2	IL-4Rα
	(% fluorescence)	
N1	2.1	11.2
N2	9.1	24.2
N3	11.2	16.4
A1	3.6	nd
A2	18.0	33.0
A3	12.7	14.9

Figure 5.2 (a): mRNA expression of APRT, IL-13Rα1, IL-13Rα2, and IL-4Rα by bronchial epithelial cell cultures of atopic asthmatic subjects. Epithelial cells were cultured for 24 hours in either medium alone (M), *Der p* (5,000 U/ml DP), IL-4 (20 ng/ml), IL-13 (20 ng/ml), or TNF-α (20 ng/ml).

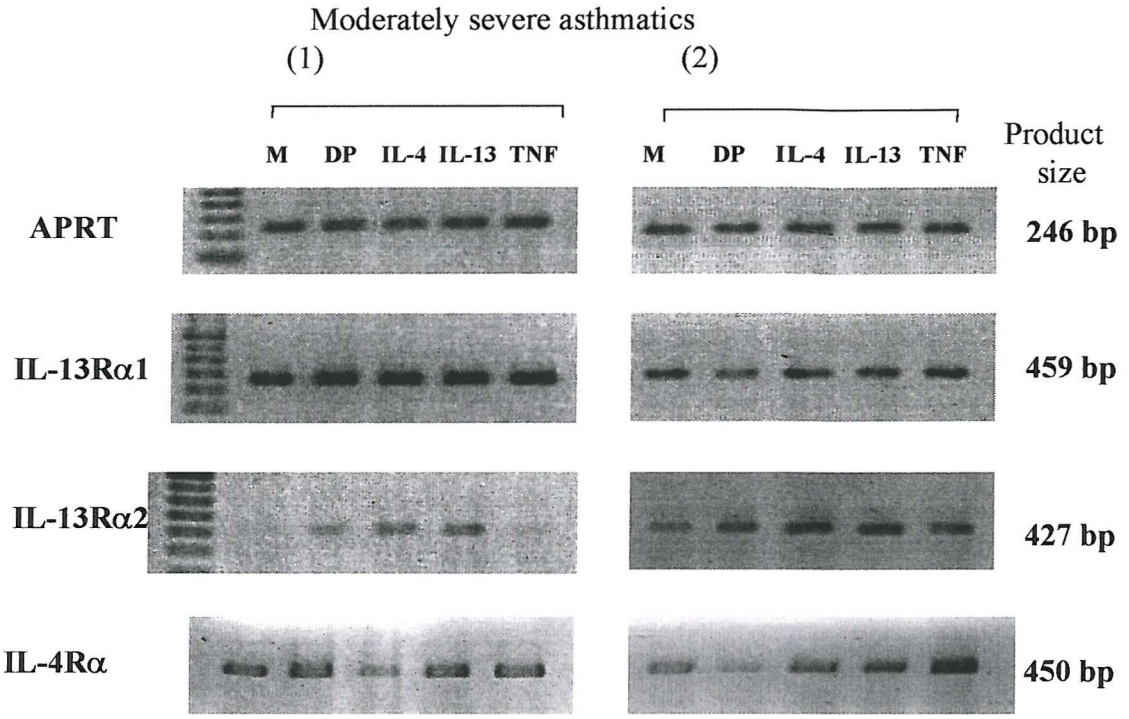


Figure 5.2 (b) RT-PCR gel showing mRNA expression of IL13 receptor subunits by primary bronchial fibroblasts,primary bronchial epithelial cells, and peripheral blood mononuclear cells. RT-PCR was performed on 0.5 μg of total RNA for IL13Rα1, IL13Rα2, and the common gamma subunit of the IL2R (γ_c) respectively. M markers, lanes 1-3 primary bronchial fibroblasts,lanes **4 - 6 primary bronchial epithelium**, lanes 7 - 9 peripheral blood mononuclear cells. Unlike PBMCs both epithelial cells & fibroblasts express the IL13Rα2 subunit.

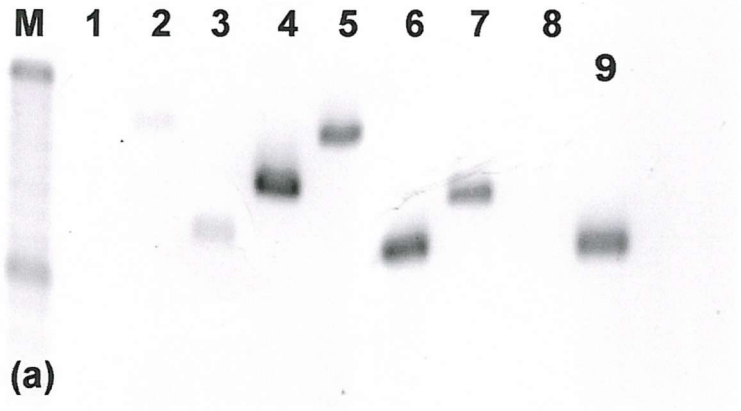


Figure 5.3 : Interleukin 4 receptor-alpha subunit expression by primary bronchial epithelial cells. IL-4Rα expression by epithelial cell cultures was assessed by flow cytometry using an IL-4 flourokine.The red curve represents the control antibody. The dark curve represents the IL-4Rα expression The cells were gated to exclude dead cells and cellular debris. 10, 000 events were obtained and events depicted on the y axis. The x axis shows the log mean flourescence intensity.

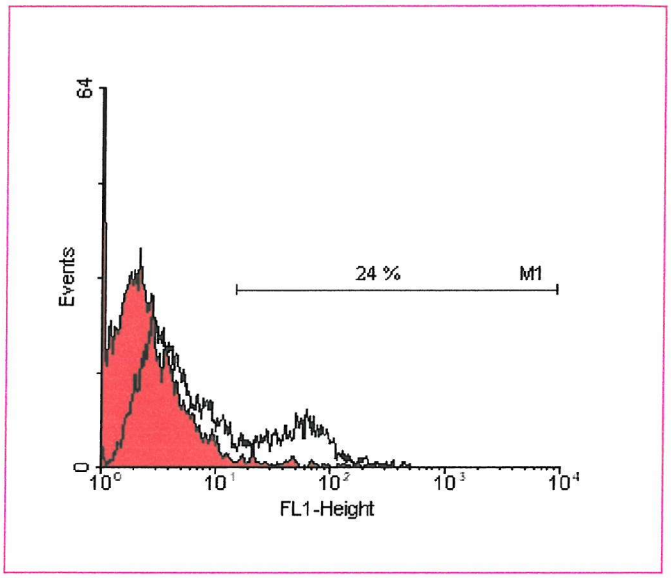


Figure 5.4 : The expression of common γ_c subunits by primary bronchial epithelial cells.The red peak represents the isotypecontrol antibody signal. The signal obtained for γ_c was extremely low.

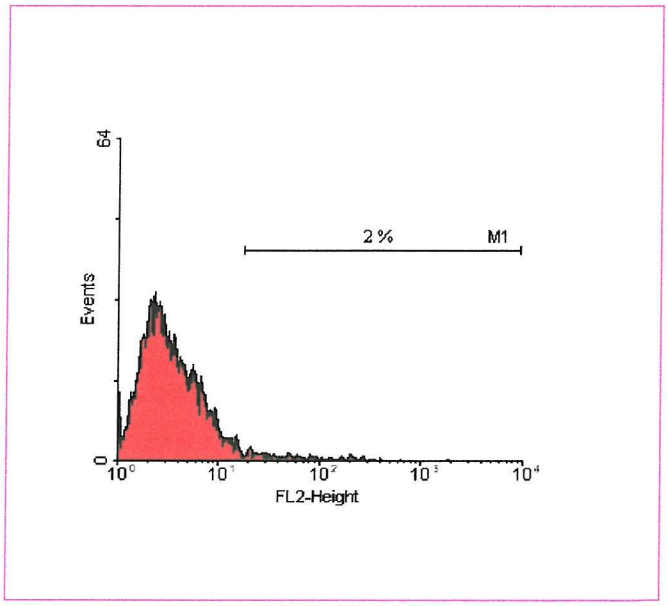
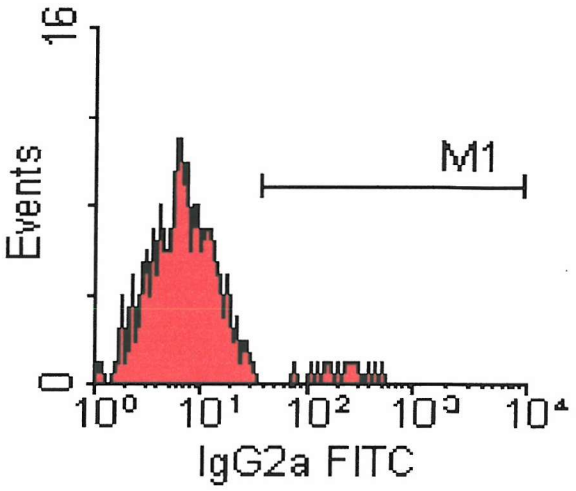
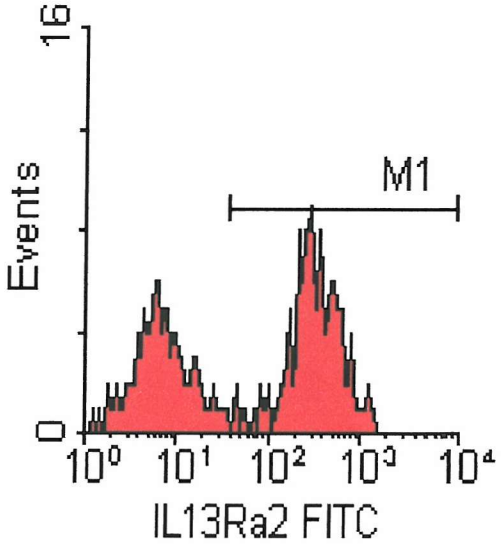


Figure 5.5: The expression of Interleukin 13 receptor-alpha2 subunits by primary lung epithelial cells. This figure illustrates the expression of IL-13Rα2 by primary bronchial epithelial cells stained with IgG2a FITC control and anti-Interleukin 13 receptor alpha 2 (IL13Rα2) FITC. 10,000 events were obtained for analysis. A different scalewas used on the y axis of this figure to demonstrate the expression of IL-13Rα2.

(a)



(b)



5.4 DISCUSSION

This study has confirmed mRNA expression for IL-4Rα, γ_c, IL-13Rα1, and IL-13Rα2 by epithelial cells taken from the airways of normal and allergic asthmatic subjects. Bronchial epithelial cells of atopic asthmatics exhibited increased IL-4Rα mRNA gene transcription after exposure to IL-13 or TNF-α compared to cells maintained in medium alone. mRNA gene transcription for IL-13Rα2 was most prominent in cells exposed to allergen, IL-4 or IL-13, suggesting that IL-13Rα2 gene transcription is up-regulated after cellular activation by IL-4 or IL-13 stimulation. To my knowledge, this is the first study to demonstrate the expression of IL-13Rα2 receptors on bronchial epithelial cells of both asthmatic and normal subjects. The application of a more sensitive semi-quantitative PCR analysis, such as Taqman, could be applied to determine the relative mRNA gene transcription of IL-4 and IL-13 receptor subunits on bronchial epithelial cells.

In this study, the application of flow cytometry has confirmed the expression of IL-4Rα by bronchial epithelial cells of asthmatic and normal subjects. Van Velden *et al* has previously used immunohistochemistry and flow cytometry to demonstrate the expression of IL-4Rα on bronchial biopsies of asthmatic subjects and human bronchial epithelial cells respectively, the bronchial epithelial cells being derived from lung resection specimens [205].

There are variable reports regarding the expression of γ_c by bronchial epithelial cells [205, 321]. Van der Velden *et al.* has previously reported the expression of mRNA gene transcripts and cell surface receptor expression of γ_c by the cell line BEAS-2B and bronchial epithelial cells obtained from resection specimens [205]. In the present study, the expression of γ_c was barely detectable above background levels using FACS analysis on epithelial cells derived from either normal or asthmatic subjects.

As human IL-13 receptors are abundant on non-immune cells, and IL-13 has been shown to confer an asthma-like phenotype on T cell-deficient mice [323], we felt it was important to characterise the expression of IL-13 receptors on non-immune pulmonary cells, in particular the bronchial epithelium [319]. A recent study has reported the expression of IL-13Rα1 on the bronchial epithelium of bronchial biopsies from asthmatic subjects using immuno-histochemistry [324]. In the present study, mRNA for IL-13Rα1 was readily demonstrated using RT-PCR. However, it was not possible to assess the expression of IL-13Rα1 receptor sub-units on epithelial cells due to the lack of a suitable antibody for use by FACS analysis. This study has confirmed the expression of IL-13Rα2 on bronchial epithelial

cells of normal and asthmatic subjects using RT-PCR and flow cytometry. The ability of *Der p* allergen, IL-4, and IL-13 to modulate the gene transcription of IL-13Rα2 is of particular interest, and merits further assessment with a more sensitive PCR assay, such as Taqman. Although a preliminary assessment did not consistently report a modulation of IL-13Rα2 receptor expression using flow cytometry, this may merit further investigation. It is possible that IL-13 signalling in bronchial epithelial cells may be regulated by a negative feedback mechanism through the IL-13Rα2 receptor subunit. It is also possible that IL-13Rα2 may have pro-inflammatory actions in the airways and merits further investigation.

Polymorphisms have been described in both the IL-4Rα and IL-13Rα1 genes [319, 324], but information on polymorphisms of IL-13Rα2 is not yet available. At least 12 common polymorphisms have been described for IL-4Rα, encoded on chromosome 16p12.1, 5 of which result in amino acid coding changes of the gene product. Some (isoleucine [Ile] 50 valine [Val]) increase STAT 6 activation [325], some (serine [Ser] 503 proline [Prol]) decrease STAT 6 activation [326], and others do not affect IL-4 receptor signalling [12, 327]. A relatively common genetic variant has also been described for the IL-13Rα1, and a significant association found with IgE in a British population [324]. As human IL-13 receptors are abundant on non-immune cells, and IL-13 confers an asthma-like phenotype on T cell-deficient mice [323], the characterisation of the expression of IL-13 receptors on non-immune pulmonary cells, and further information regarding polymorphisms will be of particular importance [319].

5.5 Conclusions

In this study, I report for the first time the presence of the recently described IL-13Rα2 receptor subunit on epithelial cells of normal and atopic asthmatic subjects using FACS analysis. Of particular interest, mRNA gene transcription for IL-13Rα2 was increased by exposure to *Der p* allergen, IL-4 and IL-13 in epithelial cells of asthmatic subjects, but not TNF-α. The modulation of mRNA gene transcription for IL-13Rα2 by *Der p* allergen and Th2 cytokines may play an important regulatory role in IL-13 mediated inflammatory responses in bronchial epithelial cells. The precise effects of IL-13Rα2 on IL-13 mediated responses on airway structural cells such as bronchial epithelial cells is of particular interest, and merits further investigation.

CHAPTER 6

The co-operative effects of Th-2 cytokines and house dust-mite allergen on normal and asthmatic bronchial epithelial cells

6.1 INTRODUCTION

Of the Th2 type cytokines, IL-4 and Il-13 are well recognised for their many effects on inflammatory cells, and are known to share many functional and structural characteristics. However, studies with animal models have recently revealed common effects of IL-4 [194] or IL-13 [195] on the structural elements of the airways. Of particular interest, the targeted over-expression of IL-13 in the airways of mice leads to sub-epithelial fibrosis, smooth muscle proliferation, and bronchial hyperresponsiveness, suggesting that IL-13 may play a key role in sustaining the asthma phenotype [195]. In animal models, overexpression of Th2 cytokines, such as IL-4 or IL-13, results in goblet cell metaplasia and mucus hypersecretion. Recent studies using immortalised bronchial epithelial cell lines (BEAS-2B) support the ability of IL-4 or IL-13 to activate the bronchial epithelium resulting in increased production of cytokines and chemokines relevant to airway inflammation [320].

In the present study, it was hypothesised that *Der p* allergen and IL-4 or IL-13 may have a direct co-operative action on the bronchial epithelium to promote the release of cytokines, chemokines, and growth factors relevant to the airway inflammation and remodelling of asthma.

6.2 OBJECTIVES

- To assess the combined ability of Th2 cytokines (IL-4 and IL-13) and *Der p* allergen to stimulate the production of cytokines (IL-8, GM-CSF, RANTES) and growth factors (TGF-α and TGF-β_2) by bronchial epithelial cell cultures of atopic asthmatics and healthy control subjects.
- To assess the ability of corticosteroids to inhibit the production of cytokines and growth factors by bronchial epithelial cell cultures from the airways of asthmatic and normal subjects after exposure to *Der p* allergen, IL-4 or IL-13.

6.3 METHODS

6.3.1 Subjects

Mild and moderately severe asthmatics and non-atopic non-asthmatic healthy control subjects were recruited and characterised as described in chapter 2 (section 2.1, Table 4.1). Bronchoscopy was performed in all individuals, and bronchial brushings of the lower airway mucosa were obtained and epithelial cell cultures established and characterised, as described in chapter 2 (section 2.2).

