UNIVERSITY OF SOUTHAMPTON

SCHOOL OF MEDICINE
DIVISIONS OF HUMAN GENETICS & INFECTION, INFLAMMATION AND REPAIR

Genetics and Airway Expression of Interleukin-13 Receptors in Asthma

by

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ABSTRACT

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Interleukin-13 (IL-13) plays a central role in asthma pathogenesis through several mechanisms. It induces IgE synthesis, causes bronchial hyperresponsiveness and goblet cell metaplasia, contributes to airway inflammation, and initiates airway remodelling. To elicit biological responses, IL-13 binds specific cell surface receptors, which contain either the IL-13Rα1 or the IL-13Rα2 subunit. IL-13Rα1 associates with IL-4Rα and forms a functional IL-13 receptor, which also serves as an alternative receptor for IL-4. IL-13Rα2 binds IL-13 with high affinity, however, it has no signalling function and is thought to act as a decoy receptor. While there have been several studies highlighting the pivotal role of IL-13 in human asthma, the role of IL-13 receptors is poorly understood. The primary objectives of this thesis were to examine the influence of polymorphisms in the *IL-13RA1* and the *IL13RA2* genes on the genetic susceptibility to asthma, and to evaluate the expression of IL-13 receptor subunits in the airways.

Using solid-phase chemical cleavage of mismatches (SP-CCM), we screened the coding regions of *IL13RA1* and *IL13RA2*, as well as the *IL13RA1* promoter region for polymorphisms. We identified a novel T>G substitution at -281 in the *IL13RA1* promoter, as well as two previously identified non-amino acid altering polymorphisms in *IL13RA1*, a C>T transition at 1050 in the coding region and an A>G substitution at 1365 in the proximal 3' UTR. No common variants in the coding region of *IL13RA2* were found. In view of the detrimental effects of IL-13 on the airways, we hypothesised that the –281T>G SNP in the *IL13RA1* promoter region, that regulates IL-13Rα1 transcription, and the 1365A>G SNP in the *IL13RA1* 3' UTR, that regulates IL-13Rα1 mRNA stability, may confer susceptibility to asthma and atopy. To test this hypothesis, we completed a large scale genetic association study of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms with asthma and asthma-associated phenotypes, by genotyping 341 asthma-enriched Caucasian families with at least two affected sibs and 180 non-asthmatic control subjects. TDT analysis showed no association of the –281T>G and the 1365A>G polymorphisms with risk of asthma, or asthma with the presence of atopy, raised serum total IgE, $PC_{20} \leq 16$ mg/ml, or $PC_{20} < 4$ mg/ml. Case-control studies showed no significant association of the –281T>G and the 1365A>G variants with asthma. In the genotype-phenotype association studies, a borderline association between the *IL13RA1* –281T to 1365A haplotype and raised serum total IgE levels in adult female asthmatics was found (odds ratio versus G-G haplotype=2.8, odds ratio versus G-A haplotype=1.07, P=0.049). No other correlations of *IL13RA1* genotypes and 2-allele haplotypes with asthma-related traits were identified. These findings demonstrate that neither of the *IL13RA1* polymorphisms constituted risk factors for the development of asthma or associated phenotypes.

Using RT-PCR analysis, basal expression of mRNA for IL-13Rα1, IL-13Rα2, and IL-4Rα was detected in primary bronchial epithelial cells, primary bronchial fibroblasts, and the bronchial epithelial cell lines 16-HBE, NCBI-H292, and A549. Immunohistochemistry demonstrated that the columnar cells of the bronchial epithelium are the major sites of IL-13Rα1, IL-13Rα2, and IL-4Rα immunoreactivity in the airway mucosa. Using flow cytometry and confocal laser scanning microscopy, we have shown the presence of a large intracellular pool of IL-13Rα2 with diffuse granular-like cytoplasmic distribution in primary bronchial epithelial cells and primary bronchial fibroblasts. This intracellular pool of IL-13Rα2 receptors in bronchial epithelial cells and bronchial fibroblasts was not mobilised to the cell surface in response to various cytokines, suggesting that the intracellular stores of IL-13Rα2 might be released following cell damage, a feature that is frequently observed in the asthmatic mucosa.

These findings will be helpful to better understand the role of the IL-13 receptor system in the development of asthma and atopy.

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DECLARATION

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I declare that the work presented in this thesis is wholly mine, except as acknowledged above, and carried out while registered as a postgraduate candidate at the University of Southampton.

I have also written this dissertation and have been first author and co-author on the following papers and abstracts:

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List of abbreviations

ADAM33	A Disintegrin and Metalloproteinase Domain 33
APRT	Adenine Phosphoribosyl Transferase
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
CD11b	Complement receptor type 3, alpha subunit (CR3A)
CD11c	Integrin, alpha-X (ITGAX)
CD18	Leukocyte adhesion molecule CD18
CD28	T cell antigen CD28
CD29	Integrin beta-1
CD40	B Cell-associated molecule CD40
CD80	B-lymphocyte activation antigen B7-1
CD86	B-lymphocyte activation antigen B7-2
CTLA-4	Cytotoxic T lymphocyte-associated 4
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FcεRI	High affinity immunoglobulin E receptors
FcγRII	Low affinity immunoglobulin gamma FC region receptor II
FEV1	Forced expiratory volume in one second
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
γc	IL-2Rγ
HBEC	Human bronchial epithelial cells
HCAM/CD44	Homing-associated cell adhesion molecule
HDM	House dust mite
ICAM-1	Intercellular adhesion molecule I
IFN	Interferon

List of abbreviations (continued)

IL	Interleukin
IL-4R	Interleukin-4 receptor
IL-13R	Interleukin-13 receptor
IRS-1,2	Insulin receptor substrate-1,2
JAKs	Janus kinases
kb	kilobase
Kd	Dissociation constant
LPS	Lipopolysaccharide
LTC_4	Leukotriene C_4
MCP-1	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP1α	Macrophage inflammatory protein 1 alpha
MUC2	Mucin 2
MUC5AC	Mucin5, subtypes A and C
MW	Molecular weight
NK T cells	Natural killer T cells
NOD2	NOD2 protein; alternative title: Caspase recruitment domain-containing protein 15 (CARD15)
OR	odds ratio
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PECAM-1	Platelet-endothelial cell adhesion molecule I
PGD_2	Prostaglandin D_2
PHF11	PHD finger protein II
PMA	Phorbol-12-myristate-13-acetate
RAST	Radioallergosorbent
SH	Src homology
SP-CCM	Solid phase chemical cleavage of mismatches
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TDT	Transmission disequilibrium test

List of abbreviations (continued)

TGF	Transforming growth factor
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Nucleotides	One letter code
adenine	A
cytosine	C
guanine	G
thymine	T

Amino acid	One letter code	Three letter code
alanine	A	Ala
arginine	R	Arg
asparagine	N	Asn
aspartic acid	D	Asp
cysteine	C	Cys
glutamic acid	E	Glu
glutamine	Q	Gln
glycine	G	Gly
histidine	H	His
isoleucine	I	Ile
leucine	L	Leu
lysine	K	Lys
methionine	M	Met
phenylalanine	F	Phe
proline	P	Pro
serine	S	Ser

List of abbreviations (continued)

Amino acid	One letter code	Three letter code
threonine	T	Thr
tryptophan	W	Trp
tyrosine	Y	Tyr
valine	V	Val

Style

The bibliographic citation style used in this thesis is the Harvard (author-date) style. The Nomenclature System for Human Gene Mutations is used to describe polymorphisms (Antonarakis, 1998, den Dunnen and Antonarakis, 2000). Gene names are given in italicised capitals (e.g. *IL13RA1*) and names of gene products are given in non-italicised capitals (e.g. IL-13Rα1). Approved names of genes were obtained from the Online Mendelian Inheritance of Man (OMIM) Database. In general, sequence variations will be described at the DNA level. Nucleotide substitution for single nucleotide polymorphisms will be designated by '>'. For example, -281T>G in the *IL13RA1* gene indicates that nucleotide at position –281 bp relative to the translation initiation codon ATG has changed from a T (thymine) to a G (guanine). In some instances, particularly when used by published reports, changes at the amino acid level will be described. For example, Gln110Arg in the *IL13* gene indicates that the Gln (glutamine) at amino acid 25 has replaced by an Arg (arginine).

CHAPTER 1

INTRODUCTION

1.1. Asthma definition

Asthma is a complex clinical syndrome characterised by variable airflow obstruction (some of which may be permanent), bronchial hyperresponsiveness (BHR), airway oedema, and eosinophilic and lymphocytic inflammation (Busse W, 1998). Asthma has become an epidemic, affecting more than 155 million individuals in the developed world (Cookson, 1999). It is the most common childhood disease in developed countries and carries a substantial economic cost world-wide (Cookson, 1999). Although asthma is frequently referred as a disease, as if it is a single nosologic entity with a unique pathogenesis, it is more likely a syndrome, one that comprises multiple disorders, manifesting common symptoms but having distinct and probably different pathogenetic and etiologic mechanisms (Fish, 1998). Concepts about asthma pathogenesis have changed considerably in the past decade. Thus, from an initial concept of airway smooth muscle contraction, the recent focus has been on airway inflammation and remodelling (Busse W, 1998). The Global Strategy for Asthma Management and Prevention Report (Report, 1995) defines asthma as "a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils, and T lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or morning. These symptoms are usually associated with widespread but variable airflow obstruction that it is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airways responsiveness to a variety of stimuli".

1.1.1. Asthma morbidity and mortality

It has been widely observed that the prevalence of asthma and other allergic diseases has increased over the past two decades in developed countries (Holgate, 1999). Asthma is nowadays an extremely common problem with an estimated prevalence of 4% to 8% in most developed western countries (Turner, 1989). According to the latest "Asthma Audit" an estimated 7.8% of adults in the UK suffer from symptomatic asthma, whereas the estimated prevalence rate for current asthma in children from large scale surveys in the UK ranges from 12.5% to 15.5% (2001). A world-wide assessment of the prevalence of self-reported asthma, allergic rhinoconjuctivitis, and atopic eczema was conducted in 56 countries with a total of 721,601 participating

children, by "The International Study of Asthma and Allergies in Childhood-ISAAC" (1998a, 1998b). The study revealed marked variations in the prevalence of asthma and allergic symptoms between countries. The range of wheeze in the past 12 months prior to the study ranged from 2.1%-32.2%, while the prevalence of asthma was greater in males in the younger age group, with a female predominance at older ages. The highest prevalence of asthma and atopic disorders were observed predominantly in English speaking Western countries and among many communities in Latin America, whereas decreasing prevalence were found in Eastern Europe and Asia. These findings are consistent with those of the European Community Respiratory Health Survey (ECRHS), which measured the prevalence of asthma in adults aged 20-44 years predominantly in centres across Western Europe, and also found high prevalence of asthma-like symptoms in the English speaking countries (Pearce et al., 2000).

Hospitalisation is also an important outcome in asthma because it often reflects the presence of severe, uncontrolled, or progressive disease. According to the latest "Asthma Survey" the rate of hospitalisation in the UK among adults aged 15-44 years, is approximately 120 per 100,000 population per year, while for children 5-14 years is approximately 200 per 100,000 persons per year. There were 73,929 admissions to hospital due to asthma in 1999 in the UK, while the annual cost for the NHS is estimated to be £850 million (2001). In the U.S., asthma is considered to be the most common clinical cause of hospitalisation in children (Weiss, 1998). By 1997, the estimated cost for asthma hospitalisations in the U.S. accounted for 29.5% of the direct costs for asthma (Weiss et al., 2000), while direct medical expenditures exceeded $6 billion per year (Weiss et al., 1992b). The rate of hospitalisation has been also increasing in frequency from the 1960s to the late 1980s in many countries, including the UK, Canada and USA; however, it now seems to be in decline in both the UK and the U.S. (Suissa and Ernst, 2001, 2001).

Rates of death of asthma vary across countries and over time and it is currently estimated to affect between 0.5 and 2.0 of every 100,000 people aged 5 to 34 years old per year (Suissa and Ernst, 2001). Death rates also vary widely by age and disease severity, and are particularly high in underprivileged populations such as inner city

African-Americans in the U.S. (Weiss et al., 1992a). Asthma mortality in the UK has appeared to increase two-fold from the mid-1970s to the early 1990s, however, part of this trend might be attributed to inaccuracy of asthma death certificate (Reid et al., 1998). Although asthma mortality in the UK has levelled off in recent years, it is estimated that 1,500 people still die from asthma each year, including around 25 children and 500 adults younger than 65 years (2001). Asthma deaths increased steadily in the U.S. during the 1980s by 6.2% annually, with asthma deaths between 1980 and 1987 increasing by 2,891 to 4,360 or from 1.3 to 1.7 per 100,000 (Weiss and Wagener, 1990).

1.1.2. Disease mechanisms underlying asthma

The aetiology of asthma is complex and multifactorial and it is thought to involve the interaction between genetic factors and environmental stimuli (Maddox and Schwartz, 2002). Although Mendelian patterns of inheritance do not apply to asthma, it is well established that inheritance plays a significant role in the development of the disease (Sandford et al., 1996, Holgate, 1997). On the other hand, environmental factors influence both development and progression of asthma, including in utero events, various aeroallergens, smoking, viral infections, air pollution, and occupational exposure (Maddox and Schwartz, 2002). Inflammation is thought to play a pivotal role in the pathophysiology of the disease, according to the latest Global Strategy for Asthma and Prevention Report (Report, 1995), which defines asthma as a chronic inflammatory disorder of the airways in which many inflammatory cells play a role. Evidence for airway inflammation in asthma have been observed in autopsy specimens of patients dying from asthma, bronchial biopsies from patients with mild or even asymptomatic disease, bronchoalveolar lavage (BAL) fluid analyses, induced sputum studies, and the identification of cytokines and adhesion molecules that regulate these processes (Busse W, 1998).

The most common form of the disease in childhood and early adulthood is thought to result from IgE-mediated responses to common allergens (Platts-Mills and Wheatley, 1996). Even in nonatopic asthma, the pathophysiological features are similar, and high affinity IgE receptor-bearing cells are found in bronchial biopsies from atopic and nonatopic asthmatics (Humbert et al., 1999, Humbert et al., 1996). IgE-mediated

inflammatory reaction takes place through binding of IgE to high-affinity receptors (FcεRI) expressed on the surface of tissue mast cells, eosinophils, monocytes, basophils, and dendritic cells. Cross-linkage of IgE with specific allergens results in the release of an array of preformed and newly generated mediators of inflammation, especially from mast cells. These include histamine, tryptase, prostaglandin D_2, leukotriene C_4, and Th2 cytokines, which induce contraction of airway smooth muscle cell, mucus secretion, and vasodilation (Burney, 1995).

The chronic inflammation of the airways in asthma is the result of recruitment of peripheral blood cells to the airways, including eosinophils, basophils, T cells, and monocytes. A number of studies have shown the presence of increased numbers of inflammatory cells in BAL fluid (Wardlaw et al., 1988, Adelroth et al., 1990) or bronchial mucosal biopsies (Beasley et al., 1989, Ollerenshaw and Woolcock, 1992) in mild asthmatics compared with normal control subjects. The recruitment of these cells is the result of adhesive interactions between circulating inflammatory cells and microvascular endothelial cells via the production of proinflammatory mediators, cytokines, and chemokines, as well as the expression of cell surface adhesion molecules (Bousquet et al., 2000). The release of preformed cytokines by mast cells such as IL-4 and IL-13 is the likely initial trigger for the early recruitment of these cells (Bradding et al., 1994, Larche et al., 2003). There is strong evidence that the chronic inflammatory process of the asthmatic leads to structural airway changes, the so called airway remodelling, which is thought to account for the irreversible airflow obstruction in severe asthmatics (Chiappara et al., 2001). This encompasses changes in the epithelium, subepithelial basement membrane deposition of fibronectin and types I, III and V collagens, increase in mucus glands, increased vascularity, as well as smooth muscle cell hypertrophy and hyperplasia (Elias et al., 1999).

1.2. Structure of the airways

Enclosed within the visceral pleura, the normal adult lungs fill most of the thoracic cavity extending from the root of the neck and the first rib above to the domes of the diaphragm (Hasleton and Curry, 1996). In the normal adult, each lung weighs approximately 500 g, but this figure is variable. They can move freely within the thoracic cavity, being attached only at the two hila. Lungs are divided into their major

subdivisions, the lobes by fissures lined by viscera pleura. The right lung has three lobes, whereas the left has two. The conducting airways, which distribute air to the gas-exchange units, begin with the trachea, which originates at the larynx. The conducting airways are muscular tubes lined by a ciliated epithelium. The airways approximately 1 mm or more in diameter have walls reinforced by cartilage and are called bronchi. Those without cartilage are called bronchioles. Within the lung, the bronchi branch dichotomously, giving rise to progressively smaller airways. The number of generations from the main bronchus to the acini varies from as few as 8 to as many as 25, depending on the region of the lung. The acinus is the basic unit of gas exchange, supplied by a single terminal bronchiole and consisting of respiratory bronchioles, alveolar ducts, alveolar sacks and alveoli.

1.3. Bronchial mucosa

The bronchi are lined by a pseudostratified ciliated columnar epithelium that rests on a homogeneous eosinophilic basement membrane 1 to 3 μm thick (Jeffery, 2000). The basement membrane, also described as a basal lamina, provides mechanical support for cells, acts as a semi permeable barrier between tissue compartments, and regulates cellular attachment, migration, and differentiation (Hasleton and Curry, 1996). As seen by electron microscopy, the 'true' basement membrane consists of a lamina rara (lucida) and a lamina densa (basal lamina) each 40-60 nm thick. There is also an additional component beneath the basal lamina, the so-called lamina reticularis that becomes thickened in asthma. Beneath the basement membrane is a subepithelial layer consisting of collagen, elastic fibers, capillaries, lymphatics, and nerves. Outside the subepithelial layer is an almost circular layer of muscle interrupted only by collagen and the ducts of bronchial mucus glands.

1.3.1. Airway epithelial cells

The bronchial epithelium consists of the surface epithelium and mucus glands. The surface epithelium consists of three principal cell types: basal, ciliated, and secretory cells (Velden and Versnel, 1998). The ciliated cells are columnar cells attached to the basal lamina and reaching the bronchial lumen. The apical surfaces of the ciliated cells are exposed to the airway lumen and have approximately 250 cilia projecting into the lumen, which are typically 6-7 μm long. The cilia propel mucus of the

tracheobronchial tree proximally to clear the airway of trapped debris. Therefore, ciliated cells play a critical role in the body's defense against inhaled and noxious particles (Proud, 1998). Microvilli, 2-3 μm in length, are interspersed between the cilia and extent from the ciliated cell surface as simple fingerlike projections or branched structures. The characteristics of the epithelium vary considerably in different airway generations. Ciliated cell types predominate in the conducting airways and represent more than 90% of the total epithelial cell population. The proportions of ciliated cells, the numbers of cilia per cell, and the length of cilia diminish from the proximal airways to the peripheral airways. In the large airways ciliated cells are interspersed with secretory cells, including goblet cells and serous cells. Goblet cell numbers can increase several fold in chronic exposure to irritants and chronic airway diseases including asthma (Aikawa et al., 1992) and COPD (Ebert and Terracio, 1975). In the bronchioles, ciliated cells are interspersed with Clara cells, endocrine cells, neuroendocrine cells, and neuroepithelial bodies.

1.3.2. Mucus secreting cells

The mucus gel is produced by specialised secretory cells, which are located in the bronchial surface epithelium and in the glands of the submucosa (Weibel, 1998). The main type of secretory cell in the surface epithelium is the goblet cell. In the bronchi of all sizes and in larger bronchioles one finds goblet cells interspersed between the ciliated cells. The apex of the goblet cell is covered by microvilli. The mucus is formed in their endoplasmic reticulum and Golgi complex, stored as droplets in their apical part, and discharged in bulk. A special secretory cell appears in the smaller bronchioles, the Clara cell, whose secretory product is still unknown.

The mucus glands are compound tubular glands that lie outside the muscle and inside or between the cartilage (Hasleton and Curry, 1996). They are connected to the bronchial surface by long and narrow ducts. Three types of cells are recognised in the secretory tubules: mucus, serous, and myoepithelial cells. Serous cells are most numerous at the ends of the secretory tubules. Their secretory granules contain glycoproteins such as lysozyme, antileukoprotease, and lactoferrin. Mucus cells are filled with secretory vesicles and their apical surface is covered with short microvilli. The myoepithelial cells have the cytoplasmic features of smooth muscle. Their

cytoplasm is filled with contractile microfilaments that express the smooth muscle isoform of alpha actin.

1.3.3. Epithelial cell as a target in asthma

Due to its physical position in the airways, the respiratory epithelium is exposed to a variety of environmental and endogenous stimuli. The response of the epithelial cell to such stimuli can range from the production of cellular mediators, modulation of ion-transport processes, and mucociliary function to cell death (Proud, 1998). There is evidence for ongoing epithelial damage in asthma and reported changes include: (i) epithelial sloughing in patients who died of status asthmaticus (Dunnill, 1971); (ii) desquamated clumps of cells in sputum from asthmatic subjects (Creola bodies), which are found in increased numbers during disease exacerbations (Tateishi et al., 1996); (iii) damage of epithelium in bronchial biopsies during bronchoscopy, which probably reflects the fragility of the asthmatic epithelium *in vivo,* and may involve disruption of the epithelial cell tight junctions (Jeffery, 2001); (iv) goblet cell hyperplasia and submucosal gland enlargement (Dunnill et al., 1969); and (v) mast cell and eosinophil infiltration (Djukanovic et al., 1990). Studies have highlighted a potential disturbance of cell-cell adhesion in chronic asthma, since cleavage of the epithelium in bronchial specimens has been observed between the superficial epithelial cells and basal cells (Lackie et al., 1997).

There are several mechanisms that may be implicated in epithelial damage. Ozone exposure and respiratory viruses are known to cause direct damage to the bronchial epithelium. Other studies have demonstrated infiltration of eosinophils and mast into the airway mucosa and release of granule proteins, histamine, and tryptase leading to epithelial fragility (Azzawi et al., 1990, Gleich et al., 1979, Takafuji et al., 1996) (Beasley et al., 1989, Wenzel et al., 1988). Macrophages can also contribute to the fragility of epithelium, through release of increased amounts of matrix metalloproteinase-9 (Mautino et al., 1997). Injury to the epithelial barrier has many effects. The protective barrier is lost; therefore irritants and allergens can reach the underlying fibroblast and smooth muscle cells. Furthermore, the sensory nerve endings are increasingly exposed to various stimuli, thereby eliciting smooth muscle contraction and vascular permeability by an axon reflex (Barnes, 1986). Epithelial

loss can also result in reduced production of epithelial-derived bronchodilators, such as nitric oxide and prostaglandin E_2 (PGE_2), as well as loss of enzymes that degrade proinflammatory peptides (Bousquet et al., 2000), which can cause the release of an array of proinflammatory and bronchoconstrictive mediators.

1.3.4. Mediator production by epithelial cells

It has now become apparent that structural cells of the airways, including epithelial cells, fibroblasts, and smooth muscle cells are involved in the inflammatory process in asthma, by secreting a variety of mediators and interacting with inflammatory cells via adhesion molecules (Bousquet et al., 2000). Epithelial cells release a wide variety of mediators such as cytokines, growth factors, and lipid mediators. Cultured epithelial cells produce a range of cytokines, such as RANTES, IL-8, GM-CSF, IL-1, TNF-α, and IL-6. RANTES is a member of the C-C chemokine family that is chemotactic to basophils, monocytes, eosinophils, and memory T cells (Schall et al., 1990, Rot et al., 1992). RANTES production is induced by IL-1, TNF-α, and IFN-γ (Stellato et al., 1995). IL-8 is a potent chemoattractant for neutrophils and eosinophils and is thought to play a significant role in severe asthma (Leonard and Yoshimura, 1990, Sehmi et al., 1993). IL-8 production by airway epithelial cells is induced by IL-1, TNF-α (Cromwell et al., 1992), asbestos (Rosenthal et al., 1994), ozone, and nitrogen dioxide (Devalia et al., 1993), IL-4 and IL-13 (Lordan et al., 2002). *In vitro* studies have shown that GM-CSF produced by epithelial cells can promote maturation of dendritic cells and prolong survival of eosinophils (Lordan et al., 2002); its production is upregulated by viruses (Subauste et al., 1995), IL-1 (Churchill et al., 1992), IL-4, and IL-13 (Lordan, 2001). IL-1 and TNF-α can influence cellular traffic by upregulating ICAM-1 expression on endothelial cells (Tosi et al., 1992b).