6.3.2 Primary bronchial epithelial cell cultures

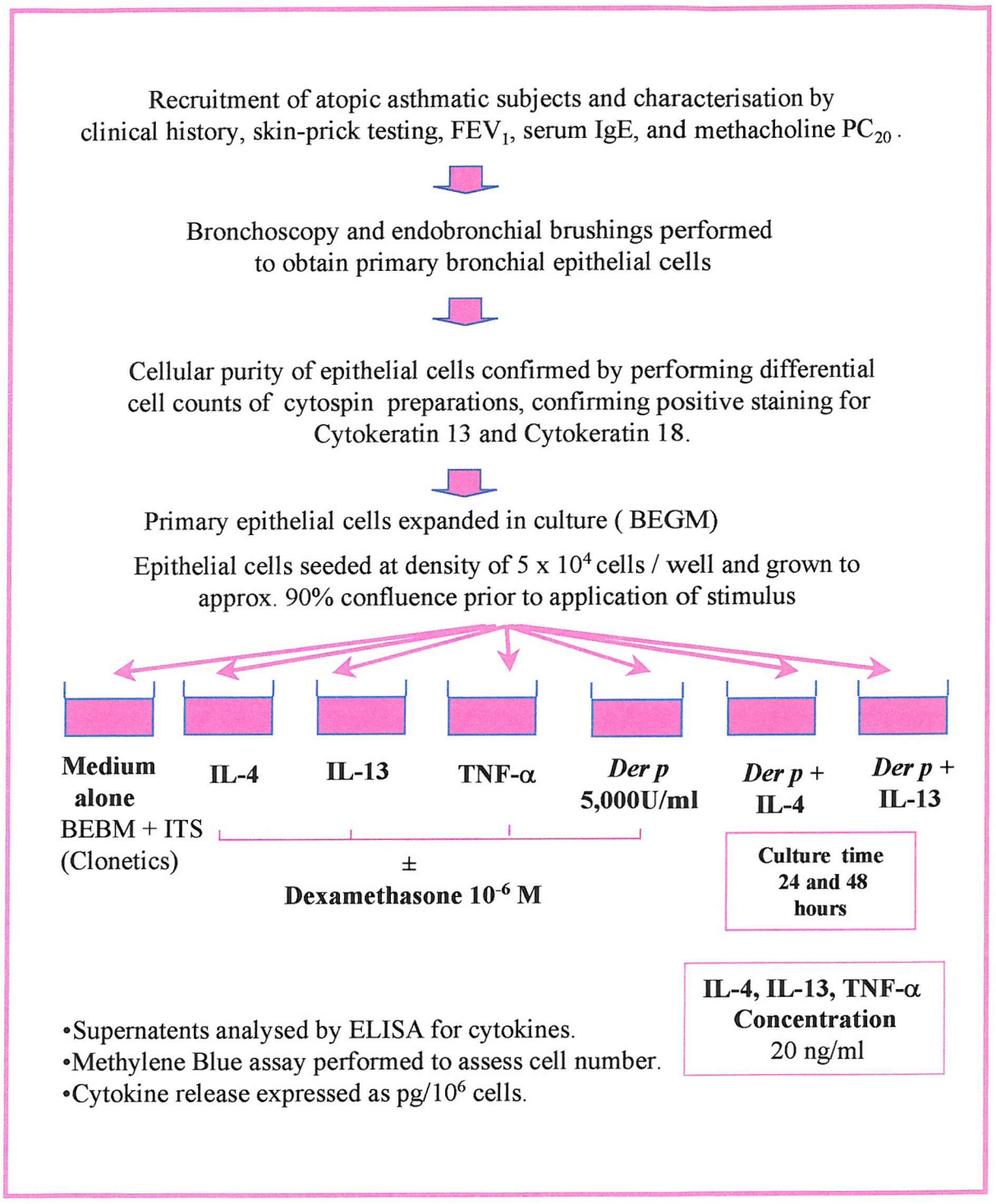
The primary epithelial cells cultures were performed as described in detail in chapter 2 (section 2.3). Confluent epithelial cells were cultured for either 24 or 48 hours in either basal medium alone, *Der p* allergen extract (Aquagen ALK, Denmark, 5,000 SQ U/ml), interleukin-4 (20 ng/ml), IL-13 (20 ng/ml), TNF-α (20ng/ml)(Peprotech EC, London, UK) (Figure 6.1). Parallel cultures were performed in the absence or presence of dexamethasone (10^{-6}M) to assess the corticosteroid-sensitivity of the different responses. In selected experiments, bronchial epithelial cells were cultured with a combination of stimuli including *Der p* (5,000 U/ml) plus IL-4 (20 ng/ml) or IL-13 (20 ng/ml) to assess the co-operative effects of these combined stimuli. Upon completion of the cultures, the supernatants were then removed, centrifuged at 4°C and stored in aliquots for later analysis for cytokines and growth factors as described in chapter 2 (section 2.8).

The density of individual epithelial cell cultures were quantified using the methylene blue assay as described in detail in chapter 2 (section 2.4), and cytokine production corrected for cell number, and expressed as picograms or nanograms of cytokine produced per 10^6 epithelial cells cultured. Data is expressed as median and range.

6.3.3 Statistical analysis

Cytokine values were analysed by non-parametric statistical tests. The Wilcoxon signed rank test for paired data was used for within-group comparison of cytokine protein levels, using SPSS 7.5 for Windows. The Mann Whitney U test was used for between-group comparison. Values of $P < 0.05$ were accepted as statistically significant.

Figure 6.1 : Study protocol for the recruitment and characterisation of subjects, fibreoptic bronchoscopy, bronchial brushings,and epithelial cell culture.



6.4 RESULTS

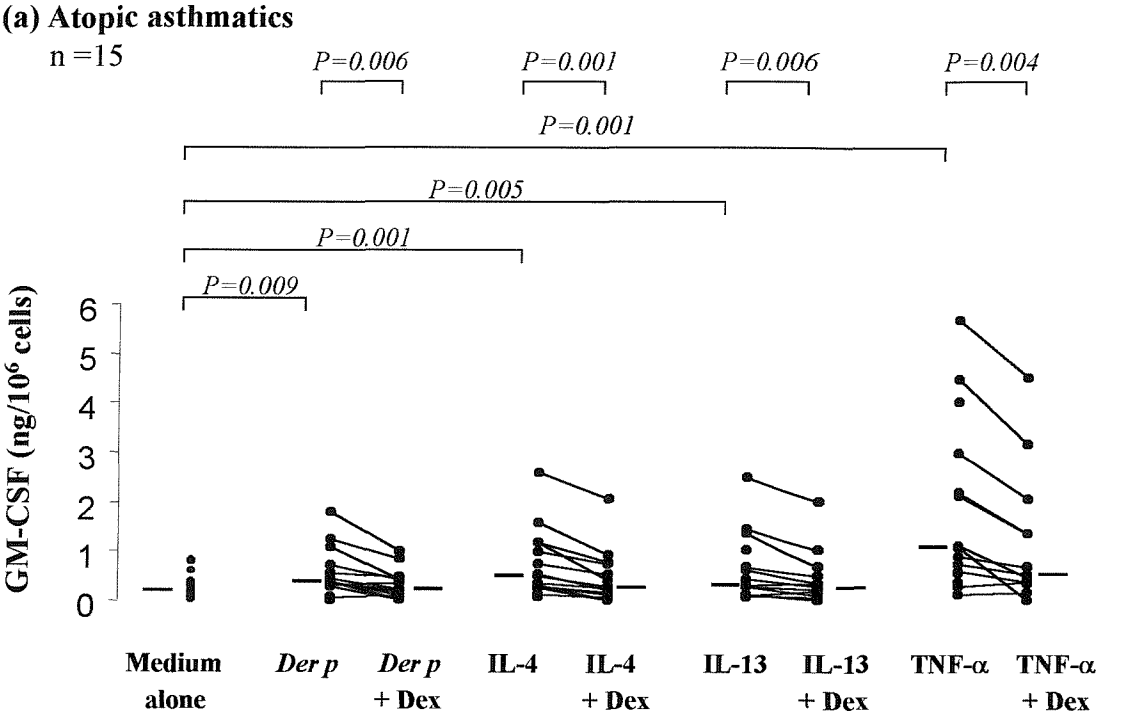
6.4.1 Interleukin-8 and GM-CSF production by bronchial epithelial cell cultures of both atopic asthmatics and normal control subjects is stimulated by IL-4, IL-13, *Der p* allergen, and TNF-α

To determine the effects of Th2 cytokines and allergen on epithelial cells, bronchial epithelial cell cultures were exposed to either IL-4, IL-13, *Der p* allergen, and TNF-α or a combination of stimuli including IL-4 plus *Der p*, or IL-13 plus *Der p* for 24 or 48 hours. Basal production of IL-8 (Figures 6.2) and GM-CSF (Figure 6.3) was noted by epithelial cells from both normal and asthmatic subjects, with no significant differences being noted between the two groups. The production of IL-8 and GM-CSF by epithelial cells of both normal and atopic asthmatic subjects was significantly increased by exposure to *Der p* allergen (Figures 6.2 & 6.3). IL-4 and IL-13 also stimulated the release of IL-8 and GM-CSF by epithelial cell cultures of both normal and asthmatic subjects, with no significant differences between the two groups (Figures 6.2 & 6.3). Cytokine production following TNF-α stimulation of parallel bronchial epithelial cell cultures is included in figures for comparative purposes. The application of combined stimulation with either IL-4 or IL-13 plus *Der p* allergen further increased the production of IL-8 and GM-CSF by epithelial cells of asthmatic subjects, when compared to the application of single stimuli (Figures 6.4). In the majority of cases, combined stimulation led to an additive response, but a degree of synergism was noted in particular cultures.

6.4.2 Corticosteroids inhibit the production of IL-8 and GM-CSF in response to stimulation with *Der p* allergen, IL-4, IL-13 or TNF-α

The release of IL-8 and GM-CSF by epithelial cell cultures of normal and asthmatic subjects was significantly reduced by pre-treatment of the epithelial cell cultures with the corticosteroid, dexamethasone (Figure 6.2 & 6.3). However, the addition of corticosteroids did not completely suppress cytokine release, suggesting that the pathways involved in cytokine release by bronchial epithelial cells are at least partially unresponsive to the effects of corticosteroids at the doses used in these studies.

Figure 6.2 : GM-CSF production by primary bronchial epithelial cells after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4, IL-13, or TNF-α (20 ng/ml) for 24 hours in the absence or presence of dexamethasone (10^{-6} M).



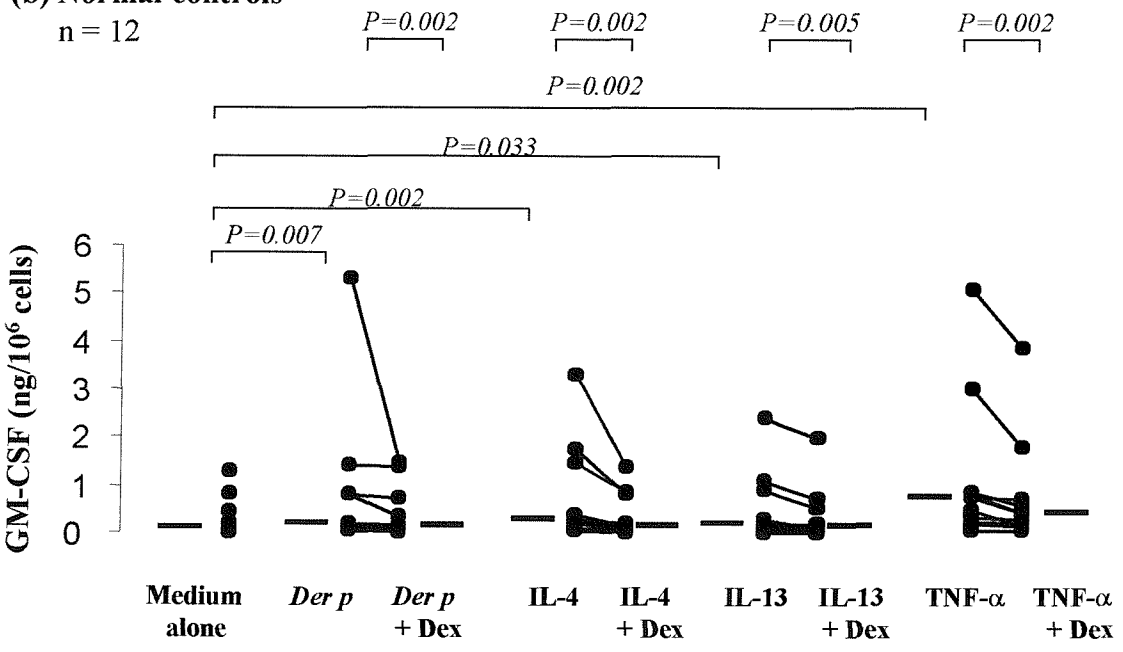
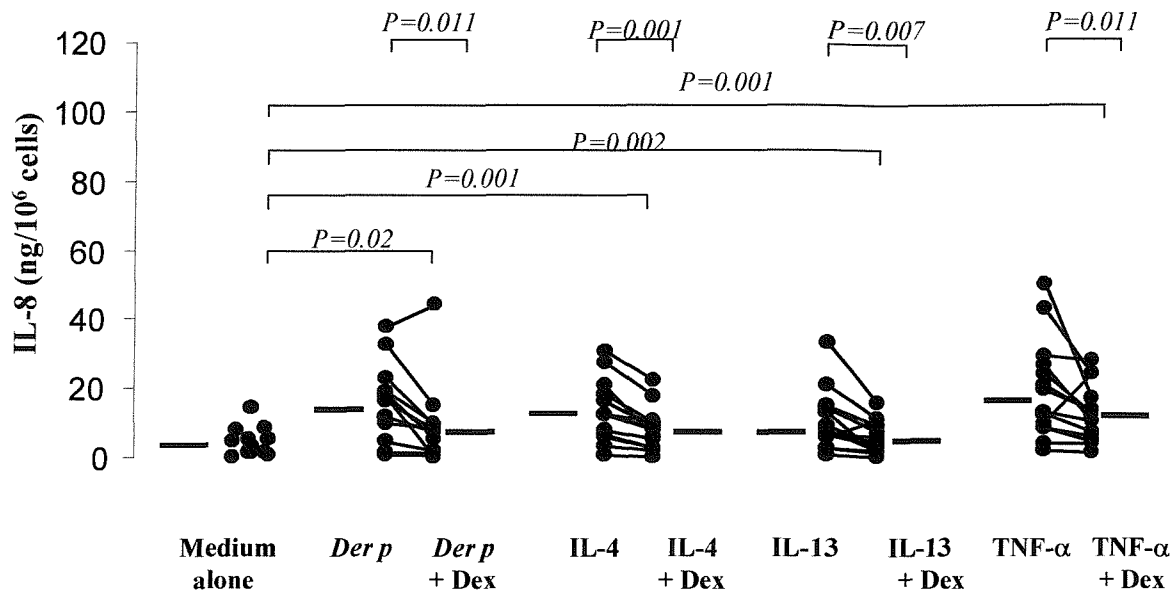


Figure 6.3 : Interleukin-8 production by primary bronchial epithelial cells after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4, IL-13, or TNF-α (20 ng/ml) for 24 hours in the absence or presence of dexamethasone (10^{-6} M).

(a) Atopic asthmatics

n = 14



(b) Normal controls

n = 12

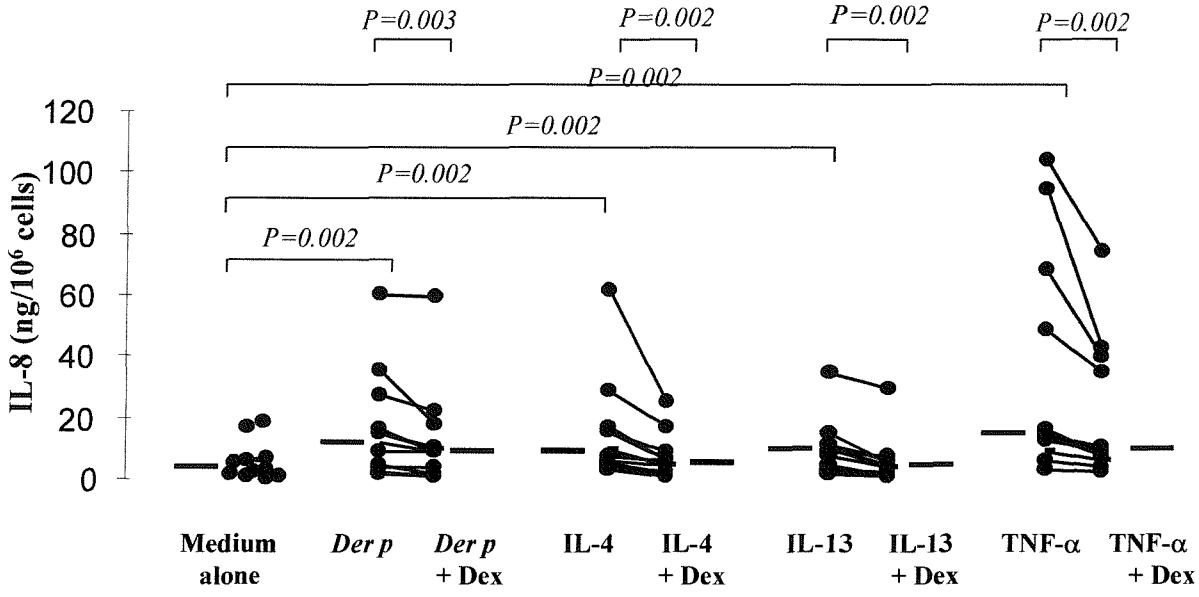
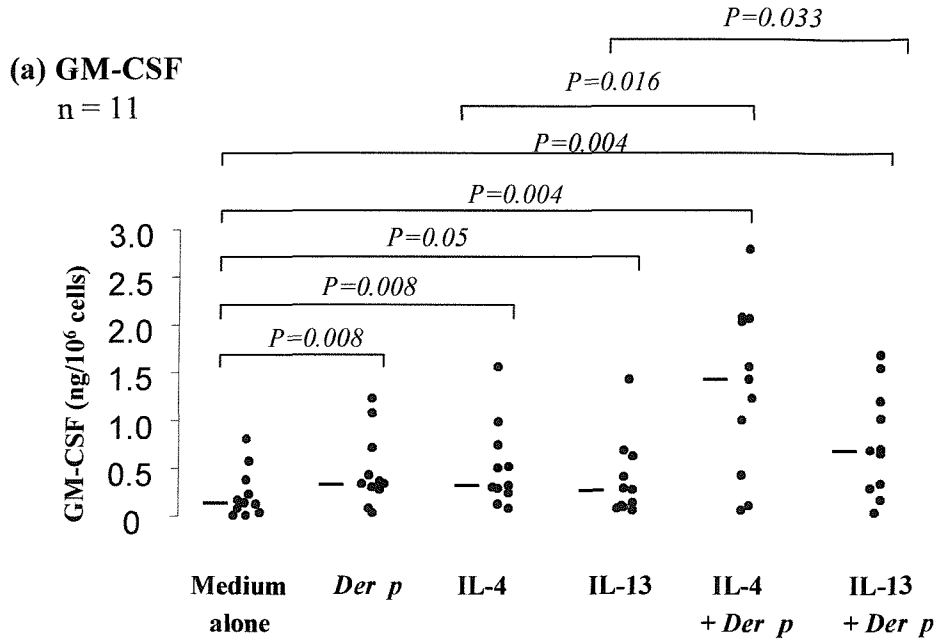


Figure 6.4 (a): GM-CSF and Interleukin-8 production by bronchial epithelial cells of atopic asthmatics after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4 (20 ng/ml), IL-13 (20 ng/ml), or combined stimulation with *Der p* allergen plus IL-4, or IL-13 for 24 hours.