Epithelial cells can also metabolise arachidonic acid by both the lipoxygenase and the cyclooxygenate pathways. In human airway epithelial cells the 15-lipoxygenate pathway is predominant producing 15-hydroperoxy-eicosatetranaenoic acid (15-HPETE), 15-hydroxy-eicosatetranaenoic acid (15-HETE) and dihydroxy-eicosatetranaenoic acids (14, 15-diHETE). 15-HETE is considered to be the major product, and has been found to increase mucus release (Kaliner and Marom, 1983), as well as the acute response after antigen challenge in asthmatics (Lai et al., 1990).

Equal amounts of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ are generated through the cyclooxygenate pathway in bronchial epithelial cells. PGE_2 mediates relaxation of the smooth muscle cells and inhibits mast cell degranulation (Peters et al., 1982). Conversely, $PGF_{2\alpha}$ enhances smooth muscle cell contraction and mast cell degranulation.

1.4. Fibroblasts

The fibroblast is the principal cell of most connective tissues, which is involved in constituting collagenous and noncollagenous components of the extracellular matrix (Takamizawa et al., 1999). Fibroblasts in the lung maintain the integrity and solidity of the organ by producing a variety of extracellular matrix (ECM) components, including collagen, laminin, fibronectin, hyaluronic acid, and proteoglycans (Spoelstra et al., 2001). Fibroblasts also play a major role in airway repair following inflammatory damage. After wound injury, fibroblasts at the wound margin proliferate and migrate to the site of injury, where they lay down large quantities of matrix proteins and form granulation tissue. During granulation-tissue formation, many fibroblasts transform into myofibroblasts that contract, reducing the size of the wound and bringing the wound margins towards one another (Aoshiba et al., 1999).

The subepithelial fibroblasts are in anatomical and functional interaction with the epithelium, neural tissue, and ECM. This complex unit is now called epithelial-mesenchymal trophic unit and was first identified as having a major role in fetal lung development where the interaction among these cells modifies their responses at various stages of airway growth and branching (Minoo and King, 1994). There is increasing evidence that pulmonary fibroblasts contribute to the airway wall remodelling in asthma. The thickened lamina reticularis seen in asthmatics consists of type I, II, and V collagen derived from subepithelial fibroblasts (Knight, 2001). The subepithelial fibrosis in asthma is also accompanied by an increase number of myofibroblasts in the subepithelium (Brewster et al., 1990). This was highlighted in a study showing that only 24h after allergen challenge, myofibroblast numbers in biopsies derived from asthmatic subjects were higher than before a bronchial challenge (Gizycki et al., 1997). Fibroblasts can transform into myofibroblasts following stimulation with transforming growth factor β (TGF-β). In the asthmatic

airways, activated eosinophils and epithelial cells are major sources of TGF-β, and as such, these cells may play a key role in the conversion of fibroblasts into myofibroblasts (Desmouliere et al., 1993).

1.4.1. Fibroblasts and airway inflammation

Fibroblasts participate in the orchestration of acute and chronic airway inflammation by releasing a variety of inflammatory mediators. Cytokines released by fibroblasts include chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), TGF-β, in response to interleukin-1, and TNF (Larsen et al., 1989, Leizer et al., 1990), as well as IL-8 and leukotriene B_4 (LTB_4) in response to smoke extract (Sato et al., 1999). Fibroblasts have also been reported to produce IL-6, IL-11, IL-8, and growth related oncogene-α (Gro-α) in response to IL-17 (Molet et al., 2001). The release of inflammatory mediators from fibroblasts is thought to contribute to the airway inflammation in asthma by modulating the cell recruitment and the survival of the inflammatory cells in the airways. This notion has been confirmed by *in vitro* studies showing that a variety of stimulants, such as smoke extract (Sato et al., 1999), lipopolysaccharides (LPS) (Koyama et al., 1999), and bleomycin stimulate lung fibroblasts to release neutrophil and monocyte chemotactic activities (Takamizawa et al., 1999). Another study has demonstrated that GM-CSF production by fibroblasts enhanced eosinophil survival when human fetal fibroblasts were co-cultured with eosinophils (Vancheri et al., 1989).

Fibroblasts express a variety of adhesion molecules, including ICAM-1, V-CAM, H-CAM, β_1 and β_2 integrins. ICAM-1 on fibroblasts acts as a receptor for surface molecules expressed on various inflammatory cells. The major ligands for ICAM-1 are the β_2 integrins, the lymphocyte function-associated antigen (LFA-1, CD11a/CD18) expressed by leukocytes, and the Mac-1 (expressed by neutrophils, monocytes and natural killer cells). The expression of ICAM-1 is associated with leukocyte transmigration over fibroblasts (Tessier et al., 1996), as well as with eosinophil activation and degranulation following adherence of eosinophils to fibroblasts through the binding of ICAM-1 to LFA-1 (Takafuji et al., 1998). Therefore, an increased expression of ICAM-1 in response to cytokines may represent

a mechanism by which fibroblasts adhere to and interact with inflammatory cells. The expression of ICAM-1 in human fibroblasts is upregulated in response to IL-1β, TNF-α and IFN-γ, whereas V-CAM expression is upregulated in response to IL-1β and TNF-α (Spoelstra et al., 2000). Interestingly, salmaterol, a long acting β-agonist, and fluticasone, a corticosteroid, have been shown to downregulate ICAM-1 expression *in vitro* (Silvestri et al., 2001).

1.5. Inflammatory cells in asthma

Bronchial inflammation is an essential component of asthma. Autopsy studies of patients who have died of asthma show infiltration of airways by eosinophils (Houston et al., 1953), mast cells, lymphocytes, and macrophages (Crepea and Harman, 1955). The same type of inflammatory cells are also present in the airways of asthmatic subjects and are found in increasing numbers in BAL fluid following an allergen challenge (Jeffery et al., 1992, Laitinen et al., 1993).

1.5.1. Eosinophils

Eosinophils are nondividing bone marrow derived cells (Kita, 1998). Their cytoplasm contains about 20 membrane-bound specific granules, which contain the basic proteins. In addition, each cell contains a number of primary granules of varying size. These granules contain Charcot-Leiden crystal protein that is found in asthmatic sputum. Eosinophils also contain a few lipid bodies, which are the principle stores for arachidonic acid (Church, 2001), presynthesised major granule proteins, newly synthesised cytokines, and lipid mediators. The major granule proteins include major basic protein, eosinophil cationic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, β-glucuronidase, acid phosphatase, and arylsulfatase B. The basic proteins are toxic to helminthic parasites and to human cells in a dose-related manner. Installation of major basic protein and eosinophil peroxidase induced bronchocostriction and increased BHR in monkeys (Gundel et al., 1992).

Eosinophils contribute to the development and maintenance of allergic reactions through the generation of various cytokines, including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL10, GM-CSF, RANTES, TNF-α, TGF-α, and TGF-β_1 (Kita, 1998). Eosinophils

are the major producers of leukotrienes, such as leukotriene C_4 (LTC_4) and 5-HETE, which are potent bronchoconstrictors. The mechanisms of eosinophil degranulation are poorly understood although it has been shown that it can be induced by IgE, IgG1, and IgG3 antibodies, as well as complement fragments C5a, C3a, and via the Mac-1 adhesion molecule (Kita, 1998). Eosinophils are found in all tissues undergoing a late-phase or chronic allergic response. They are deleterious in asthma by the release of granule proteins, which are toxic to the epithelium, and by synthesis of cysteinyl leukotrienes, which cause bronchocostriction. They can also release growth factors, such as TGF-β_1 and TGF-α, as well as elastase and metalloproteases involved in tissue remodelling (Bousquet et al., 2000). Increased numbers of eosinophils in lung tissue have been found to be significantly greater in cases of fatal asthma compared to cases of sudden death (Azzawi et al., 1992). The number of eosinophils is increased in central and peripheral airways (Azzawi et al., 1990), as well as in BAL fluid of asthmatics (Walker et al., 1991a), with large numbers of eosinophils in the asthmatic airways being degranulated (Walker et al., 1991a). Both airway eosinophilia and peripheral blood eosinophilia have been associated with both asthma severity and BHR (Laitinen et al., 1991, Kay, 1991). In a recent clinical trial, the fundamental role that eosinophils were thought to play in the late response to allergen provocation was challenged (Leckie et al., 2000). In this study, monoclonal antibodies directed against IL-5 markedly reduced blood eosinophil levels and prevented eosinophil recruitment to the airways after allergen challenge in mild asthmatic subjects; however, this treatment had no significant effect on the early or late response to allergen challenge or on baseline BHR.

1.5.2. T cell subsets

Effector T cells fall into two functional classes, CD4+ and CD8+ T cells, with each class being able to detect peptide antigens derived from different types of pathogen (Janeway et al., 1999). Peptide antigens derived from pathogens that multiply within the cytoplasm of the cell are carried to the cell surface by MHC class I molecules and presented to CD8+ T cells, which differentiate into cytotoxic T cells that kill infected target cells. Peptide antigens from pathogens multiplying in intracellular vesicles, and those derived from ingested extracellular bacterial and toxins, are carried to the cell surface by MHC class II molecules and presented to CD4+ T cells that can differentiate into two types of effector CD4+ T cells, Th1 and Th2 cells. Pathogens that accumulate in large numbers inside macrophage vesicles tend to stimulate the differentiation of Th1 cells, whereas extracellular pathogens tend to stimulate the production of Th2 cells (Janeway et al., 1999). Th1 cells are involved in delayed type hypersensitivity (DTH) reactions, whereas Th2 cytokines encourage antibody production, particularly IgE responses (Mosmann and Coffman, 1989). Production of Th1 cells leads to activation of macrophages mainly through IFN-γ production. IFN-γ has two key functions: first, it activates macrophages enhancing their microbicidal actions; second, it stimulates B cells to produce IgG antibodies, which are the principal antibodies involved in the opsonization and phagocytosis of extracellular pathogens (Boehm et al., 1997). Th2 cells initiate the humoral immune response by activating naïve antigen-specific B cells to produce IgM, IgA, and IgE, as well as neutralising and weakly opsonizing subtypes of IgG (Janeway et al., 1999).

The initial distinction between Th1 and Th2 cells was described among mouse CD4+ T-cell clones on the basis of their patterns of cytokine production by Mosmann and co-workers (Mosmann et al., 1986). Th1 clones produce IL-2, IFN-γ, and TNF-β, whereas Th2 clones produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Several other cytokines are secreted by both Th1 and Th2 cells, including IL-3, TNF-α, GM-CSF, and members of the chemokine family (Mosmann and Sad, 1996). T cells expressing cytokines of both patterns have been called Th0 (Mosmann and Coffman, 1989). The Th1 and Th2 pattern of cytokine production was later described among human T cells (Del Prete et al., 1991). Human Th1 and Th2 cells produce similar patterns, although the synthesis of IL-2, IL-6, IL-10, and IL-13 is not as tightly restricted as in mouse T

cells (Mosmann and Sad, 1996). Each T cell subset produces cytokines that serve in as its own autocrine growth factor and promote differentiation of naïve cells to that pattern (Abbas et al., 1996). In addition, the two subsets produce cytokines that suppress each other's development and activity, for example, IL-4 amplifies Th2 development and inhibits proliferation of Th1 cells (Abbas et al., 1996). However, using in-situ hybridisation and immunostaining for individual cells it has become apparent that many T cells cannot easily be classified into Th1 and Th2 subsets based on the original criteria, with some investigators arguing that cytokine-producing T cells cannot be classified into discrete subsets (Kelso, 1995). These investigators argue that individual T cells and clones display remarkable diversity in their cytokine profiles, forming a continuous spectrum in which Th1 and Th2 cells may be the two extreme phenotypes. It seems, however, that mixed (Th0) cytokines patterns are most noticeable early after lymphocyte activation and the clearest demonstration of the presence of Th1 and Th2 subsets are in chronic disease states, in which the antigens are persistent (Abbas et al., 1996). The same phenomenon is observed *in vitro,* where repeated antigenic stimulation of T cells expressing single transgenic antigen receptors leads to increasingly polarised and irreversibly committed Th1 and Th2 populations (Abbas et al., 1996).