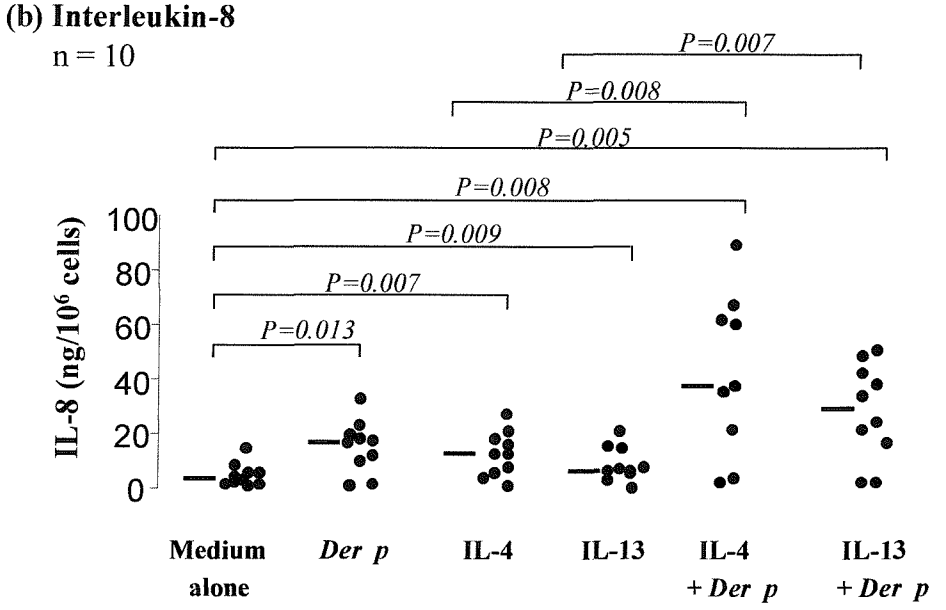
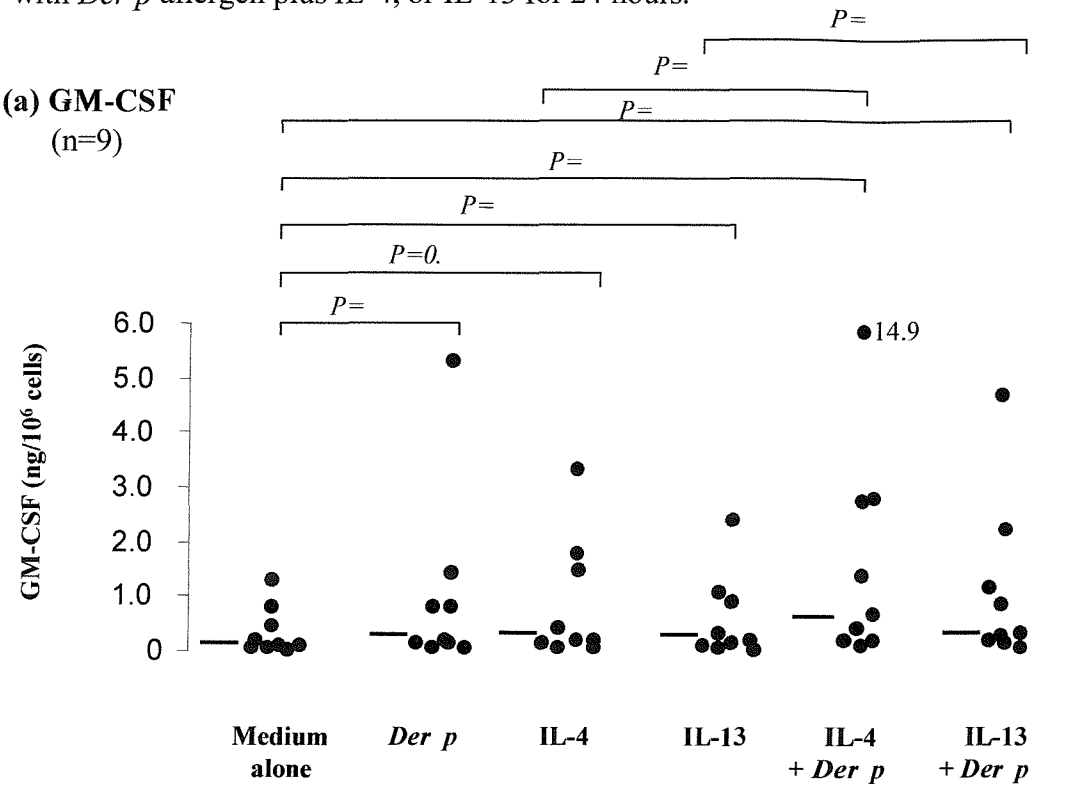
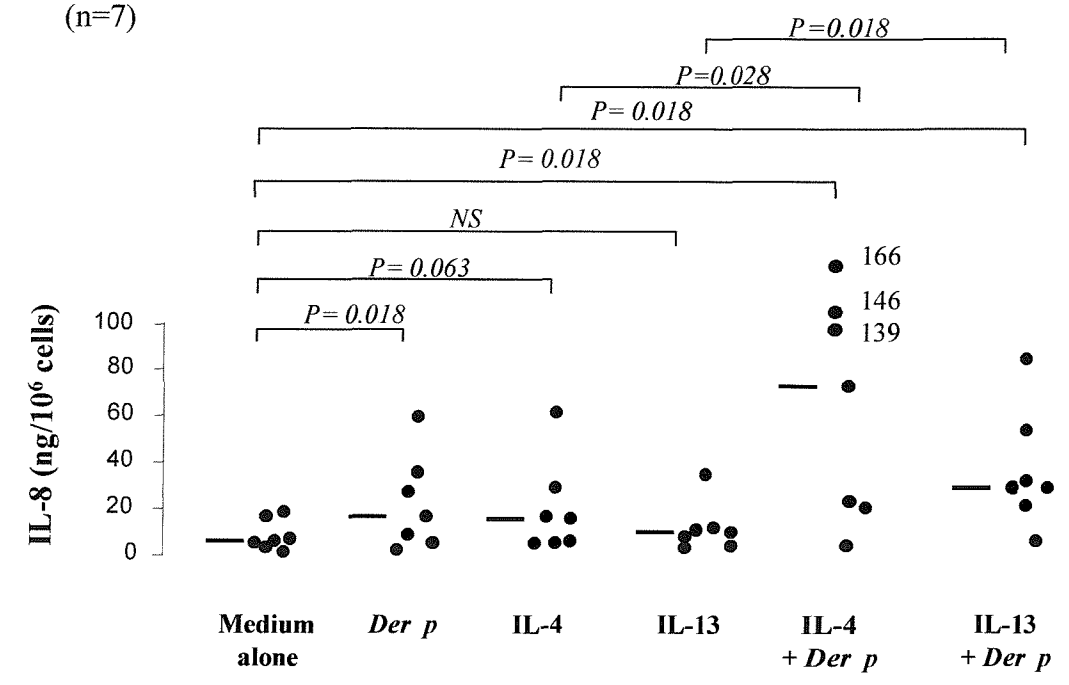


Figure 6.4 (b): GM-CSF and Interleukin-8 production by bronchial epithelial cells of normal controls after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4 (20 ng/ml), IL-13 (20 ng/ml), or combined stimulation with *Der p* allergen plus IL-4, or IL-13 for 24 hours.





6.4.3 RANTES production by epithelial cells of allergic asthmatics after stimulation with *Der p*, IL-4, IL-13, or TNF-α

To assess the effects of Th2 cytokines and allergen on the epithelial-derived release of chemokines, the production of RANTES by epithelial cells of asthmatic and healthy control subjects was measured after exposure to IL-4, IL-13, *Der p*, or TNF-α or combined stimulation with IL-4 or IL-13 plus *Der p* allergen (Figure 6.5 & 6.6).

There was no significant difference in the basal production of RANTES by bronchial epithelial cells of atopic asthmatic or normal subjects. RANTES production was significantly increased in cultures derived from asthmatic subjects after exposure to *Der p* allergen, IL-4, IL-13, or TNF-α. IL-4 was a more potent stimulus for RANTES production compared to IL-13 or indeed *Der p* allergen. However, TNF-α was an extremely potent stimulus for the production of RANTES by epithelial cells of asthmatic subjects. The production of RANTES by bronchial epithelial cells of asthmatic subjects was further increased by combined stimulation with IL-4 or IL-13 plus allergen suggesting a degree of synergism (Figure 6.6). In contrast, the release of RANTES by epithelial cell cultures of normal subjects was only increased by the application of combined stimulation with IL-4 and *Der p* allergen.

Although there was no significant difference in the basal production of RANTES between atopic asthmatic and normal control subjects, the production of RANTES by epithelial cells of healthy subjects was not significantly increased by exposure to IL-4, IL-13, allergen or TNF-α. This suggests an intrinsic difference in the effects of allergen or pro-inflammatory cytokines on RANTES production by epithelial cells of atopic asthmatics compared to healthy control subjects, which may contribute to the selective accumulation of inflammatory cells noted in the airways of asthmatic subjects, but not healthy subjects.

The potent corticosteroid, dexamethasone significantly reduced RANTES production by epithelial cells of asthmatic subjects in response to stimulation with *Der p* allergen or IL-4 (Figure 6.5). Corticosteroids did not significantly reduce the production of RANTES by epithelial cells of asthmatic subjects after stimulation with IL-13 or TNF-α, suggesting that the production of RANTES by bronchial epithelial cells are at least partially unresponsive to corticosteroids.

Figure 6.5 : RANTES production by primary bronchial epithelial cells after culture in medium, or *Der p* allergen (5,000 SQ IU/ml) IL-4, IL-13, or TNF-α (20 ng/ml) for 24 hours in the absence or presence of dexamethasone (10^{-6} M).

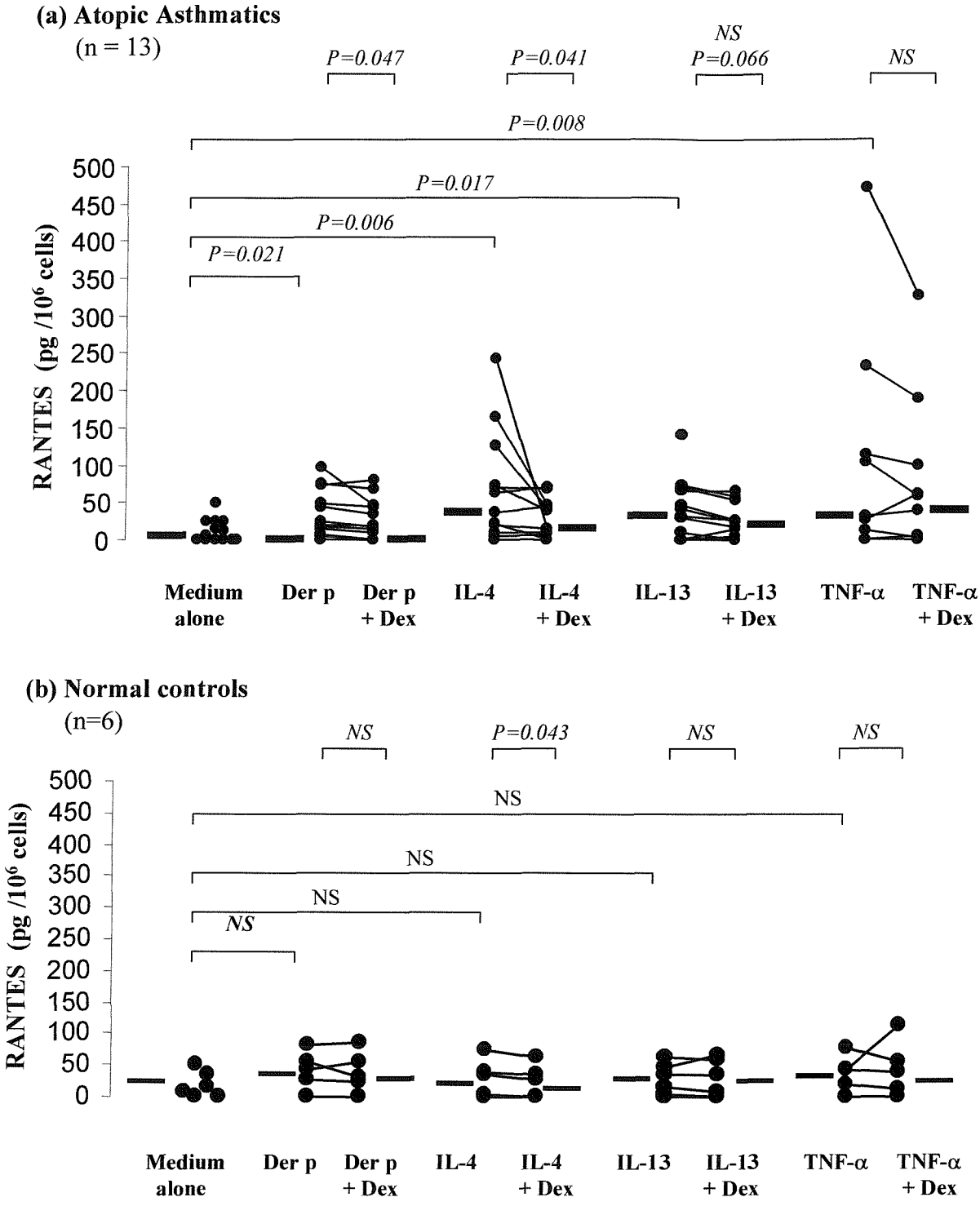
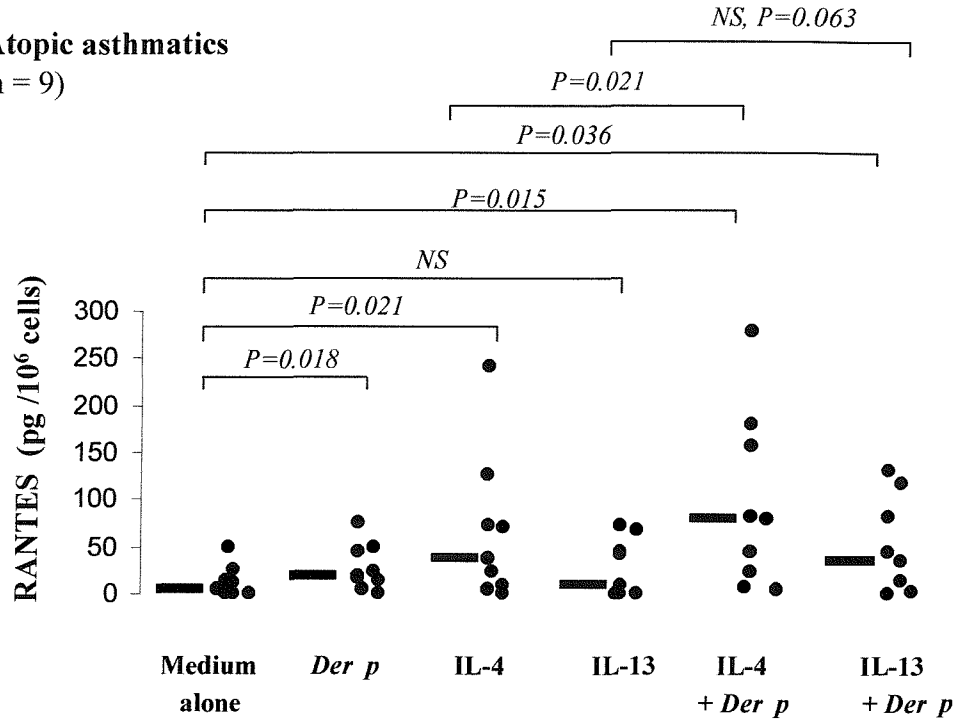
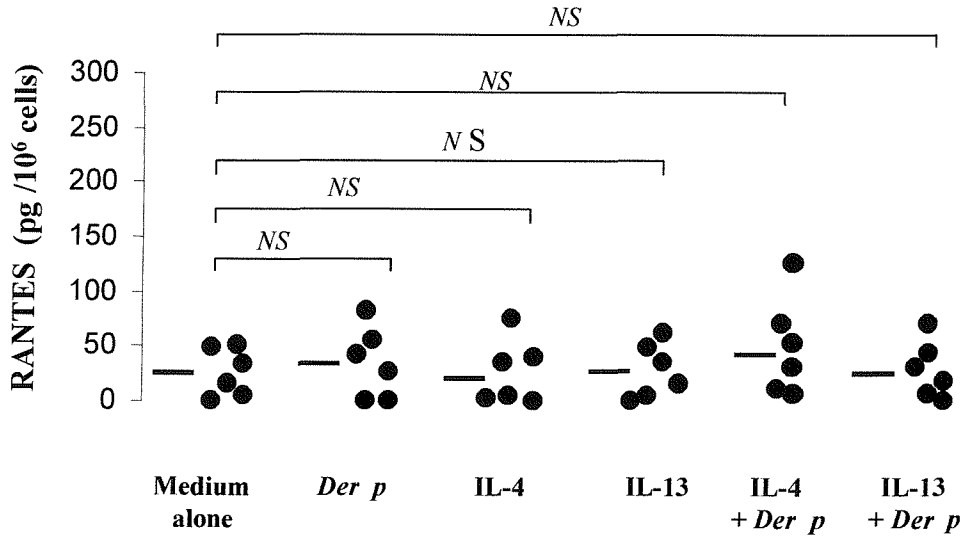


Fig 6.6: RANTES production by primary bronchial epithelial cells of (a) atopic asthmatics and (b) normal control subjects after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4 (20 ng/ml), IL-13 (20 ng/ml), or combined stimulation with *Der p* plus IL-4, or IL-13 for 24 hours.

(a) Atopic asthmatics
(n = 9)



(b) Normal controls
(n = 6)



6.4.4 TGF-β_2 production by bronchial epithelial cell cultures of asthmatic subjects

Recent animal studies have shown that the targeted over-expression of IL-13 in the airways of mice results in goblet cell hyperplasia, sub-epithelial fibrosis, smooth muscle hyperplasia, associated with marked increases in airway hyper-responsiveness. It was important to assess the ability of IL-4 and IL-13 to stimulate the release of growth factors as well as pro-inflammatory mediators by epithelial cells of asthmatic and normal control subjects.

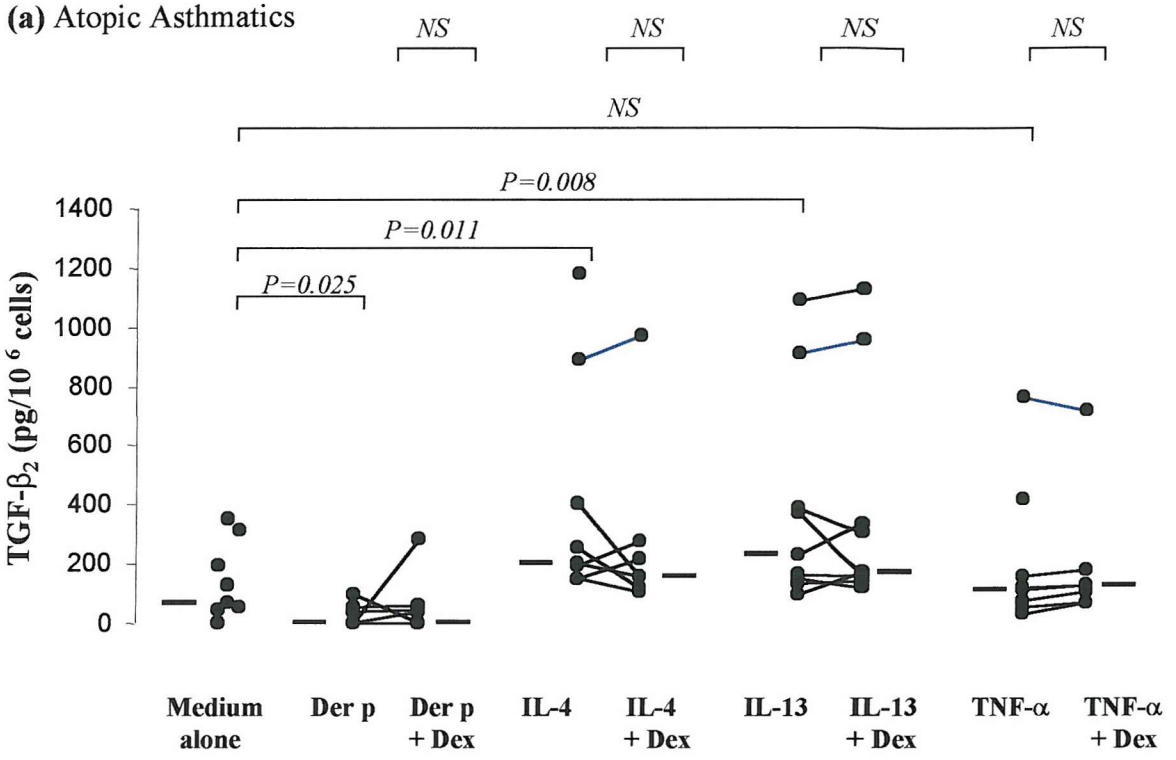
Epithelial cells of asthmatic and normal subjects were found to release TGF-β_2 in the absence of stimulation after culture for 48 hours. The basal production of TGF-β_2 by bronchial epithelial cells of atopic asthmatics (69.3(32.8-280.1) pg/10^6 cells) was decreased by exposure to *Der p* allergen (0(0-51)pg/10^6 cells; P=0.015), but was increased by exposure to IL-4 (196(94-327) pg/10^6 cells; P=0.025) and IL-13 (167(69-383) pg/10^6 cells; P=0.012) (Figure 6.7). TNF-α exposure did not significantly increase the production of TGF-β_2 by bronchial epithelial cells of atopic asthmatics (94(34-149; *NS*). In comparison to the additive and often synergistic responses noted for IL-8 and GM-CSF production, there was no increased production of TGF-β_2 with the application of combined stimulation (Figure 6.7).

In contrast to the responses noted in bronchial epithelial cells derived from atopic asthmatics, TGF-β_2 production was not significantly increased by stimulation of epithelial cells of healthy control subjects with allergen or Th2 cytokines.

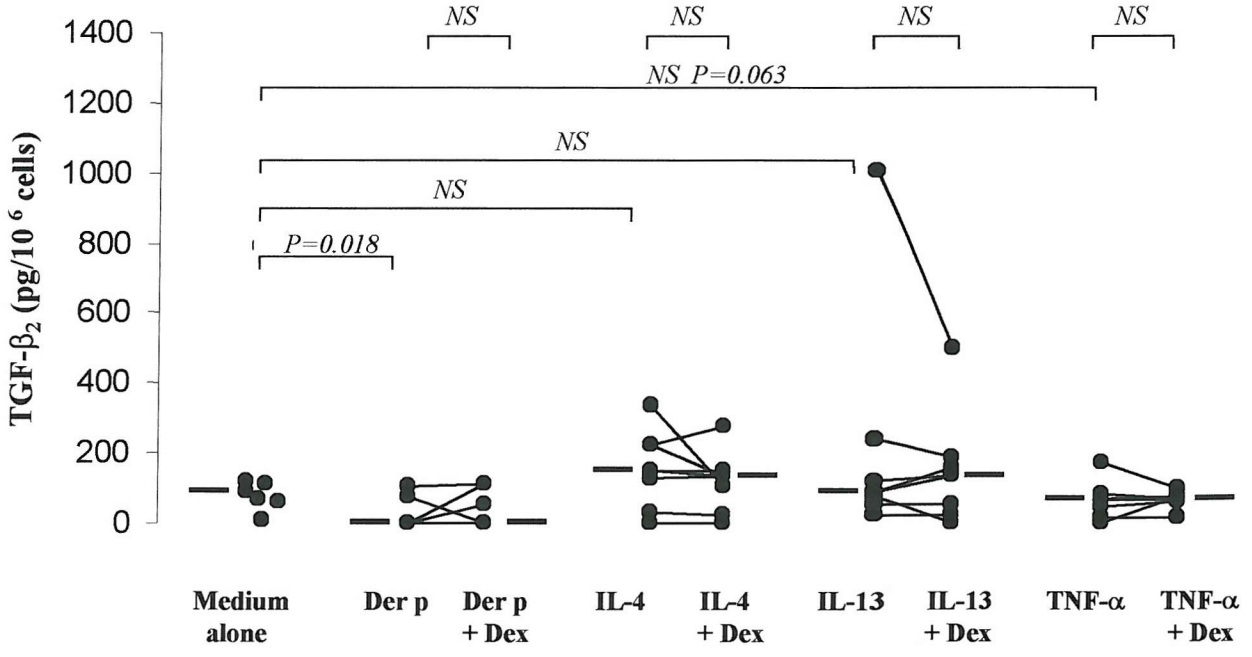
In contrast to the ability of corticosteroids to suppress GM-CSF and IL-8 production induced by exposure to allergen or Th2 cytokines, dexamethasone did not reduce the production of TGF-β_2 by epithelial cells of asthmatic subjects after stimulation with IL-4, IL-13, or *Der p* allergen (Figure 6.7). However, corticosteroids increased the production of TGF-β_2 after stimulation with TNF-α.

Figure 6.7 : Transforming Growth Factor-β_2 production by primary bronchial epithelial cells after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4, IL-13, or TNF-α (20 ng/ml) for 48 hours in the absence or presence of dexamethasone (10^{-6} M).

(a) Atopic Asthmatics



(b) Normal controls



6.4.5 TGF-α is released by bronchial epithelial cell cultures of asthmatic subjects, but not from non-atopic non-asthmatic subjects

Recent studies have focussed on the involvement of epidermal growth factor receptor (EGFR) signalling in the perpetuation of airway inflammation, damage, repair, and remodelling in the airways of asthmatics [328]. EGFR activation in response to oxidant stress has also been linked to increased expression of mucus genes and the excessive production of mucus in the airways of asthmatics [196, 197]. The ability of epithelial cells to generate the soluble ligand for the EGFR complex, TGF-α, was assessed. Basal production of TGF-α was noted by epithelial cells of both asthmatic and normal subjects in the absence of stimulation, with no significant difference being noted between normal or asthmatic cultures. Of particular interest, the production of TGF-α by bronchial epithelial cells of asthmatics but not from normal subjects was significantly increased following stimulation with *Der p* allergen, IL-4, IL-13, or TNF-α (Figure 6.8). There was a trend for increased production of TGF-α by epithelial cells of asthmatic with combined stimulation by *Der p* allergen plus either IL-4 or IL-13, but this did not reach statistical significance (Figure 6.9).

6.4.6 Corticosteroids suppress the release of TGF-α by bronchial epithelial cell cultures of asthmatic subjects

In contrast to the relative insensitivity of dexamethasone to inhibit TGF-β release, dexamethasone effectively suppressed the release of TGF-α by epithelial cells from normal or asthmatic subjects, after stimulation either with *Der p* allergen, IL-4, IL-13, or TNF-α (Figure 6.8).

Figure 6.8 : Transforming Growth Factor-α production by primary bronchial epithelial cells after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4, IL-13, or TNF-α (20 ng/ml) for 24 hours in the absence or presence of dexamethasone (10^{-6} M).

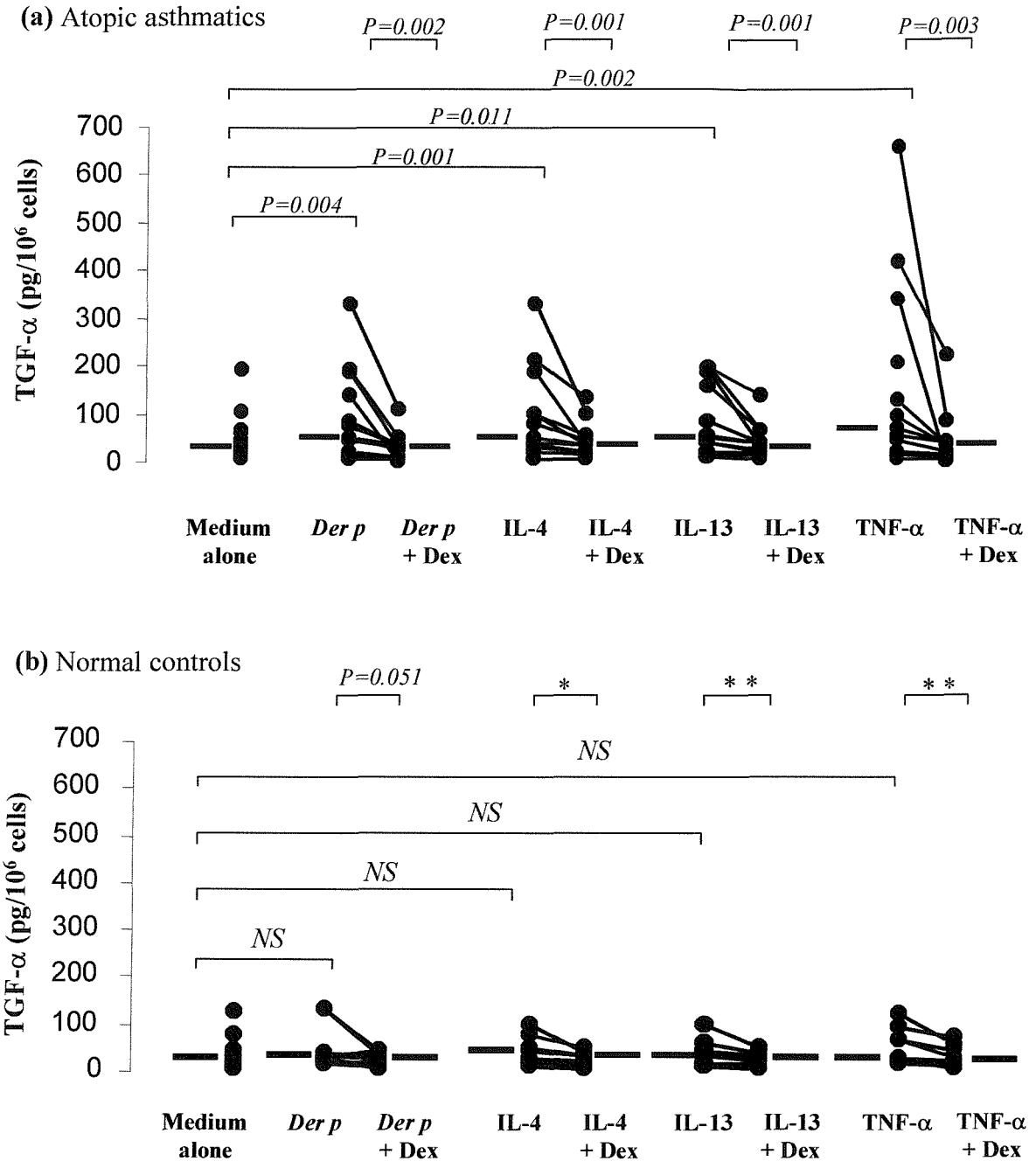
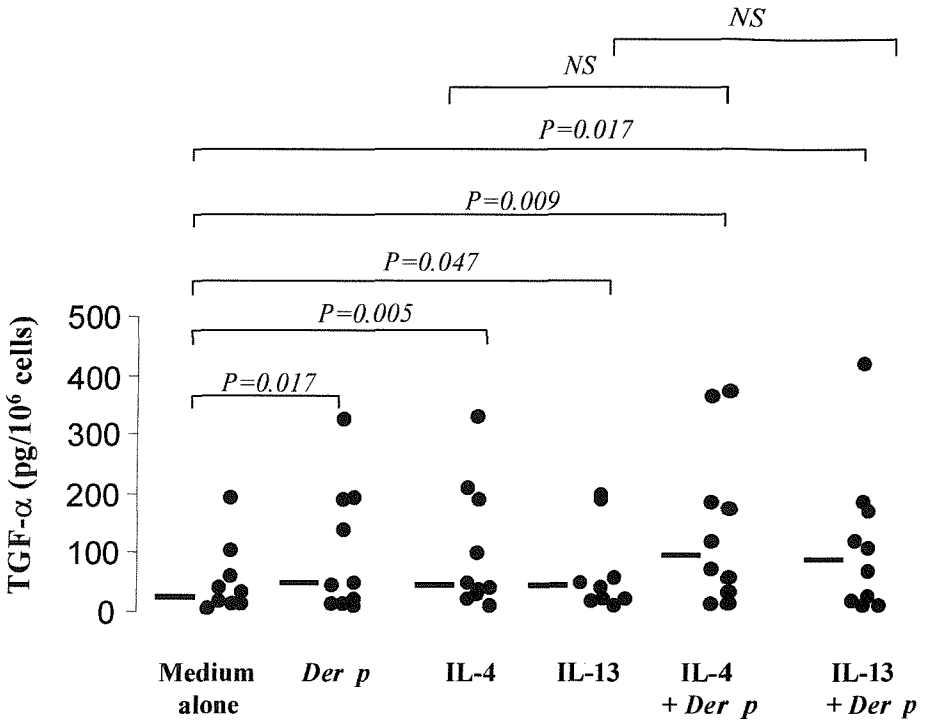


Figure 6.9: Transforming Growth Factor-α production by primary bronchial epithelial cells of atopic asthmatic subjects after culture in medium alone, *Der p* allergen (5,000 SQ IU/ml), IL-4 (20 ng/ml), IL-13 (20 ng/ml), or combined stimulation with *Der p* allergen plus IL-4 or IL-13 for 24 hours.



6.5 DISCUSSION

In chapter 5, I have reported the expression of mRNA transcripts for IL-4 and IL-13 receptor components on bronchial epithelial cells of both asthmatic and normal subjects, and confirmed the expression of IL-4 and IL-13 receptor sub-units, including IL-4Rα, common γ_c and IL-13Rα2 by flow cytometry. In the present chapter, I have extended this study to assess the functional responses of IL-4 and IL-13 receptors on bronchial epithelial cells of atopic asthmatics and normal control subjects. I have confirmed increased production of pro-inflammatory cytokines (GM-CSF), chemokines (IL-8 and RANTES), and growth factors (TGF-α and TGF-β_2) relevant to the inflammatory and remodelling process of asthma after bronchial epithelial cells were exposed to *Der p* allergen, IL-4, IL-13, or TNF-α in culture. Of particular interest, was the observation that the selective stimulation of epithelial cells from atopic asthmatics, but not normal control subjects, with IL-4 and to a lesser extent IL-13, resulted in increased release of the eosinophil chemo-attractant, RANTES, and the growth factors, TGF-β_2 and TGF-α. This study confirms the ability of primary bronchial epithelial cells to generate TGF-α, which is a potent ligand for the epidermal growth factor receptor, and has been linked with the airway production of mucus, of particular relevance to asthma [196]. The selective ability of IL-4 and IL-13 to promote the release of these mediators may perpetuate the airway inflammation, remodelling, and excess production of mucus found in the airways of asthma.

GM-CSF plays an important role in promoting the recruitment, survival, and activation of eosinophils and the maturation of dendritic cells [288, 329, 330] in the airways of asthmatics [267]. We have shown that bronchial epithelial cells respond to stimulation with *Der p* allergen, IL-4 and IL-13 with increased production of the eosinophil survival factor, GM-CSF. In the majority of cultures, IL-4 was a more potent stimulus for GM-CSF release compared to IL-13 or allergen. However, by comparison, TNF-α was a particularly potent stimulus for the release of GM-CSF. There were no significant differences in GM-CSF production by bronchial epithelial cells of normal or asthmatic subjects, in the presence or absence of stimulation. Devalia *et al* have previously noted increased basal production of GM-CSF and IL-8 by epithelial cells of asthmatics compared to healthy control subjects in primary epithelial cells established in culture from bronchial biopsies, which was further enhanced by exposure to environmental pollutants [174]. The epithelial cells used in these cultures were ciliated type epithelial cells, which may respond differently to the basal type epithelial cells used in this study.

Evidence from animal studies and *in vitro* studies using epithelial cell lines has identified the bronchial epithelium as an important source of chemokines that regulate the recruitment of inflammatory cells into the airways of asthmatics [202, 311, 331, 332]. Here, I have noted equivalent levels of constitutive IL-8 production by cultured bronchial epithelial cells of normal and atopic asthmatic subjects. However, increased IL-8 production was confirmed after stimulation of bronchial epithelial cells with *Der p* allergen, IL-4 or IL-13.

In chapter 5, the expression of IL-13Rα2 on primary epithelial cells was assessed, which is thought to act as a decoy receptor or as an antagonist of IL-13 signalling. Of particular interest, in six cultures assessed for IL-13Rα2, the cells that expressed the highest levels of IL-13Rα2 failed to respond to IL-13 and IL-4, although still responding to TNF-α. The relative amount of epithelial cytokine production (expressed as fold stimulation above baseline) induced by IL-4, IL-13 and TNF-α above baseline for IL-8 and GM-CSF were (0.6, 0.2, and 6.0) and (1.1, 0.6, and 2.2), respectively. This suggests that IL-13Rα2 may act to down regulate IL-13 mediated signalling on bronchial epithelial cells.

Although RANTES production was observed in resting bronchial epithelial cell cultures of both normal and asthmatic subjects, only bronchial epithelial cells from asthmatic subjects exhibited increased RANTES production after stimulation with *Der p*, IL-4 or IL-13. It is possible that the difference may be due to a lower number of subjects in the normal control group (n=6) compared to the asthmatic group (n=13). TNF-α was again noted to be a potent stimulus for RANTES production by epithelial cells from atopic asthmatics. The selective ability of bronchial epithelial cells originating from the airways of asthmatics to produce increased quantities of RANTES in response to Th2 cytokines may contribute to the accumulation of eosinophils in the inflammed airways of asthmatics.

Of particular interest, combined stimulation of epithelial cell cultures of atopic asthmatics with IL-4 or IL-13 plus *Der p* allergen resulted in significantly increased production of IL-8 and indeed GM-CSF. The application of combined stimulation with IL-4 or IL-13 plus allergen further augmented the release of RANTES. Thus the Th2 type cytokine mileau in the airways of asthmatics may potentiate the effects of environmental allergens on cytokine release by the bronchial epithelium.