1.5.3. Role of T cells in asthma

A number of studies have provided evidence to support a key role for T cells in asthma. Increased numbers of T lymphocytes infiltrating the lung tissue are found in bronchial biopsies from asthmatics after fatal asthma attack compared to non-asthmatics (Azzawi et al., 1992). In contrast, similar numbers of B cells are found in the airways of asthmatics and non-asthmatics in autopsy studies (Azzawi et al., 1992). Increased numbers of T cells are also found in bronchial biopsies (Bradley et al., 1991) and BAL fluid (Walker et al., 1991) of asthmatic subjects compared to normal controls. Additional studies revealed that T cells in the bronchial mucosa and BAL fluid of asthmatics have features of activation (Azzawi et al., 1990, Jeffery et al., 1989) (Robinson et al., 1993). The presence of activated T cell has been related to the severity of asthma (Walker et al., 1991, Robinson et al., 1993). Activated CD4+ T cells were also detected in the peripheral blood of patients with acute severe asthma

and their numbers were reduced following therapy (Corrigan et al., 1988, Corrigan and Kay, 1990).

Over the past few years, a working hypothesis has been that Th2 cells contribute to asthma pathology through IL-4 and IL-13 enhancement of IgE synthesis, and IL-5 promotion of eosinophil maturation and chemotaxis (Larche et al., 2003). There has been growing evidence to support the hypothesis that allergic disorders and asthma are associated with activation of Th2 cells. For example, atopic asthmatic subjects have more BAL T cells positive for mRNA for IL-3, IL-4, and IL-5 compared to normal controls, indicating that atopic asthma is associated with a Th2 phenotype (Robinson et al., 1992). Furthermore, a Th2 pattern of cytokine mRNA expression has been shown in allergen-induced late-phase cutaneous reactions (Kay et al., 1991), and allergen-specific T cell clones from donors with atopic disease have been found to preferentially release Th2 cytokines (Parronchi et al., 1991).

There has been a debate about the role of Th2 cells in nonatopic and occupational asthma. A study has found significantly higher number of T cells in bronchial biopsies of patients with atopic asthma compared to patents with nonatopic asthma (Amin et al., 2000). However, other studies have found similar numbers of eosinophils and T cells present in all forms of asthma (Bentley et al., 1992b, Bentley et al., 1992a, Humbert et al., 1999). Furthermore, local expression of mRNA for the epsilon heavy chain for IgE was demonstrated in bronchial biopsies of both atopic and nonatopic asthmatics despite the absence of specific serum IgE or positive skin responses in nonatopic asthmatic patients (Ying et al., 2001). Taken together, these data support a Th2 and IgE-mediated immunopathological process in both atopic and nonatopic asthma.

1.5.4. Mast cells

Mast cells originate from hematopoietic stem cells that enter the circulation as agranular leukocytes expressing FcεRI receptors, and settle in various tissues (Rodewald et al., 1996). Two subtypes of mast cells are found within human tissues: (i) the MC_T subtype, which contains tryptase; and (ii) the MC_{TC} subtype, which in addition to tryptase also contains chymase, mast cell carboxipeptidase, and cathepsin G. The MC_T subtype found in mucosal sites, is thought to be "immune-system related", whereas the MC_{TC} subtype, found in connective tissues, appears to be "non-immune system-related". The predominant subtype in the lung is the MC_T, containing tryptase (Schwartz, 1998). Mast cells found in tissues have intracellular granules that contain preformed inflammatory mediators including histamine, as well as mast cell proteases including tryptase, chymase, carboxipeptidase and cathepsin G. Mast cells also contain stores of cytokines, including IL-4, IL-13, IL-5, IL-6, IL-3, IL-6, TNF-α, and RANTES (Schwartz, 1998). The secretion and synthesis of these cytokines is upregulated following FcεRI activation. Immunological activation of the mast cells also induces the generation of eicosanoids, such as PGD_2 and LTC_4 (Church, 2001).

Mast cells play a pivotal role in the early-phase allergic response. Cross-linking of membrane bound IgE molecules by specific antigens results in the degranulation of the mast cell and release of its performed mediators, including histamine and proteases, as well as stimulation of the cell to synthesise newly generated eicosanoids (Benyon et al., 1989). Mast cells are found in the bronchi of both normal and asthmatic subjects; however, there is evidence of ongoing mast cell degranulation in the asthmatic airway (Beasley et al., 1989). In the upper airways, release of mast cell mediators causes nasal symptoms, such as nasal discharge and nasal blockage. In the lower airways, release of mast cell mediators causes bronchoconstriction and increased vascular permeability leading to oedema and mucus production, whereas in the skin the early response results in a wheal and flare (Church, 2001). The important role of mast cells, as effector cells in the early asthmatic response has been highlighted in many studies. BAL fluid obtained from allergic asthmatics following segmental bronchopulmonary provocation contained increased levels of histamines 5 min after allergen provocation and showed marked increase in eosinophils and IL-5 concentration at 48 h (Jarjour et al., 1997). Similarly, allergen challenge in atopic and

nonatopic asthmatics resulted in significant increase in histamine and tryptase concentrations compared with that seen in a control group (Koshino et al., 1996). Further evidence for the importance of mast cell in the early asthmatic response was provided by a study demonstrating a correlation between mast cell and basophil numbers in the airway of asthmatics and the degree of BHR (Wenzel et al., 1988, Koshino et al., 1996).

1.7. Adhesion molecules

A number of interactions of airway cells with nearby structures rely on adhesion molecules, including interaction with basement membrane and extracellular matrix components, as well as interactions with adjacent cells and infiltrating inflammatory cells (Frenette and Wagner, 1996a). These interactions between the adhesion receptor and the extracellular milieu result in the transmission of information that allows the cell to communicate with its environment. Furthermore, they play a crucial role in every step of leukocyte recruitment, including leukocyte-endothelial interactions (margination), diapedesis (transendothelial migration), and direct movement through tissues (chemotaxis) (Bochner et al., 1995). Therefore, adhesion molecules may well be implicated in tissue remodelling in asthma. To date, four families of adhesion molecules have been identified, the integrins, the selectins, the cadherins, and the immunoglobulin superfamily.

1.7.1. Integrins

The integrins are membrane glycoproteins with two subunits, designated α and β. At least 16 α subunits and 8 β subunits have been identified that can combine to generate at least 23 distinct heterodimers (Frenette and Wagner, 1996a). Epithelial cell integrins bind to various proteins in the extracellular matrix, such as collagens, fibrinogen, fibronectin, vitronectin laminin, and tenascin (Pilewski and Albelda, 1993, Smith et al., 1990, Yokosaki et al., 1994). Some integrins are involved in cell-cell interaction and binding to other cell-surface adhesion molecules, especially those of the immunoglobulin superfamily, including ICAM-1, ICAM-2, and VCAM-1 (Mutsaers et al., 1997). Integrin-mediated adhesion has been shown to prevent apoptosis (Mutsaers et al., 1997). Cell movement during tissue repair and inflammation depends on integrin-mediated interactions. The expression of integrins immobilises the cells via interaction with other cells or matrix. These immobilised cells are then primed to proliferate, migrate, or produce matrix proteins (Mutsaers et al., 1997). Integrins may also be involved in tissue repair in the airways, and expression of α5β1 and αvβ6 in the airway epithelium is induced in response to TGF-β1 (Wang et al., 1996).

1.7.2. Selectins

Selectins play a part in leukocyte rolling on stimulated endothelium, before migration of leukocytes to sites of inflammation or vascular injury (Frenette and Wagner, 1996b). The three known selectins were named after the tissue in which they were first identified. L-selectin (CD62L) is found on leukocytes and mediates the lymphocyte attachment to high endothelial venules in peripheral lymph nodes, but is also expressed in nonlymphoid vascular endothelium (Butcher and Picker, 1996). Its ligands on endothelium have been identified as mucosal addressing cell adhesion molecule-1(MAdCAM-1)(Berg et al., 1993). E-selectin is expressed on activated endothelium after exposure to various mediators, including IL-1, TNF, lipopolysaccharide (LPS) (Baumheter et al., 1993), and IFN-γ (Bevilacqua et al., 1987). Its expression is inhibited by TGF-β (Gamble et al., 1993). Its ligands have not yet been fully characterised, although in a subset of memory (CD45RO+) skin-homing lymphocytes a carbohydrate structure termed Cutaneous Lymphocyte Antigen (CLA) has been proposed as a ligand for E-selectin (Berg et al., 1991). Like E-selectin, P-selectin is expressed on activated epithelium, with induction of its expression within minutes following exposure to various stimulants such as leukotriene C_4, leukotriene C5α, throbin, and histamine (Lorant et al., 1993). The rapid induction of P-selectin is due to the fact that it is contained within cytoplasmic granules (the Weibel-Palade bodies). IL-4 and IL-3 have been shown to induce a prolonged increase in its expression (Yao et al., 1996). The ligand for P-selectin, widely expressed on leukocytes, is a glycoprotein named P-selectin glycoprotein ligand-1 (PSGL-1) (Sako et al., 1995). L-selectin-deficient mice show impaired homing of lymphocytes to lymph nodes, whereas in P-selectin-deficient mice, leukocytes do not roll along normal blood vessels, but do roll at sites of inflammation (Frenette and Wagner, 1996b). Mice lacking E-selectin do not exhibit any abnormality, but in mice deficient of both of the endothelial selectins-P and E-, leukocytes do not roll even at sites of inflammation and these mice are susceptible to bacterial infections (Frenette and Wagner, 1996b).

1.7.3. Cadherins and CD44

Cadherins establish links between adjacent cells forming zipper-like structurs at membrane regions where a cell makes contact with another cell (Frenette and Wagner, 1996a). E-cadherin in particular, plays a critical role in interactions between adjacent epithelial cells (Montefort et al., 1992). CD44 is the principal surface receptor for hyaluronic acid, collagen, and fibronectin (Lesley et al., 1993). It is involved in the adhesion of the extracellular matrix with the cytoskeleton of the cell. CD44 is found at high levels on endothelial cells, epithelial cells, and leukocytes. Alternative splicing of 10 variant exons of the CD44 can generate an array of splice variants. Bronchial epithelial cells from normal adult lung express the CD44v6 and CD44v9 isoforms, whereas the standard form of the molecule, CD44s, is found only on basal cells (Proud, 1998). CD44 in the lung plays an important role in the lung development and in pulmonary fibrosis (Kasper et al., 1995).

1.7.4. Immunoglobulin superfamily

The immunoglobulin superfamily of adhesion molecules includes ICAM-1, ICAM-2, ICAM-3, VCAM-1, PECAM-1, and MadCAM-1. The structure of these molecules demonstrates a series of globular domains, formed by disulfide bonds, resembling those found in immunoglobulins (Bochner, 1998). Like integrins, these adhesion molecules are involved in adhesion to other cell-surface ligands and exhibit signalling functions.