Other studies using airway epithelial cell lines and bronchial biopsy studies have cited the airway epithelium as a major source of IL-16 and eotaxin in the airways of asthmatics. These investigators have documented mRNA transcripts for eotaxin by airway epithelial cells using more potent stimuli (IL-4, 50 ng/ml combined with TNF-α, 100 ng/ml), although more

differentiated ciliated type epithelial cell cultures or epithelial cell lines were used for these studies [304]. In this study, there was no detectable production of IL-16 or eotaxin in the supernatants of primary epithelial cell cultures derived from normal or asthmatic subjects. The epithelial cell cultures established in this study were undifferentiated monolayers, which may behave differently to the differentiated type ciliated columnar epithelial cells studied by other investigators. It is plausible that eotaxin or IL-16 may be selectively released by more differentiated columnar-type epithelial cells, and not from basal type epithelial cells as used in the current experiments.

Sub-epithelial fibrosis, smooth muscle hypertrophy, mucus hypersecretion, and goblet cell hyperplasia are characteristic features of airway wall remodelling in asthma [333, 334]. Recent studies in mice have shown that over-expression of IL-13 in the airways results in sub-epithelial fibrosis, goblet cell metaplasia, and smooth muscle proliferation associated with marked AHR, and eosinophilic and lymphocytic inflammation [195]. A number of growth factors including members of the Transforming Growth Factor-β family are considered to play a prominent role in the airway remodelling process [334, 335]. In the airways of asthmatics, TGF-β levels have been correlated with the thickness of the subepithelial layer and the number of fibroblasts [179]. Increased levels of TGF-β_1 have been noted in the BAL fluid of atopic asthmatics compared to normal control subjects [179]. An over-expression of mRNA and protein for TGF-β_1 has also been observed in bronchial biopsies of severe and moderate asthmatics as compared to non-asthmatic control subjects, using *in situ* hybridisation [336, 335, 337] or immunohistochemistry [338, 339]. Richter *et al* has recently shown by that exposure of primary asthmatic lung fibroblasts to TGF-β_2, but not IL-4 or IL-13 results in fibroblast proliferation and myofibroblast differentiation with increased expression of α-smooth muscle actin [369]. However, exposure to IL-4 or IL-13 did stimulate increased production of eotaxin by the fibroblast cultures [369].

In this study, I have confirmed bronchial epithelial cells to be a source of TGF-β_2, and I report the selective ability of *Der p* allergen, IL-4, and IL-13 to promote the release of TGF-β_2 by bronchial epithelial cells from asthmatic subjects. However, these responses were not found in equivalent studies of bronchial epithelial cells from the airways of normal control subjects. In comparison to the experimental animal studies of asthma [195, 323], we found equivalent production of TGF-β_2 after exposure to IL-4 or IL-13. This suggests that while Th2

cytokines cause direct activation of fibroblasts with a pro-inflammatory outcome by the release of eosinophil chemotatactic factors [369], the effects on sub-epithelial fibrosis and airway wall remodelling in asthma may also be mediated indirectly by promoting the release of TGF-β_2 from bronchial epithelial cells. The ability of IL-4 or IL-13 to stimulate increased production of TGF-β_2 by primary bronchial epithelial cells in this study has been confirmed by a recent study by Wen *et al*, and was reduced by exposure to IFN-γ [396].

Atopic asthma is also characterised by excessive mucus secretion which may contribute significantly to the morbidity and mortality associated with asthma. Mucus secretion and plugging of the airways are characteristic features of patients who die from asthma [321, 340, 341]. The regulation of mucus secretion is a complex process involving cell proliferation and differentiation, mucin gene expression, and release of mature mucus from storage granules. In the respiratory tract, seven of the nine mucin (MUC) genes are expressed (MUC1, MUC5/5AC, MUC2, MUC4, MUC5B, MUC7, and MUC8) [342]. Murine models of allergic disease have been particularly useful in advancing our knowledge of mucus regulation. MUC5 expression is also markedly elevated in the lungs of allergen challenged mice [343]. MUC5 gene expression has also been confirmed in lung mucus samples collected from individuals with asthma and pooled samples from normal individuals [344, 345]. In murine studies, it is thought that mucus production and goblet cell metaplasia (GCM) are directly affected by Th2 type cytokines (IL-4, IL-5, IL-9, and IL-13). Adoptive transfer of Th2 cytokines into the airways of mice results in GCM [346], but it is considered that IL-4 or IL-13 may induce GCM and mucus secretion by promoting the release of other mediators, whereas IL-9 may induce mucus-cell changes directly [347]. Recently, it has been suggested that mucin secretion is regulated by the epidermal growth factor (EGFR) receptor system [196, 197]. In rats, activation of EGFR tyrosine kinase by its ligands leads to the synthesis of mucin MUC5AC, a major mucin in airways, at both mRNA and protein levels. However, the mechanisms of EGFR activation in the airways are unknown. It was hypothesised that the effects of IL-4 and IL-13 on mucus production may be mediated by TGF-α, a potent ligand for the EGFR, released by the bronchial epithelium. To test this hypothesis TGF-α production was measured in supernatants from primary bronchial epithelial cell cultures of asthmatic and normal subjects after exposure to allergen, IL-4, IL-13, or TNF-α. There was constitutive production of TGF-α by epithelial cells of normal and asthmatic subjects, and after

stimulation with either IL-4 or IL-13, TGF-α production was significantly increased in epithelial cell cultures from asthmatic subjects, but not from normal subjects. The production of TGF-α was particularly increased after combined stimulation with *Der p* allergen plus IL-4 or IL-13. This suggests that IL-4 or IL-13 may mediate their effects on mucus hypersecretion and goblet cell metaplasia indirectly by increasing the production of TGF-α by the bronchial epithelium.

In this study, TNF-α also significantly increased the release of TGF-α from primary bronchial epithelial cells of asthmatic subjects, but not from normal control subjects. TNF-α has recently been shown to increase the release of TGF-α from regenerating murine hepatocytes [348]. As TNF-α has also been shown to promote mucus hypersecretion and MUC2 gene expression by human airway epithelial cells, it is plausible that the increased mucus production after exposure of epithelial cells to TNF-α may also involve TGF-α mediated mechanisms [349].

Corticosteroids are the cornerstone of treatment of asthma, and have been effective in improving symptoms in a large number of asthmatic subjects. However, there is a significant proportion of asthmatic subjects that remain symptomatic despite treatment with adequate doses of inhaled corticosteroids, and a proportion are truly resistant to the anti-inflammatory effects of corticosteroids [350, 351]. The airway epithelium is a major target of inhaled corticosteroids, and indeed, corticosteroids have been shown to reduce the release of pro-inflammatory cytokines and chemokines by airway epithelial cells [201, 352]. In this study of bronchial epithelial cells from asthmatic subjects, the increased production of GM-CSF, IL-8, and RANTES after stimulation with *Der p*, IL-4, IL-13, and TNF-α was reduced by dexamethasone, but this was incomplete with cytokine production not being suppressed to basal levels. In similar fashion, the release of TGF-α was also reduced by dexamethasone, but the release of TGF-β_2 was not reduced by corticosteroid treatment. It is possible that the inability of corticosteroids to completely suppress the release of pro-inflammatory cytokines, chemokines, and growth factors by structural airway cells, such as the bronchial epithelium, may contribute to the inability of steroids to completely reverse the characteristic airway remodelling features of the asthmatic phenotype.

The inability of corticosteroids to completely suppress airway inflammation in asthma has prompted further research into more selective therapeutic strategies at targeting airway inflammation. Concerns have been raised about the possibility of long-term side effects of

inhaled corticosteroids due to systemic absorption. There has been particular recent interest in strategies to inhibit the effects of IL-4 in asthma and allergic diseases. A recent study has recently shown that the administration of a soluble IL-4 receptor to atopic asthmatic subjects led to improvements in lung function and reduced symptoms [353]. In a preliminary study, we have assessed the ability of a soluble IL-4 receptor to inhibit cytokine release by airway epithelial cells. We have shown that the release of IL-8 and GM-CSF by IL-4 stimulation of epithelial cells from asthmatic and normal subjects is significantly reduced by the soluble IL-4 receptor.

6.6 CONCLUSIONS

In this study, I have shown that IL-4 or IL-13 can stimulate the release of IL-8, GM-CSF, or RANTES by epithelial cells of asthmatic or normal subjects, similar to the responses noted for *Der p* and or TNF-α in chapter 4. Of particular interest, a co-operative effect was noted between IL-4 or IL-13 and *Der p* allergen in increasing the release of the cytokine GM-CSF, the chemokines, IL-8 and RANTES, and the growth factors, TGF-α and TGF-β_2, from epithelial cells of asthmatic subjects. The ability of IL-4 or IL-13 to stimulate the release of mediators or growth factors from structural elements of the airways such as the bronchial epithelium may play an important role in the inflammation and re-modelling process of asthma.

CHAPTER SEVEN

Discussion and Future Studies

7.1 SUMMARY OF RESULTS

The application of modern molecular and cellular biology techniques combined with the ability to obtain clinical tissue samples from the airways of patients with asthma by fibreoptic bronchoscopy has proved invaluable in this study. This has facilitated the study of the involvement of resident inflammatory cells, the structural cells, and the cytokine mediators involved in the pathogenetic mechanisms of this common yet complex airway disease. The studies presented in this thesis have examined the underlying cellular and molecular inflammatory mechanisms involved in the pathogenesis of asthma, with particular reference to the pro-inflammatory effects of the common aero-allergen, *dermatophagoides pteronissinus*, the involvement of Th2 cytokines, and the contributions made to this process by the bronchial epithelium.

The use of the novel *in vitro* integrated tissue culture model, the bronchial explant culture has proven itself as a valuable and safe technique for the initial assessment of inflammatory pathways in lower airway tissue obtained from asthmatic subjects. In this thesis, I have shown that it is safe and informative to extend these explant studies to patients with moderately severe persistent disease to determine whether inflammatory mechanisms are different in those patients with persistent symptoms despite adequate doses of corticosteroids, as compared with those with mild asthma. A particular benefit of this explant system is the ability to perform a preliminary assessment of the anti-inflammatory effects and therapeutic potential of novel compounds, which have not yet been approved for use in clinical trials. This has allowed the *in vitro* assessment and comparison between the effects of the novel antagonist of T cell co-stimulation, the humanised fusion protein CTLA-4Ig, on cytokine release by bronchial tissue and circulating mononuclear cells in moderately severe asthmatics. The differences noted in this study between the inflammatory mechanisms in the cultured bronchial tissue and the circulating peripheral blood mononuclear cells also emphasises the importance of studying airway inflammatory responses, rather than drawing conclusions from findings in peripheral blood.

The application of immunohistochemistry has helped to define the inflammatory cells resident in the bronchial mucosa of the moderately severe asthmatics involved in the bronchial explant study. The application of inflammatory biomarkers by this technique has helped to identify the presence of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocyte subsets, neutrophils, activated eosinophils, mast cells, B lymphocytes, macrophages, and occasional $CD1a^+$ dendritic cells in the bronchial mucosa of moderately severe asthmatics. This technique was

applied to document the low level of expression of co-stimulatory molecules in the bronchial mucosa of moderately severe asthmatics.

The application of molecular techniques, such as PCR to the cultured bronchial biopsies has considerably improved our knowledge of the ongoing transcription of genes for various cytokines and chemokines in the bronchial mucosa of asthmatic subjects. The application of the highly sensitive PCR-ELISA has particular advantages, as it is 100 times more sensitive than standard gel electrophoresis for the detection of PCR product, and its specificity is comparable to that of Southern blotting. As the PCR-ELISA methods offers a numerical value, it can be used to compare relative amounts of mRNA gene transcripts in different tissue samples. I have used this technique to detect and semiquantify a range of cytokines in the bronchial biopsies cultured under different conditions in the explant studies in chapter 3.

A particular development in this study was the application of a gentle bronchial brushing technique to obtain viable bronchial epithelial cells during fibreoptic bronchoscopy from the lower airways of carefully characterised normal control subjects and patients with mild and moderately severe allergic asthma. The successful establishment of primary bronchial epithelial cell cultures from these bronchial brushings of patients with real asthma has helped to document the expression of IL-4 and IL-13 receptor subunits by bronchial epithelial cells. The primary bronchial epithelial cell cultures have facilitated the investigation of the contribution of the bronchial epithelium to the cytokine mileau in normal subjects and patients with asthma. This novel culture technique has identified intrinsic differences in the profiles of cytokines, chemokines, and growth factors released by bronchial epithelial cells. It has confirmed the isolated and combined ability of house dust-mite allergen and Th2 cytokines to activate the bronchial epithelium with the release of cytokines, chemokines, and growth factors.

The studies presented in this thesis have demonstrated that:

a) Stimulation of bronchial explant cultures from moderately severe asthmatic subjects with a mixed house dust-mite allergen extract results in increased release of IL-5 and GM-CSF, but not IL-13, which are not effectively suppressed by inhibition of CD28-B7 co-stimulation with the fusion protein CTLA-4Ig. This contrasts with the efficacy of CTLA-4Ig to inhibit cytokine release in studies performed under identical conditions in mild asthma (Chapter 3).

b) Exposure of PBMC cultures of moderately severe asthmatics to *Der p* allergen extract results in increased release of IL-5 and IL-13, that is effectively suppressed by CTLA-4Ig, suggesting different requirements for CD28-mediated costimulation in PBMC cultures compared to the bronchial tissue cultures, emphasising the importance of the tissue micro-environment in pulmonary inflammatory responses in severe asthma. This also emphasises the importance of assessing inflammatory responses in an integrated bronchial tissue culture system.

c) Primary bronchial epithelial cells can be safely obtained using bronchial brushings of the airways of normal control subjects, mild asthmatics, or severe asthmatics and grown in culture to study epithelial inflammatory responses and mediator release.

d) Bronchial epithelial cells from normal and asthmatic subjects express mRNA transcripts for the IL-4 and IL-13 receptor subunits, common γ_c, IL-4Rα, IL-13Rα1, and IL-13Rα2.

e) Flow cytometry confirms the presence of IL-4Rα and IL-13Rα2 on bronchial epithelial cells from normal or asthmatic subjects, but not common γ_c. It was not possible to assess IL-13Rα1, due to the lack of availability of an effective antibody.

f) Exposure of bronchial epithelial cells of asthmatic subjects to *Der p* allergen, or the Th2 cytokines, IL-4 or IL-13 increased the production of the cytokine, GM-CSF, the chemokines, IL-8 and RANTES, and the growth factors, TGF-β and TGF-α. The pro-inflammatory cytokine TNF-α was a potent stimulus for the release of GM-CSF, IL-8, RANTES, and TGF-α by bronchial epithelial cells.

g) The application of combined stimulation with the Th2 cytokines, IL-4 or IL-13 in addition to house dust-mite allergen was noted to further augment the release of GM-CSF, IL-8, RANTES, and TGF-α by epithelial cells of asthmatic subjects.

h) The exposure of epithelial cells of normal subjects to *Der p* allergen, IL-4, IL-13 also resulted in increased release of GM-CSF and IL-8, but not RANTES, or the growth factors, TGF-β or TGF-α.

i) There was no significant difference in the production of GM-CSF or IL-8 by epithelial cell cultures of normal subjects and atopic asthmatics.

j) There was considerable heterogeneity noted for cytokine release between different individuals, which may be related to genetic variability or the presence of different polymorphisms.

k) Corticosteroids reduced but did not completely suppress the production of GM-CSF, IL-8, RANTES, and TGF-α to basal levels in bronchial epithelial cell cultures of asthmatic subjects.

l) In similar fashion, GM-CSF, IL-8, and TGF-α production was reduced but not completely suppressed by the potent corticosteroid, dexamethasone in cultures of normal subjects.

Airway inflammation is thought to be central to the pathogenesis of asthma, involving the recruitment and activation of inflammatory cells into the airways, particularly after exposure to environmental stimuli, such as aero-allergens, viruses, or airway pollutants. In sensitised individuals, allergen exposure leads to activation and influx of $CD4^+$ T lymphocytes to the airways [354, 355]. The production of Th2-type cytokines by these Th2 type lymphocytes [356] promotes mast cell and basophil development (IL-3), the synthesis of IgE by B cells (IL-4 and IL-13), and the recruitment and survival of eosinophils in the airways (IL-5 and GM-CSF).

The study presented in chapter 3 has confirmed ongoing expression of mRNA transcripts for pro-inflammatory cytokines (IL-5, IL-8, IL-13, and GM-CSF) and chemokines (RANTES, IL-16, and eotaxin) in the bronchial tissue of moderately severe asthmatics. Exposure to allergen resulted in an increase in the production of IL-5 and GM-CSF, but in contrast to the studies performed in mild asthma by Jaffar *et al* [222, 239], IL-13 production

was not confirmed at a protein level. Although this may be due to the persistent effects of prior corticosteroid treatment in these subjects, it is possible that there may be an intrinsic difference in the profile of cytokines, such as IL-13, in more severe asthma. The novel antagonist of T cell costimulation, CTLA-4Ig was used to examine the involvement of T lymphocytes in allergen-induced cytokine production. In contrast to the mild studies performed by Jaffar *et al* [222, 239], the fusion protein did not inhibit the production of IL-5 or GM-CSF. Although CTLA-4Ig effectively suppressed cytokine production by PBMC cultures established from circulating mononuclear cells, this does suggest that ongoing memory-type T cell dependent immune responses in the airways of more severe asthmatics are less dependent on CD28 mediated co-stimulation. Recent bronchial explant work performed by Dent *et al* in our laboratory has also compared inflammatory responses in mild and severe asthma, also using the bronchial explant model [253]. It was noted that the chemotactic activity of allergen-stimulated bronchial explant supernatants on T-lymphocytes was suppressed by antagonists of CD28-mediated co-stimulation in mild asthmatics, but not in moderately severe asthmatics, suggesting an intrinsic difference in inflammatory mechanisms [253].

A recent clinical trial has reported the administration of CTLA-4Ig to patients with severe refractory psoriasis, and the investigators noted a reduction in parameters of T-cell mediated inflammatory skin lesions and clinical parameters of disease severity [261]. Further studies are required to clarify the precise role of CD28-B7 costimulation in asthmatic airway inflammation and to assess the importance of the tissue microenvironment in pulmonary inflammatory responses in severe asthma. Blockade of co-stimulatory pathways using antagonists such as CTLA-4Ig has attracted significant attention in other T cell mediated inflammatory diseases, particularly in the induction of tolerance and prevention of solid organ transplant rejection [357].

The involvement of other co-stimulatory pathways, including ligation of Ox40-Ox40L [258], ICOS (Inducible costimulator) signalling [259], or T1/ST2 (member of the IL-1 receptor family) [260] in Th2 mediated immune responses in the lung mucosa merits further investigation. Although ICOS-Ig fusion protein was not available at the time of this study, it is now important that antagonists of these pathways, in particular the recently available fusion protein, human ICOS-Ig, are assessed in the severe bronchial asthmatic explant system.

Eosinophils have been closely associated with the degree of airway inflammation, epithelial damage, and disease severity in asthma [110]. The production of interleukin-5 and eotaxin in the airways in asthma has been closely associated with the recruitment and increased survival of eosinophils in the bronchial mucosa of asthmatics [116]. However, two recent clinical trials have questioned the obligate requirement for eosinophils and IL-5 in the pathogenesis of airway inflammation in asthma. The administration of a single intravenous injection of a humanised blocking monoclonal anti-IL-5 antibody (SKB-240563) to allergic asthmatics effectively suppressed the number of circulating and airway eosinophilia, as determined by induced sputum, both before and after allergen exposure [121]. In a separate clinical trial, the administration of recombinant IL-12 to asthmatic subjects also resulted in a suppression of the number of circulating and sputum eosinophils [359]. However, neither treatment resulted in a reduction of the early or late bronchoconstrictor response after allergen inhalation, improvements in AHR, or symptomatic benefit. It is possible that the number of eosinophils resident in the airway mucosa were not reduced by the therapeutic interventions, mediated by the protective effect of cytokines (e.g. GM-CSF) released by the airway epithelium and underlying myofibroblasts against the programmed cell death or apoptosis of activated eosinophils in the airway tissue. However, on the basis of current data, this does question the central role attributed to the eosinophil in the airway inflammation of asthma.

The initial studies presented in chapter 3 have assessed the effects of allergen on an integrated bronchial tissue culture system, focusing particularly on the role of inflammatory cells and their related cytokines. However, it is increasingly obvious that a major part of the bronchial hyperresponsiveness of asthma is related to the structural changes that occur in the airways, including epithelial cell damage, deposition of extra-cellular matrix in the airways, goblet cell metaplasia, smooth muscle hypertrophy, and increased angiogenesis [157, 333]. As the physical barrier between the external environment and the internal milieau, airway epithelial cells are in a key position to respond to environmental stimuli, such as allergens [54]. However, bronchial epithelial cells are also capable of responding to cytokines and mediators released by inflammatory cells recruited to the airways, with the release of pro-inflammatory cytokines and mediators [360].

In chapter 4 of this study, I have performed a detailed analysis of the responsiveness of the bronchial epithelium to house dust-mite allergen, and confirmed allergen-induced

increased production of IL-8 and GM-CSF. However, there was no significant difference in cytokine production by epithelial cells of normal or asthmatic subjects. Allergen also augmented the production of RANTES, but only in bronchial epithelial cell cultures derived from asthmatic subjects. This is interesting as Bayram *et al* have previously assessed cytokine production by primary bronchial epithelial cell cultures established from bronchial biopsies, and reported constitutively increased production of IL-8, GM-CSF, and RANTES by epithelial cells of asthmatics compared to normal subjects [173]. As the cell cultures established in their study were ciliated columnar-type epithelial cells, this may explain the differences noted for cytokine production by the epithelial cell mono-layers, that were utilised in the present study, which were not ciliated.

The effects of the Th2 cytokines, IL-4 and IL-13, on the production of IL-8, GM-CSF and RANTES by asthmatic and normal bronchial epithelial cells were also assessed in chapter 5, where equivalent production of IL-8 and GM-CSF was noted. In similar fashion to the effects of allergen, the Th2 cytokines augmented the production of RANTES by epithelial cells of asthmatics, but not normal controls. Of particular interest, the combined effects of allergen and Th2 cytokines exhibited a degree of synergism on the release of IL-8 and GM-CSF by epithelial cells derived from asthmatics and normal subjects, and again only asthmatic epithelial cells exhibited augmented production of RANTES with combined stimulation. The selective production of RANTES by bronchial epithelial cells derived from bronchial biopsies of atopic asthmatics but not normal subjects has previously been noted by Bayram *et al* [163]. Since all forms of asthma are characterised by increased airway production of Th2 cytokines [102, 214, 361, 362], the presence of these mediators in the airways of asthmatics is a key difference between asthmatic and normal subjects. The ability of IL-4 and IL-13 to augment the release of cytokines by bronchial epithelial cells in the presence of *Der p* allergen may explain the airway inflammation noted in asthmatic airways. Since allergen inhalation is equivalent for normal subjects and asthmatics, the Th2 mediated inflammation in the airways of asthmatics would amplify the epithelial release of GM-CSF, IL-8, and RANTES. This would lead to an increased influx and survival of inflammatory cells, including eosinophils [277], neutrophils [283, 285], and T lymphocytes resulting in an exaggerated airway inflammatory response [286]. RANTES has been identified as a major eosinophil in the BAL fluid of asthmatic subjects exposed to allergen challenge [221]. The levels of RANTES were

noted to correlate with eosinophil numbers in lavage fluid, and concentrations of BAL RANTES as low as 0.5 ng/ml induced eosinophil chemotaxis *in vitro*. Neutralising the effects of chemokines such as RANTES by the development of chemokine mutants such as Met-RANTES and Met-CK-β7 which antagonize chemokine receptors (CCR3 and CCR1) to inhibit eosinophil recruitment represents a novel therapeutic strategy in asthma [221].

Distinct from their effects on the immune inflammatory cascade, a prominent role has been identified for, IL-4 and IL-13 in asthma, by their effects on mucus hypersecretion and airway wall remodelling, as demonstrated by recent transgenic animal studies [194, 195, 363-365]. *In vitro* studies have also confirmed direct effects of IL-4 and IL-13 on epithelial and fibroblast function [194, 366, 367], and more recently on airway smooth muscle cells [368]. In our laboratory at Southampton, we have recently established primary lung fibroblast cultures from atopic asthmatic subjects, and shown that IL-4 and IL-13 stimulate the production of eotaxin by lung fibroblasts [369]. Unlike the growth factor TGF-β, the Th2 cytokines did not promote the transformation of these cells into myofibroblasts, and they did not stimulate collagen I gene expression [369]. However, in chapter 6 of this thesis, I have shown that exposure of bronchial epithelial cells of asthmatic subjects to IL-4 and IL-13 increased the production of TGF-β_2 by these cells, which may promote the restoration of a damaged epithelium in asthma, but also provides an indirect mechanism through which these cytokines may promote airway remodelling in asthma. Hashimoto and colleagues have recently shown that human lung fibroblast cultures do exhibit increased expression of α-smooth muscle actin in a concentration and time dependent manner after exposure to either IL-4 or IL-13 by pathways involving c-Jun NH_2-terminal kinase [397].

A central role has been reported for the EGFR in the establishment of the muco-secretory phenotype, as removal of EGF prevents mucin gene expression and mucus secretion [370]. Exposure of epithelial cells to cigarette smoke causes induction of mucus gene expression by an EGF receptor pathway (EGFR/cerbB1) [371]. Th2 cytokines have recently been implicated in goblet cell metaplasia and excess production of mucus [195, 321], which have been attributed to an indirect mechanism involving IL-13 induced IL-8 production, increased neutrophil chemotaxis, and increased neutrophil-derived oxidant-induced activation of the EGFR [196, 372]. However, airway epithelial cells are a source of transforming growth

factor-α (TGF-α), a ligand for the EGF, which has been shown to induce the expression of mucus genes in airway epithelial cell lines [196, 197]. IL-4 has also been shown to induce mucin gene (MUC5AC) expression by airway epithelial cells *in vitro* and *in vivo*, even in the absence of neutrophils [194, 373]. In chapter 6, I have documented IL-4 and IL-13-induced production of TGF-α by bronchial epithelial cells of asthmatic subjects, but not of normal controls, supporting the hypothesis that the effects of IL-4 and IL-13 on mucus production in asthmatics may be indirectly mediated by increased production of the EGFR ligand, TGF-α, by epithelial cells. Consistent with the effects of TNF-α on hepatocytes [348], I also report for the first time that TNF-α increases the release of TGF-α by asthmatic bronchial epithelial cell cultures, suggesting a novel mechanism by which TNF-α can also impact on the airway remodelling process in asthma.

The poor response of the normal bronchial epithelial cells to release TGF-α may be due to a difference in the expression, processing and utilisation of TGF-α, rather than a selective suppression of responses to the Th2 cytokines. Consistent with this proposal, the epithelial cells exhibited increased production of IL-8 and GM-CSF in response to IL-4 and IL-13. TGF-α is synthesised as a trans-membrane precursor, whose cleavage is promoted by metalloproteinases including TNF-α converting enzyme (TACE). The inability to detect TGF-α in normal epithelial cell supernatants may be due to a lack of gene expression, an inability to process the growth factor, or alternatively may be due to increased autocrine utilisation of the growth factor TGF-α by the epithelial cells resulting in apparently reduced production. Thus, the increased production of TGF-α by the asthmatic epithelium may contribute to airway remodelling by augmented fibroblast proliferation and goblet cell metaplasia. Alternatively, the reduced utilisation of TGF-α by the asthmatic epithelial cells may reflect an abnormal repair response in the asthmatic epithelium, and explain the epithelial disruption that is so characteristic of asthma.

In chapter 6, I report the ability of *Der p* allergen to promote the release of TGF-α by epithelial cells of asthmatic subjects, which has not been documented previously. *Der p* allergens are known to have enzymatic activity, both cysteine and serine proteinase activity [48], but it is not known whether allergens are directly capable of cleaving the membrane-bound precursor of TGF-α or activating cell surface metalloproteinases. It will be interesting

to determine the ability of allergen proteinases to promote the release of biologically active molecules from cell surface precursors, or to cause disruption of intercellular adhesion junction proteins.

The overlapping, but not identical, effector profiles of the Th2 cytokines, IL-4 and IL-13, have been described earlier in chapter 1, and are likely related to the shared use of the IL-4Rα and the IL-13Rα1 in the multimeric IL-4 and IL-13 receptor complex [314, 315, 374]. However IL-4 can also bind to IL-4Rα complexed with the common γ chain of the IL-2R (γ_c), while IL-13 can interact with the IL-13Rα2, which appears to have a negative impact on IL-13 signal transduction [324]. IL-4 and IL-13 signalling is negatively controlled by a variety of regulators, including silencing of cytokine signalling (SOCS) proteins, shp phosphatase, a dominant negative splice variant of STAT-6, which has recently been described on epithelial cells of normal and asthmatic epithelial cells in our laboratory [375], and the IL-13Rα2 [376, 377]. The high-affinity IL-13Rα2 receptor sub-unit negatively regulates IL-13 function by competitive binding of IL-13 and promoting receptor internalisation. This is the first study to report the expression of IL-13Rα2 on human bronchial epithelial cells, suggesting it may be involved in regulating epithelial responses to IL-4 and IL-13. This suggests that IL-13Rα2 may be an important modifier of epithelial responses to Th2 cytokines, and that further work to analyse the regulation and function of IL-13Rα2 expression in normal and asthmatic bronchial epithelium is warranted.

In chapter 5, I have applied RT-PCR to confirm gene transcription for IL-4Rα, γ_c, IL-13Rα1, and IL-13Rα2 by bronchial epithelial cells of normal and asthmatic subjects. Cell surface expression of IL-4Rα, IL-13Rα2 and γ_c was confirmed by FACS analysis using epithelial cell cultures of normal control subjects (n=3) and atopic asthmatics (n=3). IL-4Rα and IL-13Rα2 were readily detectable in every case, but in the case of IL-13Rα2, the levels of expression were found to be variable. Of particular interest, in the six cultures assessed for IL-13Rα2, the cells that expressed the highest levels of IL-13Rα2 failed to respond to IL-13 and IL-4, although still responding to TNF-α. The relative amount of epithelial cytokine production (expressed as fold stimulation above baseline) induced by IL-4, IL-13 and TNF-α above baseline for IL-8 and GM-CSF were (0.6, 0.2, and 6.0) and (1.1, 0.6, and 2.2), respectively. In contrast to IL-4Rα and IL-13Rα2, only low levels of γ_c were detectable on

the epithelial cells. It was not possible to evaluate the presence of IL-13Rα1 due to the lack of a suitable antibody for use by FACS analysis.

7.2 FUTURE DIRECTIONS

The studies performed in this thesis have investigated the airway inflammatory responses in allergic asthma following allergen exposure, and the involvement of Th2 cytokines in this process, with particular emphasis on the role of T cell co-stimulation and the involvement of structural elements of the airways, in particular the bronchial epithelium. The involvement of co-stimulation in T cell dependent pulmonary immune responses in severe asthma should now be extended to further assess the role of CD28 mediated costimulation in this disease. The recent safety studies and clinical results of the phase 1 clinical trial of the fusion protein CTLA-4Ig in patients with psoriasis is encouraging for the application of this compound in the management of other T cell mediated conditions, such as severe asthma or the induction of tolerance in clinical solid organ transplantation [262, 357]. The identification of other co-stimulatory pathways, including Ox40-Ox40L [258], ICOS (Inducible costimulator) signalling [259], or T1/ST2 (member of the IL-1 receptor family) [260] that may be specifically implicated in ongoing pulmonary immune responses also merit further investigation in similar bronchial explant culture systems in moderately severe asthma. The recent development of antagonists such as the ICOS-Ig fusion protein will be of particular use in this context [358].

The epithelial culture studies in this thesis have shed considerable light on the direct effects of house dust-mite allergen on mediator release by epithelial cells of asthmatic subjects and normal subjects. It has also reported the production of cytokines, chemokines and growth factors induced by Th2 cytokines that are likely to contribute significantly to airway inflammation and remodelling, with particular reference to the EGFR ligand TGF-α which has been closely linked to airway mucus hypersecretion and goblet cell metaplasia.

In this study, I have developed a reliable technique for the growth of primary cultures of normal and asthmatic bronchial epithelial cells using bronchial brushings, obtained at fibreoptic bronchoscopy, with no side effects. The ability to reliably establish bronchial epithelial cell cultures by this technique has paved the way towards further dissecting the underlying mechanisms that regulate bronchial epithelial cell function in allergic asthma. The methodology has now been extended at Southampton to differentiate epithelial cell cultures of

normal subjects and asthmatics on an air-liquid interface to form a polarised stratified epithelium, with basal cells supporting functionally active ciliated cells and mucus secreting goblet cells. This is an ideal model to extend these studies to assess the mechanisms involved in goblet cell metaplasia and mucus hypersecretion in asthma.

The involvement of the Th2 cytokines, IL-4 and IL-13 in goblet cell metaplasia and the induction of mucus gene expression and excess mucus secretion should now be assessed by the application of recently developed novel selective antagonists for IL-4 and IL-13 (i.e. solIL-4R (Immunex), solIL-13Rα1 and solIL-13Rα2 (both from Genetics Institute)). The involvement of the EGFR/c-erbB receptor and ligand family in this process should also be assessed using selective neutralising antibodies to EGF or its ligand TGF-α. By the application of transgenic mouse technology, it would be important to investigate these mechanisms in parallel using an integrated *in vivo* mouse model.

The ability to obtain epithelial cells from the airways of asthmatic subjects may also be applied to perform a more detailed examination of the numerous genes expressed by the structural elements of the airways using techniques such as cDNA expression arrays. The identification of ongoing transcription of genes for IL-13Rα1 and IL-13Rα2 by bronchial epithelial cells may be of particular value to identify functional variants of IL-13 signalling. Further studies should be performed to identify variants of IL-4 and IL-13 signalling in the development of asthma and atopy in humans, which has considerable potential for the development of novel preventive or therapeutic strategies.

The ability to establish primary cultures of bronchial epithelial cells and other cells, should be further utilised to assess the effects of soluble mediators released into epithelial supernatants on *in vitro* inflammatory responses by structural cells including myofibroblasts, and inflammatory cells including T lymphocytes, esosinophils, mast cells, or dendritic cells. The establishment of *in vitro* co-cultures of epithelial cells and inflammatory cells may also help to further dissect the ability of the bronchial epithelium to modulate inflammatory responses in these cells.

The ability to grow epithelial cell monolayers on a collagen gel seeded with myofibroblasts has been a particular strength of the co-culture model developed by Zhang *et al* [193]. This has the interaction and signalling between epithelial cells and the underlying

myofibroblast layer. Chakir *et al* have recently performed similar co-culture studies using primary cultures of epithelial cells and primary lung fibroblasts from the airways of normal subjects and atopic asthmatics [400]. The fibroblasts were grown in a collagen matrix and epithelial cells seeded on top and cultured on an air-liquid interface resulting in a pseudostratified ciliated epithelium and the presence of mucus secreting cells. The ability to produce an *in vitro* model of the bronchial mucosa, using cells derived from the airways of patients with inflammatory lung disease, is a valuable tool to improve our understanding of the involvement of the epithelial-mesenchymal trophic unit in the pathogenesis of airway remodelling and inflammation in asthma [157].

7.3 INTEGRATED MODEL OF ASTHMA

In response to environmental allergen exposure, the polarization and activation of T lymphocytes to a Th2 phenotype by the cognate interaction of intra-epithelial dendritic type antigen presenting cells and T lymphocytes involving MHC II-peptide-TCR ligation, appropriate costimulatory signalling, and cytokine mileau. This leads to the generation of Th2-type cytokines that promote chronic airway inflammation by mechanisms involving the switching of B lymphocytes to the synthesis of IgE, promoting the airway recruitment and activation of inflammatory cells including T lymphocytes, eosinophils and mast cells. The subsequent release of pro-inflammatory mediators results in airway inflammation and damage.