ICAM-1

ICAM-1 (CD54) is a 90-kDa protein with a 453 amino acid extracellular domain organised into five Ig-like domains, a putative 24 amino acid transmembrane domain and a 28 amino acid intracytoplasmic tail (Bochner, 1998). ICAM-1 is widely distributed in haemopoietic and nonhaemopoietic cells and plays an essential role in neutrophil adhesion to the endothelium and transendothelial migration (Oppenheimer-Marks et al., 1991). ICAM-1 is constitutively expressed along the luminal, intercellular, and subluminal surfaces of vascular endothelial cells, epithelial cells, and fibroblasts. It is also expressed in low amounts in peripheral blood leukocytes (Dustin et al., 1986). Ligands for ICAM-1 include leukocyte function-associated antigen-1 (LFA-1), fibrinogen, most serotypes of rhinovirus, and the β2 integrin Mac-

1 ($\alpha_M\beta_2$), which is expressed on the surface of leukocytes (Kishimoto et al., 1989). Several mediators, including IL-1β, IFN-γ, TNF-α, histamine, and PMA have been shown to increase ICAM-1 expression in primary bronchial epithelial cells, immortalised epithelial cell lines, and fibroblasts (Tosi et al., 1992b, Look et al., 1992, Bloemen et al., 1993, Spoelstra et al., 1999).

There have been many studies highlighting the role of ICAM-1 during cell migration in asthma. Monoclonal antibodies to ICAM-1 attenuate T cell adhesion to vascular endothelium and inhibit transendothelial migration of T cells (Oppenheimer-Marks et al., 1991). An anti-ICAM-1 monoclonal antibody inhibits the adhesion of neutrophils to airway epithelial cells, suggesting that ICAM-1 plays a significant role in leukocyte adhesion to the airway epithelium (Tosi et al., 1992a). Antibodies to ICAM-1 in sensitised animal models, prior to multiple allergen inhalations, reduced the number of eosinophils in the bronchoalveolar lavage and attenuated BHR (Wegner et al., 1990). In the same study, antigen inhalation resulted in increased ICAM-1 expression in both vascular endothelial and epithelial cells in the airways. In another study assessing the expression of ICAM-1 in bronchial biopsies from asthmatic and non-asthmatic subjects by immunohistochemistry, the epithelium was positive for ICAM-1 protein in 26 out of 33 biopsies derived from asthmatics, whereas no expression was seen in all 13 samples derived from non-asthmatics (Manolitsas et al., 1994). Similarly, the percentage of bronchial epithelial cells expressing ICAM-1 in asthmatics was found to be significantly increased by comparison with control subjects (Vignola et al., 1993).

ICAM-2 and ICAM-3

ICAM-2 (CD102) binds to LFA-1 (CD11a/CD18), expressed on the surface of leukocytes, causing inhibition of endothelial cell adhesion (Li et al., 1993). ICAM-3 (CD50) is expressed on endothelial cells, mononuclear cells, basophils, mast cells, and platelets. ICAM-3 is constitutively expressed on leukocytes and mast cells, but not on endothelial cells. ICAM-3 acts as a signalling molecule inducing calcium mobilisation and tyrosine phosphorylation (Juan et al., 1994).

VCAM-1

VCAM-1 (CD106) is expressed on endothelial cells, macrophages, dendritic cells, bone marrow stromal cells, and astrocytes (Rice et al., 1991, Rosenman et al., 1995). Its major ligand is the integrin VLA-4 ($\alpha_4\beta_1$), which is expressed in monocytes and mast cells (Chan and Aruffo, 1993). Several inflammatory mediators can induce VCAM-1 expression, including IL-1, TNF, LPS, IL-4, and IL-13 (Wellicome et al., 1990, Masinovsky et al., 1990, Bochner et al., 1995). VCAM-1 has been shown to play an important role in asthma. Endothelial VCAM-1 and epithelial ICAM-1 staining was found to be increased after endobronchial allergen challenge, and both parameters were significantly correlated with eosinophil influx (Bentley et al., 1993). Increased levels of soluble forms of ICAM-1 and VCAM-1 were also found in BAL fluid following segmental antigen challenge (Takahashi et al., 1994). In addition, increased levels of serum soluble VCAM-1 were measured in asthmatic subjects compared to normal subjects (Koizumi et al., 1995).

1.8. Cytokines involved in asthma

Cytokines are a diverse group of small, secreted proteins that are produced by a wide variety of cells and serve as signal molecules. Cytokines influence or activate adjacent cell movement, differentiation, growth, and death (Toews, 2001). They are involved in every aspect of immunity and inflammation, including antigen presentation, bone marrow differentiation, cellular recruitment, activation, adhesion molecule expression, and acute phase responses (Borish, 1998). Cytokines exhibit tremendous redundancy both in the variety of cell sources and the range of biologic activity. This makes it difficult to accurately group cytokines according to unique tissue sources or biological activities.

Interleukin-4

Interleukin-4 (IL-4) is crucial in its effect on the differentiation of naïve T lymphocytes to Th2 effector cells (Seder and Paul, 1994). It is also critical to the induction of the immunoglobulin isotype switch to IgE by initiating the transcription of ε heavy chain transcripts (Del Prete et al., 1988). IL-4 enhances the antigen presenting capacity of B cells by stimulating the surface expression of MHC class II antigens, CD80, CD86, CD40, and low-affinity IgE receptor (CD23) (Paul, 1991). It has also some macrophage-activating activities by enhancing the expression of MHC class I and II antigens and CD23 on these cells. In contrast to these proinflammatory effects, IL-4 exhibits anti-inflammatory effects on monocytes by inhibiting the expression of Fc receptors, differentiation into macrophages, and antibody-dependent cellular cytotoxicity. Moreover, IL-4 strongly inhibits the cytokine production by monocytes, including the production of IL1α, IL1β, IL6, IL8, IL10, TNFα, GM-CSF, G-CSF, and MIP1α (de Waal Malefyt et al., 1993a). Another important activity of IL-4 in allergic inflammation is the induction of the VCAM-1 on endothelial cells resulting in enhanced adhesiveness of endothelium to T cells, eosinophils, basophils, and monocytes (Moser et al., 1992).

Because of its properties, IL-4 has long been considered as a key molecule in asthma and several studies have highlighted this role. Inhaled IL-4 caused increased BHR to metacholine and increased sputum eosinophilia in asthmatics (Shi et al., 1998). In animal models, IL-4 induced allergic-like disease in transgenic mice causing

increased IgE values and occular lesions infiltrated with mast cells and eosinophils (Tepper et al., 1990). Moreover, studies in mice with parasitic infections have demonstrated the fundamental role of IL-4 in the regulation of IgE levels (Finkelman et al., 1988). Although several studies in animal models have confirmed the critical role of IL-4 in Th2 cell development, recruitment of inflammatory cells to the airways, and IgE regulation, they failed to demonstrate a clear role in mucus production (Cohn et al., 1997) and development of BHR (Brusselle et al., 1995). Thus, in a mouse model, overexpression of IL-4 in the lungs of transgenic mice although led to eosinophilic and lymphocytic inflammation, it did not cause increased BHR (Rankin et al., 1996). In contrast, a crucial role of IL-4Rα, but not IL-4, in the development of BHR and mucus production has been shown. Thus, IL-4Rα blocking antibodies prevented allergen induced BHR in an experimental mouse model (Gavett et al., 1997), and IL-4Rα deficient mice failed to develop any parameters of the asthma phenotype even after administration of IL-13, IL-4, or adoptive transfer of OVA-specific Th2 cells (Grunig et al., 1998).

Interleukin-5

Interleukin-5 (IL-5) was originally identified for its stimulatory actions on mouse B cells (Takatsu, 1992). However, in humans its action is restricted to eosinophils. IL-5 stimulates eosinophil production in the bone marrow by inducing maturation of precursor cells (Clutterbuck et al., 1989), and is also a potent chemotactic for eosinophils (Tominaga et al., 1991). Therefore, IL-5 is considered as the key effector molecule in orchestrating the eosinophilic inflammation in asthma. Initial studies in IL-5 knock-out mice, demonstrated that these animals abolish eosinophilia, BHR, and lung damage after allergen sensitisation (Foster et al., 1996). In contrast, a later study showed that blockage of IL-5 in sensitised mice, although reduced airway eosinophilia, it failed to inhibit BHR (Hogan et al., 1998). Similarly, a monoclonal antibody against IL-5 markedly reduced blood eosinophil levels and prevented eosinophil recruitment to the airways after allergen challenge in mild asthmatics, however, it had no significant effect on the early or late response to allergen challenge or on baseline BHR (Leckie et al., 2000). Taken together, these studies suggest that airway eosinophilia is not a requirement for allergen induced BHR, however,

eosinophils are thought to play a crucial role in the long term airway remodelling (Barnes, 2001).

Interleukin-9

Interleukin-9 (IL-9) is mainly produced by Th2 cells and eosinophils (Nicolaides et al., 1997). IL-9 was shown to promote, in synergy with IL-3, the proliferation of bone marrow derived mast cells (Hultner et al., 1990) and play a key role in regulating mast cell protease expression (Eklund et al., 1993). Many studies have highlighted the significant role of IL-9 in asthma. IL-9 transgenic mice demonstrated an increase number of mast cells in the airways, as well as induction of BHR (Temann et al., 1998). A distinct requirement for IL-9 in the generation of pulmonary goblet cell hyperplasia and mastocytosis in response to lung challenge was demonstrated in IL-9-deficient mice with pulmonary granuloma (Townsend et al., 2000). Similarly, IL-9 was found to induce mucus expression in the airways of mice (Louahed et al., 2000), and intratracheal instillation of IL-9 resulted in enhanced mucus production in animal models (Longphre et al., 1999). In human primary lung cultures and the human muccoepidermoid cell line NCI-H292 IL-9 upregulates the expression of the MUC2 and the MUC5AC gene (Townsend et al., 2000). Furthermore, increased expression of IL-9 and its receptor was found in the airways of asthmatic subjects compared with normal individuals, while IL-9 mRNA expression was found to correlate with FEV1 and BHR (Shimbara et al., 2000).

Interleukin-13

See section 1.11.

Tumor necrosis factor-α

Tumor necrosis factor (TNF) represents two homologous 17-kDa proteins primarily derived from mononuclear phagocytes (TNF-α) and lymphocytes (TNF-β). Apart from mononuclear phagocytes, TNF-α is also produced by neutrophils, activated lymphocytes, natural killer cells, endothelial cells, and smooth muscle cells (Borish, 1998). TNF-β is processed as a typical secretory protein (Nedwin et al., 1985), whereas TNF-α is processed as a membrane bound protein from which the active soluble protein is derived following cleavage of the extracellular domain (Perez et al.,

1990). TNF-α and TNF-β display about 30% homology at the amino acid level and bind to the same two distinct cell surface receptors TNFR I (p75) and TNFR II (p55) with similar affinities producing similar effects (Borish, 1998). TNF transcription is induced by IL-1, IL-3, GM-CSF, and IFN-γ, but the most potent inducer is bacterial derived endotoxin. TNF has diverse effects on a variety of cells types. It is directly cytotoxic toward cancerous cells and stimulates antitumor responses by immune cells (Wegner et al., 1990). Its main proinflammatory activity lies on its ability to induce intercellular adhesion molecule-1 (ICAM-1) expression in endothelial and epithelial cells, an activity shared with IL-1 and IFN-γ, permitting the influx of granulocytes into inflammatory sites (Bochner, 1998). TNF induces production of IL-1, IL-6, and IL-8, as well as upregulation of MHC class I and II expression by monocytes. During septicemia, TNF induces vascular leakage and toxic shock (Tracey et al., 1987). It is also responsible for the wasting in chronic infections and cancer (Beutler and Cerami, 1989).

There is strong evidence to suggest that TNF-α may play a crucial role in severe asthma. Increased levels of TNF-α were detected in induced sputum from asthmatics (Keatings et al., 1996), and inhalation of TNF-α caused BHR and increased sputum neutrophil counts in healthy volunteer subjects (Thomas et al., 1995). The *TNFA* -308G>A polymorphism, in the promoter region of the *TNFA* gene, has been associated with BHR and increased releasability of TNF-α (Li Kam Wa et al., 1999). IL-8 is a known potent chemoatractant for neutrophils and increased neutrophil counts and IL-8 levels in sputum from patients who experienced an acute asthma attack has been reported (Ordonez et al., 2000). The fact that TNF-α is a potent activator of IL-8 release from primary bronchial epithelial cells (Lordan et al., 2002) is suggestive of TNF-α being a crucial molecule in the regulation of neutrophil influx in the asthmatic airways. Clinical trials involving patients with severe asthma using antibodies and soluble TNF-α receptors are currently under way (Barnes, 2001). These therapies have shown remarkable clinical responses among patients suffering from rheumatoid arthritis and inflammatory bowel disease (Ghezzi and Cerami, 2004). In addition, the metalloproteinase-related TNF-α-converting enzyme, which is critical for the release of TNF-α form the cell surface, is a therapeutic target. Low-molecular-weight TNF-

α-converting enzyme inhibitors have also been in development, as oral regiments (Barlaam et al., 1999).