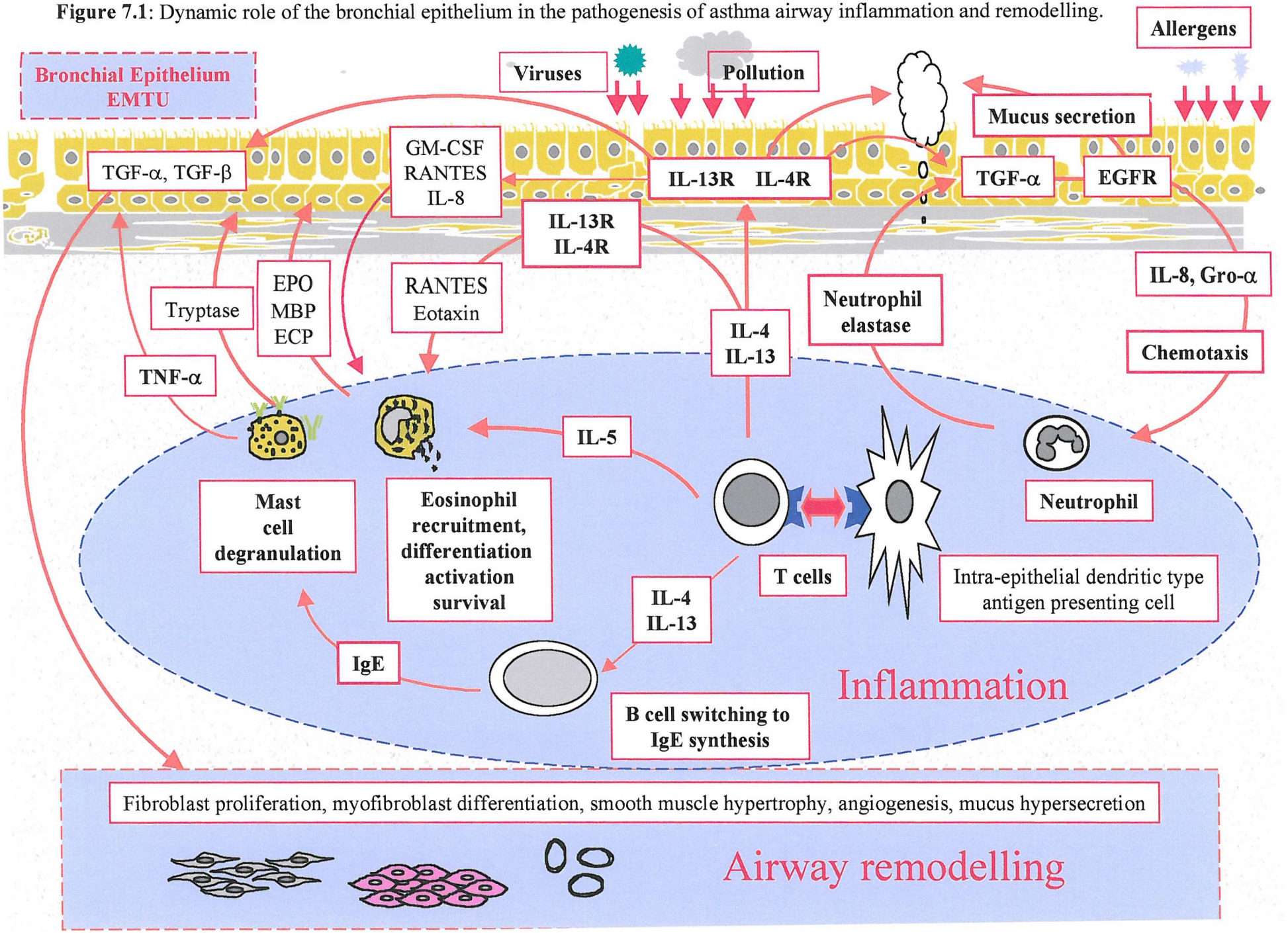
The data presented in this thesis and other studies from our laboratory support the hypothesis that the clinical expression of asthma results from an intrinsic susceptibility of the airway epithelium to Th2 mediated inflammation and environmental stimuli, such as environmental allergens (Figure 7.1). Airway epithelial cells of asthmatics can be activated by environmental stimuli such as house dust mite allergens resulting in increased generation of cytokines, chemokines and growth factors. The actions of IL-4 and IL-13 on bronchial epithelial cell function by the generation of cytokines (GM-CSF), and chemokines (IL-8 and RANTES) promote the recruitment and activation of inflammatory cells, including T lymphocytes, eosinophils, mast cells and neutrophils, resulting in increased airway inflammation.

A close relationship also exists between Th2 cytokines and the formed elements of the airway in asthma, such as the bronchial epithelium or the underlying myofibroblast layer. IL-4 and IL-13 promote the release of growth factors from bronchial epithelial cells of atopic

asthmatics with increased release of TGF-β_2, which stimulates the proliferation of asthmatic mucosal fibroblasts, cause their differentiation into myofibroblasts, and increase the expression of interstitial collagen genes. By this means, the direct actions of environmental stimuli and Th2 cytokines on the susceptible bronchial epithelium, and the indirect activation of the underlying subepithelial myofibroblast layer of the epithelial mesenchymal trophic unit propogate and amplify inflammatory and remodelling responses in the airways of asthmatics.

Goblet cell hyperplasia and mucus hypersecretion are characteristic features of asthma, particularly at the severe end of the disease spectrum. IL-4, IL-9 and IL-13 have been shown to stimulate goblet cell hyperplasia as does the EGFR ligand, TGF-α. The increased production of TGF-α by bronchial epithelial cells of atopic asthmatics after exposure to IL-4 or IL-13 implicates TGF-α as a mediator in IL-4 and IL-13 induced mucus production in asthma. The ability of house dust mite allergen and TNF-α to increase the production of TGF-α by bronchial epithelial cells of asthmatics also supports an indirect effect of these stimuli on mucin production. The increased production of neutrophil chemotactic factors including IL-8

Figure 7.1: Dynamic role of the bronchial epithelium in the pathogenesis of asthma airway inflammation and remodelling.



by the bronchial epithelium would contribute to the recruitment and activation of neutrophils, oxidant injury, activation of EGFR and mucus hypersecretion by mechanisms involving neutrophil elastase in the airways of asthmatic subjects. The ability of a susceptible bronchial epithelium to perpetuate neutrophil mediated airway inflammation, mucus hypersecretion and the inability of corticosteroids to completely suppress these responses are likely to contribute to the persistence of chronic airway inflammation and remodelling in severe asthma.

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APPENDIX

APPENDIX

Materials

Bronchoscopy

Bronchoscope (Olympus FB-20D, Tokyo, Japan)

Biopsy forceps (Olympus, Tokyo, Japan)

Bronchial brush (Olympus, Tokyo, Japan)

Medications obtained from hospital pharmacy

Lignocaine spray (10%)

Lignocaine solution (1%)

Midazelam (0-6 mg IV)

Atropine (0.6mg)

Salbutamol (2.5mg)

Ipratropium Bromide (0.5mg)

Histamine

Methacholine

RT-PCR ANALYSIS

RNA extraction

- Mini glass homogenizers (100µl) — Jencons Scientific Ltd
- Trizol Reagent — LifeTechnologies (Cat.No. 15596)
- Glycogen — Roche Diagnostics (Cat. No. 901393)
- Chloroform
- Isopropranolol
- DEPC water
  (Diethyl pyrocarbonate is added to ultra-high quality water 0.01 % v/v in RNase–free glass bottles, shaken, kept overnight, and then autoclaved twice prior to use).
- 80 % ethanol (made up with DEPC)
- Sterile Mini-Pastettes — Alpha Laboratories
- Sterile filter tips — Promega
- Sterile RNase –free Eppendorf tubes

RNA measurement

Gene Quant RNA / DNA calculator — Pharmacia, UK

Reverse Transcription

Reverse Transcription System Kit Promega (Cat. No. A3500)

Contains:

- AMV Reverse Transcriptase (1500 Units) Cat. No. M900B
  - Isolated from the virions of purified AVIAN Myeloblastosis Virus
  - 1 unit defined as the amount of enzyme required to catalyze the incorporation of 1 nmol of dTTP into acid insoluble form in 10 mins at 37°C using poly(A)-oligo(dT) as a template.
  - Suplied in 200mM potassium phosphate, pH 7.2, 0.2% Triton ® X-100,2mM DTT and 50% glycerol.
- Reverse Transcription Buffer (1.4ml) Cat.No.A356A
  (Composition: 100mM Tris-HCl, pH 8.8 at 25°C, 500mM KCl,1% Triton ® X-100).
- Recombinant Rnasein ® Ribonuclease inhibitor (2500 units) Cat.No. N251A
  -Recombinant protein isolated from *E.Coli.*
- Oligo$(dT)_{15}$ Primer (50μg) Cat.No C110B
  -0.5 mg/ml in water and TE buffer.
- dNTP mixture (10mM) Cat.No. C114B
  10mM each of dATP, dCTP, dGTP, dTTP in water, neutralised to pH 7.0
- Magnesium Chloride 25 mM (1.2ml) Cat. No. A315A
- DEPC water
- Perkin Elmer Thermocycler
- Sterile filter tips and Eppendorf tubes

Solutions used for Methylene Blue Assay

Formal Buffered Saline

$NaH_2PO_4.2H_2O$	4.0 g
Na_2HPO_4	6.93g
NaCl	8.3g
40% Formaldehyde	100mls

Add tap water to a final volume of one litre and mix well.

ELISA PROTOCOLS

Interleukin-8 ELISA

CLB PeliKine Compact ™ Human Interleukin-8 ELISA kit Cat. No. M1918

- **Coating antibody**

  Coating antibody for each 96 well microtiter plate was prepared by adding 120 µl of coating antibody to 12 mls coating buffer and mixing. 100µl was added to each well and the plate was sealed and incubated overnight at room temperature (18-25 °C).

- **Initial washing step**

  The following morning, all reagents, with the exception of streptavidin-HRP, were brought to room temperature and working strength PBS solution was prepared. The wells were emptied by inversion and tapping dry on lint-free paper towels and washed 4 times with PBS.

- **Blocking procedure**

  Blocking buffer was prepared by adding 500 ml of blocking reagent to 25 mls working strength PBS. 200 µl of blocking buffer was added to each well, and the microtitre plate was covered with adhesive seal. The micritoter plate was gently agitated by tapping the side of the plate for a few seconds to mix contents. The plate was then incubated for 1 hour at room temperature.

- **IL-8 standard curve preparation**

  IL-8 standards were prepared by serial dilutions of the 10 ng/ml IL-8 standard provided. The standard curve tubes were labelled 240, 96, 38.4, 15.4, 6.1, 2.5, and 1 pg/ml. 610 µl of working-strength dilution buffer was added to the first tube labelled 240 pg/ml, and 300 µl of working-strength dilution buffer added to the other tubes. 15 µl of the 10 ng/ml IL-8 standard was added to the 240 pg/ml tube and mixed well. 200 µl of this solution was then added to the next tube, labelled 96 pg/ml. Serial dilutions were performed six more times by adding 200 µl of the previous tube of diluted standard to the 300 µl of dilution buffer. Dilution buffer alone was used for 0 pg/ml.

- **Samples**

  Due to the high concentration of IL-8 in epithelial culture supernatants, it was necessary to dilute samples by 1: 75 or 1:150 with working-strength dilution buffer prior to analysis by the IL-8 ELISA.

- **Washing step**

  The microtiter plate was emptied by inversion, tapped dry on paper towels. The plate was then washed five times by completely filling the wells with working-strength washing buffer, emptied by inversion, and tapped dry on paper towels.

- **Standards and samples** **First incubation step**

  100 µl of the prepared standards and samples were transferred in duplicate into the appropriate wells. The plate was sealed, tapped gently for a few seconds to mix contents and incubated for two hours at room temperature.

- **Washing step**

  A second washing step (x 5) was performed, as described above.

- **Biotinylated IL-8 antibody** **Second incubation step**

  Working-strength biotinylated IL-8 antibody solution was prepared by adding 120 µl of biotinylated IL-8 antibody to 12 mls working-strength dilution buffer for each 96 well ELISA plate. 100 µl of the biotin conjugate solution was then added to each well, leaving the substrate blank wells empty. The plate was covered with adhesive seal, gently agitated by tapping the side of the plate to mix contents and incubated for one hour at room temperature.

- **Washing step**

  A third washing step (x 5) was performed, as described above.

- **Streptavidin-HRP conjugate** **Third incubation step**

  Concentrated streptavidin-HRP conjugate is stored at minus 20 °C to maintain maximal stability. 3 µl of streptavidin-HRP conjugate was added to 30 mls working-strength dilution buffer immediately prior to use. Leaving the substrate wells blank, 100 µl of streptavidin-HRP conjugate was added to all wells, except the substrate blank wells. The plate was covered with adhesive seal, gently agitated by tapping the side of the plate to mix contents and incubated for 30 minutes at room temperature.

- **Washing step**

  A fourth washing step (x 5) was performed, as described above.

- **Substrate colour development** **Fourth incubation step**

  The substrate solution was prepared approximately ten minutes prior to use, as described below. 100 µl of substrate solution was added to all wells, including the substrate blank wells. The plate was covered with adhesive seal, gently agitated by tapping the side of the plate to mix contents and incubated for up to 30 minutes at room temperature.

- **Stop enzymatic reaction**

  The enzymatic substrate colour reaction was stopped by the addition of 100 µl of stop solution (see below) to all wells.

- **ELISA plate reading and calculation of IL-8 concentrations**

  The absorbance at 450 nm for the standards and samples were measured on an ELISA plate reader. A standard curve was generated from the duplicate standard absorbance readings, corrected by subtraction of the average of the substrate blank wells. The average absorbance readings of supernatant samples in the duplicate wells were noted and the concentration of IL-8 in each sample read from the standard curve. The levels were then corrected for initial dilutions and recorded. Some samples required repeat analysis, as the readings were above the range of the standard curve.

Buffers and solutions

All buffers and solutions were prepared in the laboratory in accordance with the manufacturer's instructions.

Coating buffer: 0.1 M Carbonate/bicarbonate buffer pH 9.6

Solution A: 1.24 g $Na_2CO_3.H_20$ in 100 ml distilled water

Solution B: 1.68 g $NaHCO_3$ in 200ml distilled water

70 ml of solution A, and add solution B until the pH is 9.6

The prepared buffer can be stored for one week at 2-8 °C.

PBS stock solution [20 x]: 0.2 M Phosphate buffered saline (PBS)

Dissolved 32 g $Na_2HPO_4.2H_2O$
6 g $NaH_2PO_4.2H_2O$
164 g NaCl
in 900 ml distilled water

Dissolved by intensive stirring, and pH adjusted to 6.8-6.9 with concentrated HCl or NaOH, and distilled water added to a volume of 1 liter. When diluted 20 fold, the obtained buffer will have a pH of 7.2-7.4.

Washing Buffer: PBS with 0.005 % TWEEN 20

50 µl of TWEEN 20 added to 1 liter of working strength PBS.

Substrate Buffer: 0.11M acetate buffer pH 5.5.

15 g sodium acetate dissolved in 800 ml distilled water.

pH adjusted to 5.5 with glacial acetic acid, distilled water added to a volume of 1 liter.

3,5,3',5'-tetramethylbenzidine (TMB) stock solution: 6 mg/ml TMB in DMSO

30 mg 3,5,3',5'-tetramethylbenzidine (TMB) dissolved in 5 ml dimethylsulfoxide (DMSO).

Stored at room temperature. Protected against light.

Hydrogen peroxide stock solution: 3% H_2O_2 solution in distilled water.

Substrate solution:

The following reagents were mixed for each ELISA plate.

12 ml substrate buffer

200 µl TMB stock solution

12 µl H_2O_2 stock solution

Substrate solution was prepared immediately before use and was stored at room temperature for optimal reproducible results.

Stop solution: 1.8 M H_2SO_4 solution in distilled water.

Transforming Growth Factor, TGF-β_2 ELISA

TGF-β_2 E $_{max}$® Immunoassay System, Promega

Product G3400. Technical Bulletin No. 224.

TGF-β_2 *in vivo* is processed from a latent form to a bioactive form of the protein. This processing is performed *in vitro* by acid treatment. Total TGF-β_2 measurement of samples was performed by initial acid treatment of samples to a pH of approximately 2.6 for 15 minutes. This was achieved by the addition of 4 µl of 1 N HCl to each 200 µl epithelial cell culture supernatant sample with mixing. After 15 minutes, 4 µl of 1 N NaOH was added to each sample and the samples stored on ice until analysis. The analysis of TGF-β_2 by ELISA was performed in accordance with the manufacturer's instructions.

Protocol

- **Plate preparation**

  A 96 well Costar® (Cat. # 3590) ELISA plate was coated with antibody solution, by adding 10 µl of the TGF-β_2 coating monoclonal antibody to 10 mls of carbonate coating buffer in a 15 ml polypropylene tube. This was mixed thoroughly, avoiding bubbles. 100µl of the coating antibody solution was then added to each well of the polystyrene ELISA plate. The wells were sealed with a plate sealer and incubated overnight (16-20 hours) at 4 °C.

- **Preparing TGF-β_2 blocking buffer**

  On the day of analysis, TGF-β_2 blocking buffer was prepared by adding 22.4 mls of deionized water and 5.6 mls of the TGF-β_2 block (5x) buffer in a 50ml polypropylene tube with a sterile pipette. This was mixed well by inversion prior to use. Each plate requires approximately 28 mls of TGF-β_2 block buffer (x1, working concentration) to be used.

- **Blocking the plate**

  The pre-coated plate was then removed from the refrigerator and allowed to warm to room temperature (approximately 15 minutes). The contents of the wells were drained by inversion and the inverted plates tapped on lint free paper to help clear the wells. 270 ml of working strength TGF-β_2 block (x 1) buffer solution was added to each well with a multichannel pipettor, taking care to avoid scratching off the antibody bound to the well surface. The wells were sealed with a 96 well plate sealer and incubated at 37 °C for 35 minutes without shaking.

- **Preparing the sample buffer**

Each 96 well plate requires approximately 23 mls of TGF-β_2 sample (1 x) buffer. This volume includes 3 mls to determine the TGF-β_2 standard curve. For each 96 well plate, 20.7 mls of de-ionized water was combined with 2.3 mls of TGF-β_2 sample (10 x) buffer in a 50 ml polypropylene tube using a sterile pipette, and mixing gently and completely by inversion.

- **Preparing the TGF-β_2 standard curve**

The TGF-β_2 standard curve is linear between 32 and 1,000 pg/ml. TGF-β_2 standard is provided at a concentration of 1µg/ml and is already acid treated. The supplied TGF-β_2 standard was accurately diluted 1:1,000 in TGF-β_2 sample (1 x) buffer to achieve a concentration of 1,000 pg/ml.

Following plate blocking, the contents of the wells were flicked out over a sink, and slapping the inverted plates three times on lint free paper to remove residual liquid. The standard curve was prepared in the assay plate by adding 200 µl of TGF-β_2 standard (1,000pg/ml) to duplicate wells (A1, A2). 100ml of TGF-β_2 sample (1 x) buffer was added to each of wells (B1, B2 through to H1, H2). Serial 1:2 dilutions (100 µl/well) were then performed in the two columns designated for the standard curve. No TGF-β_2 was added to wells H1, H2.

- **Addition of sample**

100 µl of the acid-treated supernatant samples were added to the remaining duplicate wells of the 96 well plate. The wells were sealed with a 96 well plate sealer and incubated for 1.5 hours at room temperature with shaking (500 ± 100 rpm).

The wells were drained by inversion and tapping 3 times on lint free paper, and washed by filling each well with TBST wash buffer. The wells were washed 4 times and drained by inversion and tapping 3 times on lint free paper, as above.

- **Addition of secondary Anti-TGF-β_2 pAntibody**

Anti-TGF-β_2 pAntibody was prepared by adding 5 µl of the anti-TGF-β_2 pAb to 10 mls of TGF-β_2 sample (1 x) buffer (1: 2,000 dilution) to prepare enough reagent for a full 96 well plate. This was mixed thoroughly by gentle inversion. 100 ml of the working strength anti-TGF-β_2 pAb was then added to each well using a multichannel pipettor. The wells were sealed with a plate sealer and incubated for 2 hours at room temperature with shaking. The washing step was repeated (x 4) as described above.