Interferon-γ

There are three members of the Interferon (IFN) family (α, β, γ) and their nomenclature is based on their ability to "interfere" with viral growth (Borish, 1998). IFNs are probably the most potent cytokines involved in cytotoxic immunity. They stimulate killing of virus infected and transformed neoplastic cells by CD8+ cytotoxic cells and natural killer cells. They are mainly produced by Th1 lymphocytes, cytotoxic T cells, and NK cells. IFN-α is well established as an antiviral agent in the treatment of hepatitis (Karayiannis, 2003, Foster, 2003). There is overwhelming data from large clinical trials that supports the use of interferon-β in relapsing-remitting multiple sclerosis (Corboy et al., 2003). IFN-γ is considered to be the most important cytokine for cell-mediated immunity, through induction of macrophage activation (Borish, 1998). It increases cytokine production and class I and II MHC expression by monocytes, as well as antigen-specific B cell maturation. Its action on monocytes results in the accumulation of monocytes at the site of inflammation and their activation into macrophages. It also induces adherence of granulocytes to endothelial cells through upregulation of ICAM-1 expression by endothelial cells (Wegner et al., 1990).

IFN-γ is one of the most important Th1 cytokines and its production is an absolute requirement for the full expression of the Th1 phenotype (Schmitt et al., 1994). The initial signals for the generation of Th1 cells are delivered by IL-12 and IL-18, which stimulate secretion of IFN-γ from immune cells. A milieu rich in IFN-γ induces the differentiation of Th0 cells into Th1 cells, capable of producing even more IFN-γ, as well as TNF-β (Mosmann and Sad, 1996). IFN-γ reduces the allergic responses by inhibiting IL-4-mediated isotype switch to IgE and expression of low-affinity IgE receptors (Romagnani, 1990). Further evidence for its inhibitory role in allergic responses has been provided by studies in mouse models. In sensitised mice, nebulised IFN-γ inhibited eosinophilic inflammation (Lack et al., 1996). Similarly,

mice lacking the IFN-γ receptor demonstrated impaired ability to resolve a pulmonary eosinophilic inflammation (Coyle et al., 1996).

Transforming growth factor-β

There are five known isoforms of Transforming Growth Factor-β (TGF-β), three of which are expressed by mammalian cells (TGF-β_{1-3}) (Roberts and Sporn, 1993). TGF-β is mainly produced by mesenchymal cells, such as fibroblasts, chondrocytes, and osteocytes, epithelial cells, and haematopoietic cells, including platelets, monocytes, and T cells. It is secreted in a latent, nonactive form that requires proteolytic cleavage to become active, and mediates a wide range of activities through its receptors, which are found in many cell types (Borish, 1998). TGF-β regulates cellular proliferation and differentiation in several settings, including embryonic development, wound healing, and angiogenesis (Wen et al., 2002) TGF-β is a stimulator of extracellular matrix (ECM) production, promoting synthesis of collagen, fibronectin, and elastin in fibroblasts. It also decreases ECM degradation by induction of TIMP (Tissue Inhibitors of Metallo-Proteinases) production and inhibition of protease synthesis (Edwards et al., 1987). Due to these actions, TGF-β is implicated in several disease states, including atherosclerosis and fibrotic disorders of the lung, liver, and kidney (Grainger et al., 1994, Border and Noble, 1994). TGF-β is also linked to airway remodelling in asthma, and TGF-β immunoreactivity was found to be increased in epithelial cells and submucosa in both asthmatic subjects and those with chronic bronchitis (Vignola et al., 1997). Blood neutrophils from asthmatics spontaneously release higher amounts of TGF-β than those from normal subjects, indicating that neutrophils are also involved in the airway remodelling process through increased release of TGF-β (Chu et al., 2000). TGF-β is also involved in immunity by inhibiting the proliferation of B cells, mast cells, and T helper cells (Sporn and Roberts, 1992), and by down-regulating unrearranged (germline) immunoglobulin ε heavy chain transcription by B cells (Sonoda et al., 1989).

The Epidermal growth factor (EGF) family

The EGF family of molecules with EGF-like regions includes EGF, transforming growth factor alpha (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, and epiregulin (Puddicombe et al., 2000). EGF is 53 amino acid long, initially synthesised as a prepro-EGF molecule consisting of approximately 1,200 amino acids, which is subsequently processed to EGF through a pro-EGF molecule stage (Amishima et al., 1998). The EGF family molecules have a direct mitogenic effect on many cell types, such as fibroblasts, smooth muscle cells, and keratinocytes (Mutsaers et al., 1997). The EGF receptor (EGFR) is a single 170-kDa polypeptide chain of 1,186 amino acids. The cytoplasmic domain of the EGF receptor has tyrosine kinase activity and is capable of initiating several pathways for signal transduction (Carpenter and Cohen, 1990). EGFR is expressed in the fetal airway, where it plays an important role in cell proliferation, branching, morphogenesis, and epithelial differentiation (Ruocco et al., 1996).

Several studies have highlighted the prominent role of the EGF molecules and the EGF receptor in the development of asthma. In a Japanese study, the immunoreactivity of EGF and EGFR was examined in bronchial tissue derived from asthmatic and non-asthmatic subjects and found considerably higher in the asthmatic group (Amishima et al., 1998). Increased EGFR immunoreactivity and rapid EGFR phosphorylation has been shown at the wound edge of injured epithelial cells in an *in vitro* model (Amishima et al., 1998). There is also evidence that the epidermal growth factor system plays a significant role in the regulation of mucin production in the airways (Guzman et al., 1995). In a mouse model, intratracheal instillation of TNF-α induced EGF-R in airway epithelial cells, and subsequent instillation of EGF and TGF-α increased the number of goblet cells, mucus production, and the MUC5AC gene expression (Amishima et al., 1998).

Insulin-like growth factor

The insulin growth factors (IGF-I and IGF-II) are single polypeptide chains that are mainly produced by macrophages (Rom et al., 1988). Although they are abundant in the systemic circulation, due to continuous liver production and secretion, they are also produced locally in the tissues. IGF-I is a potent mitogen for fibroblasts and

smooth muscle cells and is able to induce collagen synthesis in these cells (Cambrey et al., 1995, Stiles and Moats-Staats, 1989, Goldstein et al., 1989, Cherniack et al., 1991).

Platelet derived growth factor (PDGF)

The PDGF family constists of dimers of two polypeptide chains, A and B, which form the dimers PDGF –AA, -AB, and –BB. In addition to platelets, which store large amounts of PDGF-AB in α granules, other PDGF isoforms are synthesised by fibroblasts, smooth muscle cells, endothelial cells, and macrophages (Mutsaers et al., 1997). PDGF is a chemoattractant for monocytes and neutrophils (Deuel et al., 1982). It also stimulates the proliferation and chemotaxis of fibroblasts (Ross et al., 1986) and smooth muscle cells (Grotendorst et al., 1982), and was found to play an important role in pulmonary fibrosis (Cherniack et al., 1991), therefore, PDGF may play a role in airway remodelling in asthma. There is evidence to suggest that some of the proliferative effects of PDGF are mediated through TGF-β_1 production by fibroblasts (Pierce et al., 1989).

1.9. Airway remodelling in asthma

Traditional definitions of asthma have included the concept of reversible airflow obstruction. However, recent studies have shown that asthmatics experience an accelerated rate of lung function decline (Maddox and Schwartz, 2002), with some patients demonstrating a reversible or partially reversible airway obstruction and others manifesting incomplete β_2 agonist responses even after prolonged corticosteroid therapy (Elias et al., 1999). In a large 15-year follow-up study, a group of 1095 self-reported asthmatics had a decline in FEV1 of 38 ml per year as compared with 22 ml per year in those without asthma (Lange et al., 1998). The decline in lung function is the result of structural changes in the airways, secondary to the chronic inflammation in asthma resulting in tissue injury. Repair of injured tissue involves regeneration of parenchymal components that restore normal structure and replacement of damaged tissue by connective tissue (Maddox and Schwartz, 2002). The airway wall of asthmatics is characterised by increased thickness, involving an increase in muscle mass, mucous gland, and subepithelial fibrosis leading to reduced airway calibre (Bousquet et al., 2000). The magnitude of airway wall thickening is thought to be a significant contributor to airflow obstruction. Data from autopsy studies show that airway wall thickness is increased by 50-300% in cases of fatal asthma, and 10-100% in asthmatics who died of other reasons compared with non-asthmatic controls (Elias et al., 1999).

1.9.1. Thickening of the reticular basement membrane

Thickening of the reticular basement membrane (lamina reticularis) is a fundamental component of the airway pathology in asthma, and recent data suggest that the thickening occurs early in the nature course of the disease (Jeffery, 2000). The thickening is due to enhanced accumulation of tenascin, fibronectin, and types I, III, and V collagens produced by fibroblasts (Elias, 2000). A study by Payne and co-workers compared the thickness of reticular basement membrane (RBM) in endobronchial biopsy sections from children with difficult asthma, children without asthma, adults with mild and life-threatening asthma, and healthy adult individuals (Payne et al., 2003). They found that RBM thickening was similar between children and adult asthmatic individuals and was not associated with age, duration of symptoms, FEV1, or eosinophilic inflammation. The number of myofibroblasts in the

submucosa has been reported to correlate with the thickening of the reticular area and indicates that myofibroblasts may play a major role in this process (Brewster et al., 1990). Interestingly, neither short nor long-term treatment with inhaled corticosteroids reduced the thickening of the reticular basement membrane (Jeffery et al., 1992). However, it is not clear whether the thickening of lamina reticularis has functional consequences. No correlation between the subepithelial fibrosis and asthma severity was found in endobronchial biopsy specimens from asthmatic subjects examined by immunohistochemistry and electron microscopy (Roche et al., 1989). Other investigators, however, have suggested that the thickening of lamina reticularis may reduce its capacity to fold during bronchial smooth muscle contraction resulting in partially reversible or fixed airway obstruction (Lambert, 1991).

1.9.2. Increased myocyte muscle mass

An increase in muscle mass has been reported in fatal and non-fatal asthma and it is thought to be an important contributor to the thickening of the airways (Jeffery, 2001). Increased smooth muscle volume in the airways of asthmatics who died of fatal asthma were found in several studies. Approximately 12% of the wall in segmental bronchi was found to be occupied by muscle compared with about 5% in control subjects (Dunnill et al., 1969). In another study, the increase in muscle area in asthmatics was approximately three-fold in medium-sized airways and two-fold in the largest cartilaginous airways (James et al., 1989). Two patterns of distribution of increased muscle mass in fatal asthma were found (Ebina et al., 1993). In type I cases, the increase was associated only with hyperplasia in the large central airways, whereas in type II cases, there was mild hyperplasia in the large airways and hypertrophy throughout the bronchial tree, especially in small peripheral airways.

1.9.3. Increase in mucus glands

There is a well-documented mucus hypersecretion in fatal asthma, which is due to hypertrophy of the submucosal glands and increased numbers of goblet cells. Mucous glands are distributed throughout the cartilaginous airways in normal airways, however, in asthma they are present in peripheral bronchioles where normally they are absent (Bousquet et al., 2000). In fatal attacks of asthma, large and small airways become occluded by mucous plugs, which become very viscous due to the presence of

inflammatory cells and their secretory products (Jeffery, 2001). Apart from submucosal gland hypertrophy, there is evidence for an increased number of goblet cells in the airways of asthmatics (Ordonez et al., 2001). The mechanism by which goblet cells increase in number in asthma is uncertain, but it has been suggested that goblet cells metaplasia occurs by conversion of nongranulated secretory cells to goblet cells (Lee et al., 2000). Th2 cytokines play a critical role in goblet cell metaplasia in animal models (Cohn et al., 1997). Thus, overexpression of Th2 cytokines in the airways of mouse models of experimental asthma was found following allergen sensitisation, including IL-4 (Dabbagh et al., 1999), IL-5 (Lee et al., 1997), IL-9 (Louahed et al., 2000), and IL-13 (Zhu et al., 1999).

1.9.4. Remodelling of airway vasculature

An increase in both number and size of vessels was demonstrated in biopsy specimens of mild asthmatics compared to control individuals (Li and Wilson, 1997), and even more extensive angiogenesis was found in severe glucocorticoid-dependent asthma (Vrugt et al., 2000). This expansion of the microvasculature is necessary in order to accommodate the extra tissue mass of the thickened asthmatic airways (McDonald, 2001). The abundant and congested blood vessels in the airway mucosa contribute to the wall thickness together with the increased bronchial smooth muscle mass and submucosal glands. The endothelial cell-specific growth factors, vascular endothelial growth factor (VEGF) and angiopoietin 1 (Ang1), are thought to play a prominent role in the formation of new vessels (McDonald, 2001). VEGF expression in particular, was found to be increased in the airways of asthmatics and was correlated with mucosal vascularity (Hoshino et al., 2001b, Hoshino et al., 2001a).

Conclusions

In summary, there is strong evidence that remodelling in asthma usually begins early in the natural history of the disease and is characterised by thickening of the reticular basement membrane, enlargement of bronchial smooth muscle mass, submucosal gland hypertrophy, epithelial goblet cell hyperplasia, and angiogenesis (Jeffery, 2001). Remodelling may lead to accelerated decline in lung function, and may be the result of chronic inflammation although the latter is not yet very clear. There are many ongoing studies on mechanisms responsible for structural alterations in the

asthmatic airways. These studies will address many issues on airway remodelling in asthma, including the contribution of each of the features of the remodelled airway to the symptoms and natural history of the disease, and the types of interventions that may alter these various features (Elias, 2000).

1.10. Genetics of asthma and allergy

It has long been suggested that allergic disorders may have a genetic basis. In 1916 Robert Cooke and Albert Van der Veer examined 621 atopic probands and 76 non atopic controls and their families in order to assess the frequency of 'atopic' symptoms (hay fever, bronchial asthma, urticaria, angio-neurotic oedema, and acute gastro-enteritis following specific food ingestion) (Cooke, 1916). They established that 48.4% of their atopic probands had a family history of allergy, compared with 14.5% in the control population. More recently, a questionnaire study involving 6,665 families in Southern Bavaria found that children with atopic diseases had a positive family history in 55% of cases compared with 35% in children without atopic disease (Dold et al., 1992). Further analysis on the same population found that the prevalence of asthma alone increased significantly if the nearest of kin suffered from asthma alone (11.7% versus 4.7%) (von Mutius and T., 1996). A number of subsequent studies have also shown that the risk of asthma and allergic disease in offspring is higher when one or both parents are asthmatic than when neither parent is affected (Jenkins et al., 1993).

Complex genetic disorders such as asthma, however, do not exhibit classic Mendelian recessive, dominant, or X-linked inheritance attributable to a single gene locus. Disease expression is influenced by interactions between multiple major genes and modulated by interacting non-genetic factors (e.g. environmental) (Sandford et al., 1996). There is also genetic heterogeneity, where mutations in any of several genes can result in identical phenotypes, or, conversely, phenotypic heterogeneity, where the same genotype can result in different phenotypes (Barnes, 1999). In addition, an individual may inherit a genotype that results in a predisposition to develop asthma, but may never develop the disease in the absence of environmental factors. This is referred to as 'incomplete penentrance'. Another complicating factor is the presence of phenocopies within the population. Phenocopies are individuals who have a phenotype identical to the genetic disorder purely as a result of environmental factors (Sandford et al., 1996). Complex genetic disorders also exhibit 'variable expression of phenotype', that is, individuals with the same genetic susceptibility may have different expression of disease, with respect to age of onset, disease severity, and associated phenotypes (Hoffjan and Ober, 2002). Complex genetic disorders are also

more prevalent in the population than Mendelian disorders, which are relatively rare. For example, asthma occurs in at least 10% of children in the UK, whereas the frequency of cystic fibrosis, the most common Mendelian disorder, is 1 in every 2,000 live Caucasian births (Bleecker, 1997).

1.10.1. The hereditary contribution to asthma and allergy

The first step to quantifying the hereditary contribution to a complex disorder is to calculate the risk ratio (Lander and Schork, 1994). The risk ratio (λ) for a disorder is defined as the prevalence of the disorder in first-degree relatives of an affected individual divided by the prevalence in the general population. The higher the value of λ, the greater the genetic contribution to the disorder. For asthma the population prevalence is approximately 4%, while first degree relatives of asthmatics have a prevalence of 20-25%. This results in a value of λ of approximately 5-6 (Sandford et al., 1996). For cystic fibrosis λ is approximately 500, 15 for type I diabetes, 8.6 for schizophrenia, and 3.5 for type II diabetes. However, since the calculation of λ is affected by the population prevalence of the disorder, the genetic contribution to common diseases such as asthma may be underestimated (Sandford et al., 1996).

1.10.2. Twin concordance for asthma and allergy

Although family studies are consistent in pointing to the importance of shared family factors in the development of allergic disease, they are not sufficient to prove genetic causation. Shared exposure to a common environmental provoking agents might equally well explains much of the clustering of allergic disorders within families (Sandford et al., 1996). Twin studies are needed to distinguish the genetic from the environmental effects. The assumption is made that the effect of a shared family environment on the development of an allergic disorder will be the same for monozygotic and dizygotic twins. Monozygotic twins are genetically identical, whereas dizygotic twins share on average half their genes. Therefore, a disease that has a genetical component is expected to show a higher rate of concordance in monozygotic twins than in dizygotic twins (Sibbald, 1997). In a large survey involving 7,000 Swedish twin pairs, concordance rates for asthma, eczema, and hay fever were all substantially higher in mono- than in dizygotic twins (Edfors-Lubs, 1971). The concordance rate for asthma in monozygotic twins was 19%, whereas in

dizygotic was 4.8%; for hay fever, 21.4% in the monozygotic twins, and 13.6% in dizygotic; for eczema, 15.4% in monozygotic twins and 4.5% in dizygotic; for contact dermatitis, 9.6% in monozygotic twins and 6.1% in dizygotic. The inheritance of asthma was determined in 1,648 families of 16-year-old twins and their parents in a nation-wide Finnish Twin Cohort Study (Laitinen et al., 1998). The probandwise concordance rate for asthma was 42% in monozygotic twins and 21% in dizygotic twins. In the same study, asthma was reported in 11% of children of asthmatic mothers and 10% of children of asthmatic fathers, whereas only 3% of the children of non-asthmatic parents had asthma. The authors concluded that the risk of a child to be asthmatic was four times greater if either the mother or the father had asthma than if the child had been born to non-asthmatic parents. In a Danish study based on the 1953-1982 birth cohorts of the Danish Twin Registry, 11,688 twin pairs were studied (Skadhauge et al., 1999). The incidence of self-reported asthma was 6.2%. Substantially higher concordance rates, odds ratios, and correlations for asthma were estimated in monozygotic than in dizygotic twins. Thus, in the age group of 12-26 year-old the probandwise concordance for asthma (meaning the probability for one twin having asthma given the partner twin was affected) was 0.42 for monozygotic females and 0.48 for monozygotic males. By comparison, the probandwise concordance was 0.26 for dizygotic females, 0.19 for dizygotic males, and 0.16 for opposite-sex twins. In the same study, by using biometric modelling, a model including additive genetic, and nonshared environmental effects provided the best overall fit to that data. According to that model, 73% of the variation to liability to asthma was explained by genetic factors, and no sex difference or age-dependency in the magnitude of genetic effects was observed.

Concordance of a trait in twins will also be influenced by the common environment they share. One way to separate the effect of shared genes and environment in twin studies is to determine concordance in twins reared apart and compare it with the twins reared together (Sandford et al., 1996). Hanson and co-workers (Hanson et al., 1991) determined the prevalence of asthma and seasonal rhinitis, as well as blood levels of serum total and specific IgE in pairs of mono- and dizygotic twins reared apart and together. Sixty-eight reared-apart twin pairs and two hundred and twenty-one reared-together twin pairs were studied, utilising the University of Minnesota

Twin Registry and the Finnish National Public Health Institute Registry. There was not a high enough prevalence of asthma in the subjects to accurately compare concordance. For rhinitis, concordance in mono- and dizygotic twins was similar between those raised together and those raised apart. Similarly, the values for serum total IgE levels as well as blood levels for specific IgE, were not significantly different between the mono- and dizygotic twins reared together than in those reared apart. These data suggest a major genetic contribution to twin concordance.

1.10.3. Asthma phenotypes

Intermediate phenotypes in asthma such as BHR, serum total and specific IgE are regarded as contributing risk factors for the development of asthma (Wiesch et al., 1999). They are used in genetic studies because each of those is thought to be under the control by a smaller repertoire of genes compared to the complex phenotype of asthma. They also provide quantitative data for genetic analysis, which significantly increases the power in linkage and association studies (Cookson and Palmer, 1998).

Bronchial hyperresponsiveness

Bronchial hyperresponsiveness (BHR) is a cardinal feature of asthma (2000). Compared with normal subjects the bronchi of asthmatics tend to have both a decreased stimulus threshold and an increased response for bronchoconstriction. The asthmatic airway therefore contracts too much and too easily to provocation. BHR is measured by bronchoprovocation testing. The agents used for inhalation challenges include methacholine, histamine, carbacholine, and specific antigens chosen in accord with the patient's history. A positive test results when the FEV_1 of an individual falls 20% from the baseline at a standard concentration of inhaled agonist. BHR is associated with both asthma and atopy, as seen by the close association of BHR and serum total IgE levels with asthma in children (Wiesch et al., 1999). According to a study, approximately 30% of the variance in bronchial responsiveness is explained by an individual's serum total IgE level (Sears et al., 1991). The provocation challenge test has excellent sensitivity but a mediocre specificity for asthma, since it is also seen in a wide variety of other disease including COPD, congestive heart failure, cystic fibrosis, bronchitis, and allergic rhinitis (2000). A further complicating factor is the variability of BHR within subjects. BHR can vary over time with the presence of

seasonal changes in allergen exposure, with the presence of respiratory infection and with treatment (O'Byrne, 1988). Thus, most subjects with current asthma symptoms will have BHR, however, provocation challenge testing is more useful in excluding a diagnosis of asthma than in establishing one, as its negative predictive power is greater than its positive one (2000).

There is strong evidence that familial factors are involved in the development of BHR. This was illustrated in a study, which found that the incidence of BHR among monozygotic twins was increased compared to dizygotic twins (Hopp et al., 1984). In another study among relatives of asthmatic and nonasthmatic subjects, there was increased prevalence of BHR among the relatives of asthmatic compared with non-asthmatic individuals, although a high proportion of those with BHR had no clinical symptoms (Konig and Godfrey, 1973). In a separate study, bronchial response to metacholine challenge was measured in families in which there was a proband with asthma and in families without a history of allergic disease (Townley et al., 1986). Segregation analysis indicated that pure environmental models could be rejected and that a familial component to the transmission of BHR existed.

Atopy

Atopy is a predisposition towards the development of immediate hypersensitivity against common environmental antigens, defined by presence of elevated levels of serum total IgE levels, specific IgE levels, or positive skin tests (Jarvis and Burney, 1998). Atopy is a strong risk factor for the development of asthma, although the percentage of asthma cases attributable to atopy in different studies varies from as low as 8% to as high as 80% (Pearce et al., 1999). In a general population study involving 2,657 subjects, the prevalence of asthma was closely related to the serum IgE level standardised for age and sex, whereas allergic rhinitis appeared to be associated primarily with skin-test reactions to common aeroallergens, independently of the serum IgE level (Burrows et al., 1989). Although atopy and asthma interact, they are not interchangeable. Even in countries with a high prevalence of atopy (eg, UK, Australia and New Zealand) full-blown chronic asthma will develop in only 1 in 5 atopic patients (Holgate, 1999). Segregation analysis used to test genetic models for atopy inheritance provided evidence for a wide variety of genetic models in atopy,

including recessive (Marsh et al., 1974), dominant (Postma et al., 1995), co-dominant (Meyers et al., 1982), and maternal inheritence (Cookson et al., 1992). These discrepant results are consistent with the hypothesis that more than one genetic locus confers susceptibility to atopy and that alleles at each locus may have different effects (i.e., dominant, recessive, additive) (Ober, 1997).