- **Addition of TGF-β_2 horse radish peroxidase (HRP) conjugate**

The TGF-β_2 HRP conjugate solution was prepared by adding 5 µl of the stock TGF-β_2 HRP conjugate to 10 mls of TGF-b2 sample (1 x) buffer (1:2,000 dilution) in a 15 ml polypropylene tube, to prepare enough reagent for a full 96 well plate. This was mixed thoroughly. 100 µl of the diluted TGF-β_2 HRP conjugate solution was then added to each well using a multichannel pipettor. The wells were sealed and incubated for 2 hours at room temperature.

Washing (x 4) as described.

- **Colour development**

The chromogenic substrate, 3,3',5, 5'-tetramethyl benzidine (TMB), was prepared 1-2 hours prior to colour development by combining 5 mls of TMB solution with 5 mls of peroxidase substrate for each 96 well plate, and mixed gently. This was protected from light and kept at room temperature prior to use.

100µl of the TMB substrate solution was added to each well using a multichannel pipettor. The plate was incubated at room temperature for approximately 15 minutes without shaking. A blue colour developed in the sample and standard curve wells. The reaction was stopped immediately once colour development was noted in the negative control wells, by the addition of 100 µl of 1 M phosphoric acid to the wells in the same order in which substrate solution was added. A yellow colour developed.

The absorbance at 450 nm was then determined by an ELISA plate reader, and a TGF- β_2 standard curve was generated. The quantity of TGF-β_2 in the supernatant samples was then read from the standard curve.

- **Composition of buffers and solutions**

1 M Phosphoric acid

Combine 67.6 mls 85% phosphoric acid (14.8 M) and deionized water to 1 liter.

TBST wash buffer

20 mM Tris-HCl (pH 7.6).	6.304 g Tris-HCl in 2 litres deionized water.
150 mM NaCl	17.54 g NaCl
0.05% (v/v)	1 ml Tween® 20

Transforming Growth Factor alpha (TGF-α) ELISA

Oncogene Research Products, CN Biosciences, UK Catalogue number QIA 61.

A sandwich enzyme immunoassay, which utilises affinity purified goat polyclonal antibodies specific for mammalian TGF-α (Stored at minus 20 °C prior to use). Analysis was performed in accordance with instruction protocols from the manufacturers. Sensitivity less than 10 pg/ml.

Protocol

- All reagents were brought to room temperature prior to analysis.
- A working solution (1 x) of rinse buffer was prepared by adding 30 mls of (10 x) concentrated solution to 270 mls of deionized water, and mixing well.
- A working solution (1 x) of wash buffer was prepared by adding 50 mls of (20 x) concentrated solution to 950 mls of deionized water, and mixing well.
- The lyophilized standard was reconstituted to yield a stock solution of 1,000 pg/ml and mixed by occasional gentle swirling for 15 minutes. In six tubes, serial dilutions (1: 2) of the standards was made with assay buffer ranging from 1,000, 500, 250, 125, 62.5, 0 pg/ml.
- The concentrated reporter antibody (biotinylated goat TGF-α antibody) was diluted (1:20) in assay buffer to provide 55 µl of working strength biotinylated TGF-α antibody solution for each well. 50 µl of diluted working strength biotinylated TGF-α antibody solution was added to the wells, already precoated with TGF-α polyclonal antibody by the manufacturers.
- 50 µl of standards or samples were then added to appropriate wells, in duplicate.
- The plate was covered with a plate sealer and incubated at room temperature for 3 hours.
- The wells were washed 3 times with wash buffer, and once with 200 µl of rinse buffer (x 1) added to each well. It was recommended that the rinse buffer should not be incubated on the plate for more than 5 minutes. The plate was emptied by inverting it over a sink and tapping it dry on paper towels.
- 100 µl of the peroxidase conjugate (x 1) was added to each well with a multi-channel pipettor. The plate was covered and incubated for 30 minutes at room temperature.
- Substrate solution was prepared to provide 100 µl for each well. The substrate solution was prepared by dissolving one O-Phenylenediamine (OPD) tablet per 4 mls of substrate

diluent and mixing gently. This was protected from light, and once prepared, was used within 30 minutes as recommended by the manufacturers.

- The wells were washed 3 times by filling each well with wash buffer (x 1). The plate was then washed once by adding 200 µl of (1 x) rinse buffer to each well. The plate was emptied by inverting it over a sink and tapping it dry on paper towels.
- 100 µl of substrate solution was added to each well and incubated at room temperature for up to 30 minutes.
- 100 µl of stop solution (2.5 Sulphuric acid) was added to each well in the same order as the previously added substrate solution.
- The absorbance in each well was measured using a spectrophotometric ELISA plate reader at a wavelength of 490 nm. The readings in duplicate wells for each supernatant sample and standards were averaged respectively.
- A standard curve was generated from the standards, and the TGF-α concentration of the samples was determined by interpolation from the standard curve.

RANTES ELISA

human RANTES Cytoscreen ™ Immunoassay kit Cat No#KHC1032/1032-SB

- Minimum detectable dose less than 3 pg/ml.
- Standard curve dilutions 2,000, 1,000, 500, 250, 125, 62.5, 31.2, and 0 pg/ml
- Epithelial supernatant samples were analysed neat.

Assay protocol

All reagents warmed to room temperature prior to use.

- **Reconstitution and dilution of hRANTES standard**

  10,000 pg/ml standard was completely reconstituted with standard diluent buffer, by mixing gently for ten minutes.

  2,000 pg/ml standard was prepared by diluting 100µl of 10,000 pg/ml standard with 400 µl standard diluent buffer.

  Subsequent standards were then prepared by serial dilution (1:2) as shown below :

  150 µl standard diluent buffer with 150 µl 2,000 pg/ml standard to obtain 1,000 pg/ml.

  150 µl standard diluent buffer with 150 µl 1,000 pg/ml standard to obtain 500 pg/ml.

  150 µl standard diluent buffer with 150 µl 500 pg/ml standard to obtain 250 pg/ml.

  150 µl standard diluent buffer with 150 µl 250 pg/ml standard to obtain 125 pg/ml.

  150 µl standard diluent buffer with 150 µl 125 pg/ml standard to obtain 62.5 pg/ml.

  150 µl standard diluent buffer with 150 µl 62.5 pg/ml standard to obtain 32.5 pg/ml.

  150 µl standard diluent buffer was used for 0 pg/ml standard.

- **Dilution of wash buffer**

  At room temperature, dilute 50 mls of (25 x) wash buffer concentrate with deionized water to make a final volume of 1.25 liters.

- **Assay method**

- 50 µl of standard diluent buffer was added to zero wells of a 96 well ELISA plate, pre-coated by the manufacturers with a hRANTES antibody. The wells reserved for chromogen blank were left empty. 50 µl of standards, samples, or controls was added to appropriate duplicate wells.
- 50 µl of biotinylated anti-RANTES (Biotin conjugate) solution was added to each well, except the chromogen blank wells. The side of the plate was tapped gently to mix contents.

- The plate was covered with an adhesive plate cover and incubated for one hour at room temperature.
- The liquid was discarded from wells and tapped dry on lint free paper and the ELISA plate washed four times by carefully filling wells with wash solution and draining.
- Streptavidin-HRP working solution was prepared by the addition of 120 µl of (100 x) streptavidin-HRP concentrate to 12 mls Streptavidin-HRP diluent. As the concentrate is viscous, it was pipetted slowly and the excess wiped from the pipette tip. The remainder of (100 x) streptavidin-HRP concentrate was returned to the refrigerator.
- 100 µl of the streptavidin-HRP working solution was added to each well, except the chromogen blank wells. The plate was then covered with an adhesive plate cover and incubated for 30 minutes at room temperature.
- The liquid was discarded from wells and tapped dry on lint free paper and the ELISA plate washed four times by carefully filling wells with wash solution and draining.
- 100 µl of stabilised chromogen was added to each well, with the development of a blue colour. The colour development was monitored while being incubated at room temperature.
- 100 µl of stop solution was added to each well and the side of the plate tapped gently to mix. The colour changed from blue to yellow.
- The absorbance of each well was read at 450 nm by an ELISA plate reader, and the plate was blanked against duplicate chromogen blank wells composed of 100 µl each of stabilised chromogen and stop solution.
- A standard curve was generated plotting the absorbance readings of standards against the standard concentrations. The hRANTES concentrations of epithelial supernatant samples were read from the standard curve.

Interleukin-5 ELISA

human IL-5Cytoscreen ™ Immunoassay kit Cat No#KHC0052/KHC0050-SB

- Minimum detectable dose less than 4 pg/ml.
- Standard curve dilutions 750, 375, 187.5, 93.7, 46.8, 23.4, 11.7, and 0 pg/ml
- Epithelial supernatant samples were analysed neat.

Assay protocol

All reagents warmed to room temperature prior to use.

- **Reconstitution and dilution of hIL-5 standard**

  7,500 pg/ml standard was completely reconstituted with standard diluent buffer, by mixing gently for ten minutes.

  750 pg/ml standard was prepared by diluting 100µl of 7,500 pg/ml standard with 900 µl standard diluent buffer.

  Subsequent standards were then prepared by serial dilution (1:2) as shown below :

  300 µl standard diluent buffer with 300 µl 750 pg/ml standard to obtain 375 pg/ml.

  300 µl standard diluent buffer with 300 µl 375 pg/ml standard to obtain 187.5 pg/ml.

  300 µl standard diluent buffer with 300 µl 187.5 pg/ml standard to obtain 93.7 pg/ml.

  300 µl standard diluent buffer with 300 µl 93.7 pg/ml standard to obtain 46.8 pg/ml.

  300µl standard diluent buffer with 300 µl 46.8 pg/ml standard to obtain 23.4 pg/ml.

  300 µl standard diluent buffer with 300 µl 23.4pg/ml standard to obtain 11.7 pg/ml.

  300 µl standard diluent buffer used for 0 pg/ml.

- **Dilution of wash buffer**

  At room temperature, dilute 50 mls of (25 x) wash buffer concentrate with deionized water to make a final volume of 1.25 liters.

- **Assay method**
- 100 µl of standard diluent buffer was added to zero wells. Wells reserved for chromogen blank were left empty. 100 µl of standards, samples, or controls was added to appropriate duplicate wells of hIL-5 antibody pre-coated 96 well ELISA plate.
- The plate was covered with an adhesive plate cover and incubated for two hours at room temperature.
- The liquid was discarded from wells and tapped dry on lint free paper and the ELISA plate washed four times by carefully filling wells with wash solution and draining.
- 100 µl of biotinylated anti-IL-5 (Biotin conjugate) solution was added to each well, except the chromogen blank wells. The side of the plate was tapped gently to mix contents.

- The plate was covered with an adhesive plate cover and incubated for 30 minutes at room temperature.
- The liquid was discarded from wells and tapped dry on lint free paper and the ELISA plate washed four times by carefully filling wells with wash solution and draining.
- Streptavidin-HRP working solution was prepared by the addition of 120 µl of (100 x) streptavidin-HRP concentrate to 12 mls Streptavidin-HRP diluent. As the concentrate is viscous, it was pipetted slowly and the excess wiped from the pipette tip. The remainder of (100 x) streptavidin-HRP concentrate was returned to the refrigerator.
- 100 µl of the streptavidin-HRP working solution was added to each well, except the chromogen blank wells. The plate was then covered with an adhesive plate cover and incubated for 30 minutes at room temperature.
- The liquid was discarded from wells and tapped dry on lint free paper and the ELISA plate washed four times by carefully filling wells with wash solution and draining.
- 100 µl of stabilised chromogen was added to each well, with the development of a blue colour. The colour development was monitored while being incubated at room temperature for up to 25 minutes.
- 100 µl of stop solution was added to each well and the side of the plate tapped gently to mix. The colour changed from blue to yellow.
- The absorbance of each well was read at 450 nm by an ELISA plate reader, and the plate was blanked against duplicate chromogen blank wells composed of 100 µl each of stabilised chromogen and stop solution.

A standard curve was generated plotting the absorbance readings of standards against the standard concentrations. The hIL-5 concentrations of epithelial supernatant samples were then read from the standard curve and corrected for any dilutions of initial supernatant samples.

Flow cytometry analysis for IL-4Rα expression on primary bronchial epithelial cells

Fluorokine™ Biotinylated Human IL-4

Catalog Number: NF400

R&D SYSTEMS, Inc.

Reagents

- Biotinylated recombinant human rhIL-4. 1.5μg (Lyophilized). Reconstituted by addition of 1 ml of sterile distilled water.
- Avidin-Fluorescein: Avidin conjugated with fluoroscein (10μg/ml), f:p ratio of 5:1.
- Negative control reagent (60 reactions): A protein (Soybean trypsin inhibitor) biotinylated to the same degree as the cytokine (5 μg/ml).
- Blocking antibody (300 ml, 15 reactions): A polyclonal goat IgG anti-human IL-4 antibody at 1.5 mg/ml.
- RDF1 (10 x) Cell wash buffer concentrate (60 mls): A 10 x concentrated buffered-saline-protein solution specifically designed to minimise background staining and stabilise specific binding. The working-strength cell wash buffer solution was prepared by adding 1 ml of (10 x) concentrate to 9 mls of sterile distilled water. The reagent was maintained on ice during use and stored at 4 °C.
- 10 mM PBS (Dulbecco's PBS).
- Human or mouse IgG.

Principle of assay

Washed cells are incubated with the biotinylated cytokine that in turn binds to the cells via the specific cell surface receptors. The cells are then directly incubated with avidin-fluoroscein, which attaches to the receptor-bound biotinylated cytokine. Unbound biotinylated cytokine participates in an amplification reaction with the bound cytokine that results in an enhanced signal without compromising specificity. Cells expressing the specific cytokine receptors are fluorescently stained, with the intensity proportional to the density of the receptors. Relative receptor density is then determined by flow cytometry analysis using 488 nm wavelength excitation.

Flow cytometry IL-4Rα Assay

- After completion of the epithelial cell cultures, the cells were detached from the culture plates by the addition of trypsin. A single cell suspension was then centrifuged at 500 x g for 5 minutes and then washed twice in staining buffer (PBS, 2 % Foetal calf serum) to remove any growth factors that may be present in the culture medium, and re-suspended at a concentration of 1 x 10^7 cells/ml.
- 100 µl of this cell suspension (1 x 10^6 cells) was then aliquoted into a 12 x 75 mm polypropylene FACS tube.
- 10 µl of biotinylated cytokine reagent was then added to 25 µl of the washed cell suspension for a total reaction volume of 35 µl. As a negative staining control, an identical sample of cells were stained with 10µl of biotinylated negative control reagent. The cells were incubated for one hour at 4 °C (on ice).
- 10 µl of Avidin-FITC was added to each tube, and the reaction mixture incubated for a further 30 minutes at 4 °C (on ice) in the dark.
- The cells were washed twice with 2 mls of working-strength (x 1) RDF1 buffer to remove unreacted avidin-fluoroscein and resuspend the cells in approximately 200 µl of (1 x) RDF1 for final flow cytometry analysis.

Specificity testing

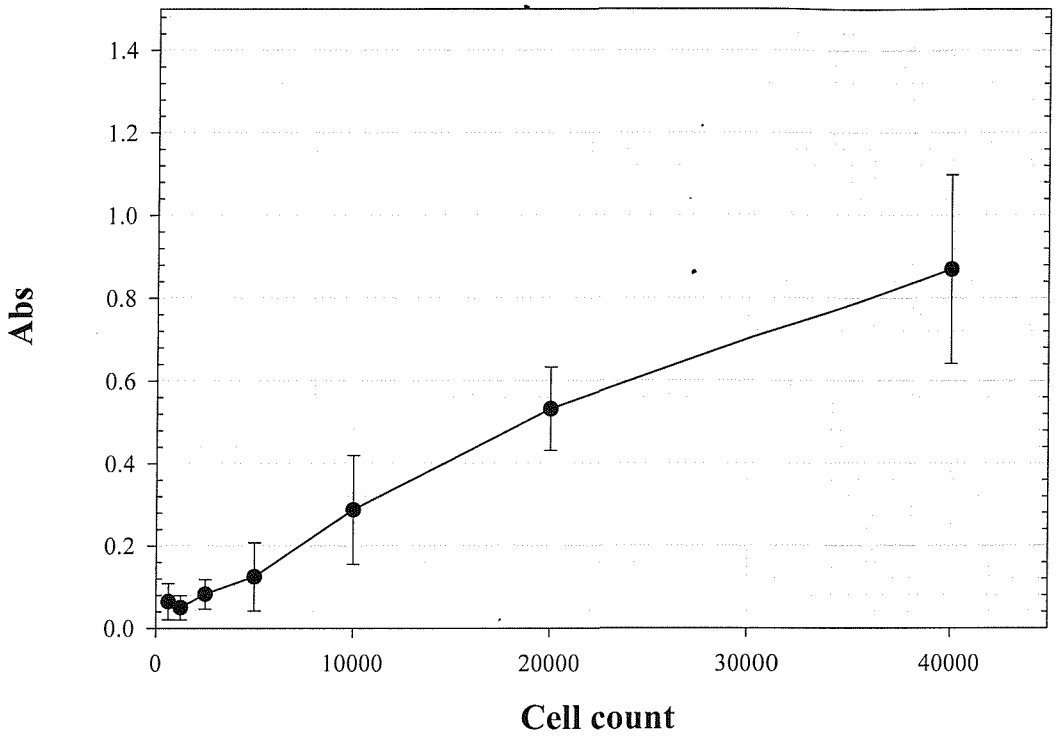
- The cells for staining were pretreated with purified mouse or human IgG (10µl of 1mg/ml / 10^6 cells) for 15 minutes at room temperature to block Fc-mediated reactions. (Care was taken not to wash cells of excess IgG for that assay).
- In a separate tube, 20 µl of anti-human IL-4 blocking antibody was mixed with 10 µl of IL-4-biotin and allowed to incubate for 15 minutes at room temperature.
- 1 x 10^5 Fc-blocked cells were then added, in a volume not more than 25 µl, to the tube containing the anti-human IL-4 blocking antibody and labelled cytokine mixture.
- The reaction is then allowed to proceed as described above.

Notes

- Some cells exhibit unusual background staining with labelled cytokines. This can be resolved by limiting the amount of labelled cytokine in the staining reaction. The manufacturers recommend that the optimal concentration is determined for the test system.

- It is recommended that cells that require trypsinization to enable removal from substrate should be further incubated in medium for 6 –10 hours to enable regeneration of the receptors. The use of the rocker platform prevents reattachment of the cells to the substrate.

Appendix Figure 1: Methylene Blue Standard curve



Mean absorbance and Standard deviation shown

Methylene blue readings were obtained from epithelial cell cultures plated at increasing cellular density by cell count, and absorbance plotted after a one in five dilution. A standard curve was subsequently generated. Epithelial cell cultures with methylene blue readings above the limits of the standard curve were corrected using Appendix Figure 2. The validity of this standard curve has been confirmed by recent work by Dr Sarah Puddicombe in our laboratory (Unpublished observations).

Appendix

Figure 2: Methylene Blue 1 in 5 correction curve