Serum total IgE

It is important to consider atopy in terms of serum total IgE levels, since it has been suggested that it provides an overall estimate of the allergic component in asthma (Burrows et al., 1989). The association of total serum IgE and childhood asthma was highlighted in the Tucson Children's Respiratory Study among 263 boys and 277 girls, which found that both persistent wheezing and early sensitisation to local aeroallergens were associated with high serum IgE (Sherrill et al., 1999). In the same study, children with persistent wheezing and early sensitisation had high levels of IgE as early as 9 months. IgE is produced by B cells following stimulation by IL-4 and IL-13 and engagement of CD40 on B cells by CD40L expressed on activated T cells (Vercelli, 1996). Serum total IgE levels can be affected by factors such as sex, age, cigarette smoking, and recent allergen exposure, with males having higher IgE levels than females throughout the entire age range (Barbee et al., 1981). Serum IgE reaches a maximum at 10-13 years and then declines markedly with age (Hanneuse et al., 1978). There is annual variation in serum total IgE levels, and patients allergic to pollen demonstrate about two-fold annual changes in total IgE levels and a significant increase in their serum total IgE levels after exposure to pollen (Yunginger and Gleich, 1973). Immunotherapy with pollen extracts has been shown to influence serum total IgE levels by initially causing a twofold increase and subsequently a decrease after several years of therapy (Marsh et al., 1974). Smoking also leads to an increase of serum total IgE levels (Gerrard et al., 1980).

Apart from environmental influences, a heritable component to IgE regulation has long been recognised. Meyers and co-workers investigated the association of total serum IgE levels in two hundred and seventy-eight individuals from forty-two random familes and found a significant correlation between parents and offspring and an even stronger correlation among siblings (Meyers et al., 1986). In a separate study,

Lebowitz and co-workers (Lebowitz et al., 1984) found that total serum IgE demonstrated a significant family concordance among 344 nuclear families. Recently, positional cloning have identified an association between total serum concentration and several alleles of the *PHF11* gene on chromosome 13q14. These variants were also found to be associated with asthma (Zhang et al., 2003). The gene product (*PHF11*) contains two PHD (plant homeodomain) zinc fingers and probably regulates transcription.

Specific IgE

Although serum total IgE is an attractive parameter for genetic study, almost 45% of the variation in the serum total IgE is attributable to the specific IgE to house dust mite (HDM) or grass pollen (Cookson et al., 1991). Allergens consistently shown to be associated with asthma and allergic rhinitis in many studies include house dust mite, dog and cat allergens, fungal species, and pollen. In a longitudinal study of a birth cohort of New Zealand children up to the age of 13 years, among 714 children skin-tested, multiple regression analysis revealed that house dust mite and cat dander were highly significant independent risk factors associated with the development of asthma (Sears et al., 1989).

Traditional views of allergens consider them to interact with the immune system to trigger the synthesis of specific IgE, which lead to hypersensitivity through binding of the Fc portion of IgE to high-affinity receptors (FcεRI) on the surface of mast cells and basophils (Robinson, 2000). When the allergen itself binds to its specific IgE on the cell surface, it results in clustering of FcεRI receptors and thereby initiates a signal transduction cascade that releases the mediators responsible for the development of an acute allergic response. There is increasing evidence that several allergens possess enzymatic activity, including dust mites (Robinson et al., 1998), cockroach (Arruda et al., 1995), stinging insects (Hoffman and Jacobson, 1984), and pollen (Matheson and Travis, 1998). House dust mites are arthropods and the term is used to designate ten species of the family Pyroglyphida, four of which are dominant: Dermatophagoides pteronyssinus, D. farinae, D. microceras, and Euroglyphus maynei (PJ Thompson, 2001). The mite allergens have been categorised in three groups; group 1 allergens (Der p1, Der f 1 and Eur m1) are proteinases of approximate molecular weight 25-

kDa, which are secreted from the digestive tract and found in high concentrations in mite faeces; group 2 allergens (Der p 2, Der f 2) are found in both faecal pellets and mite bodies; and, group 3 allergens (Der p 3, Der f 3) are digestive enzymes. Many studies have shown a significant association between sensitisation to house dust mite and the development of asthma among different populations (Peat et al., 1996, Squillace et al., 1997, Sporik et al., 1999). Furthermore, inhalation of dust mite allergen in a group of 12 asthmatics sensitised to house dust mite caused an FEV_1 drop larger than 20% in all subjects, whereas the FEV_1 remained unchanged in 7 nonallergic asthmatics (M'Raihi et al., 1990). The major cat allergen is the Fel d 1, a 36-kDa glycoprotein found in saliva and sebaceous glands of the skin (Ohman et al., 1984). The major dog allergen is the Can f 1, a 27-kDa protein whose function is unkown (de Groot et al., 1991). The relationship between exposure to cat and dog allergens and the development of asthma is not clear, since exposure to cat or dog during the first year of life is was found to be associated with a decreased rather than an increased risk for development of asthma and allergic rhinitis later in life (Hesselmar et al., 1999). Children clinically tolerant to cats can produce a protective IgG and IgG_4 antibody response to Fel d 1 without sensitisation or risk of asthma (Platts-Mills et al., 2001). Fungal species, especially those belonging to the Deuteromycotina (Aspergillus, Cladosporium, Penicillium, Alternaria), and Basidiomycotina (mushrooma, puffballs, rusts, smuts) are thought to be important allergen sources (PJ Thompson, 2001). Although it has been difficult to establish the allergenic importance of airborne fungi, since there are thousands of fungal species, in a study of children raised in a desert environment, persistent asthma (diagnosed before age 6) was independently associated with Alternaria skin tests at both ages 6 and 11, whereas new asthma (diagnosed after age 6) was associated with Alternaria skin tests at age 6 but not at age 11 (Halonen et al., 1997). In the above study, the prevalence of physician-diagnosed asthma was 9.8% at age 6 (n = 948) and 15.5% at age 11 (n = 895). Airborne pollen produced by a wide variety of wind-pollinated plants can act as a source of allergen and contribute greatly to allergic disease, including asthma and allergic rhinitis in susceptible individuals (Reid et al., 1986). A wide variety of grass, weed, and tree-pollen allergens have been described (Stewart, 2000). Pollen grains are frequently released when hot, dry conditions prevail, with late spring and summer being times of high pollen.

1.10.4. Methods for identifying disease susceptibility genes

Two fundamental approaches are being used to detect susceptibility genes in asthma and atopy: linkage analysis with functional cloning and association analysis for polymorphisms of 'candidate' genes thought to be involved in disease pathogenesis (Holgate, 1999).

Linkage analysis

Linkage analysis uses family data to follow the transmission of genetic information between generations (Sandford et al., 1996). This information is used to determine if a genetic marker is close (linked to) a gene involved in a particular disease. Genetic markers are segments of DNA found throughout the human genome that vary between individuals. Any polymorphism can be used as a marker for linkage analysis, including SNPs. The most common markers are 'microsatellite' markers, short segments of DNA in which the nucleotide sequence consists of repeats of specific base pairs (e.g., $[CT]_n$]. These repeats are found throughout the genome and can be amplified by PCR. The location of more than 5,000 microsatellite markers have been determined to date (Dib et al., 1996). Microsatellites are more informative than SNPs, as they have high heterozygosity. This means that any two individuals are more likely to have different alleles on a particular locus. Whole genome screens usually use 300 to 500 dinucleotide, trinucleotide, or tetranucleotide markers spaced 5 to 10 centiMorgan (cM) across the genome (Holgate, 1999). In a genome screen, markers are tested sequentially until one is found that shows low levels of recombination with the disease (Sandford et al., 1996). This marker is said to be linked to the disease gene, i.e., close to the disease gene on the chromosome. Thus, if a large number of families are studied and a specific marker is found to be transmitted with the disease phenotype more frequently than should occur by chance (i.e., it is linked), then the disease-causing gene must be close to that specific marker gene (Sandford et al., 1996). The proximity of a marker to a gene can be estimated by measuring the number of recombination events between them, measured as recombination fraction (θ). The statistical significance of the linkage is measured by the lod score, which is the log of the ratio of the data's likelihood given linkage to the likelihood of no linkage (Morton, 1955). Linkage is confirmed when the odds ratio drops to 1,000 to 1 in favour of linkage (Z=3.0), and rejected when the odds ratio drops to 100 to 1 in

favour of no linkage (Z=-2.0) (Schork et al., 1993). However, if many markers have been used, the chance of a spurious positive result is greater than if only one marker is used. It has been suggested that a LOD score of 3.3, corresponding to a *P* value of 5 x 10^{-5}, represents a 5% false positive in genome-wide scans and has been recommended as the appropriate threshold in genome searches of complex traits (Lander and Kruglyak, 1995). A LOD score of 2.2 (P=7 x 10^{-4}) is suggestive of linkage, 3.6 (P=2 x 10^{-5}) corresponds to significant linkage and a score of 5.4 (P = 3 x 10^{-7}) is highly significant linkage (Lander and Kruglyak, 1995). After a chromosomal region has been linked to a particular trait, finer mapping of the region can be achieved using markers spaced at 1-2 cM apart. The Human Genome Projects provides an almost complete catalogue of all genes in a relevant region, so construction of physical maps may not be needed. Next, association studies are undertaken to demonstrate a clear association between the phenotype and allelic variations within candidate genes in the region. The high frequency of asthma in the general population has adverse effects on the power to detect linkage using affected sibling pairs. Simulation studies indicate that 500 affected sibling pairs will give less than 50% power to detect linkage to a given marker, and that over 1,000 affected pairs will be required to achieve 90% power (Cookson, 1999). Once a specific gene and its mutations have been identified, additional studies, including differential expression in diseased tissue, knock-out and transgenic animal studies provide further information on its role (Holgate, 1999).

Allele sharing methods

A major problem using linkage analysis in complex disease is the need to propose a model to explain the inheritance pattern of phenotypes and genotypes observed in a pedigree. Allele sharing methods, instead, assume no model for inheritance of the trait and therefore tend to be more robust than linkage analysis (Lander and Schork, 1994). In allele-sharing methods one is trying to prove that the inheritance pattern of a chromosomal region is not consistent with random Mendelial segregation, by showing that affected relatives inherit identical copies of the region more often than expected by chance. Excess allelic sharing can be estimated with a simple χ^2. Affected sib pair analysis is the simplest form of this method (Lander and Schork, 1994). The analysis is done on pairs of affected siblings within a family. Because both siblings are affected, the disease genes are assumed to have acted and nonpenentrant individuals

are excluded from the analysis. In a large group of asthmatic pairs, more than 50% will share a specific parental at any locus if that locus is linked to a gene causing the disease. If both parents are available the data could be analysed separately, for the maternal and paternal chromosome. Until recently, only a single marker was studied using allele sharing methods. It is now possible to perform complete multipoint sib pair analysis using the information by all genetic markers to infer the full probability distribution of genetic markers along the genome (Kruglyak and Lander, 1995).

Genome scans in asthma and atopy

A search of three databases, Online Mendelian Inheritance in Man (OMIM), Human Gene Mutation Database (HGMD), and LocusLink produce estimates of approximately 1,200 genes causing disease (Tabor et al., 2002). As of June 2002, HGMD listed over 27,000 mutations associated with human diseases and traits (Botstein and Risch, 2003). Of the genes that have been positionally cloned, most (~90%) involve traits with Mendelian inheritance (Tabor et al., 2002). In studies of complex disease though, the real challenge has not been the identification of regions of linkage, but identifying the genes underlying the observed linkage. However, to date, the *ADAM33* gene (Van Eerdewegh et al., 2002) and the *PHF11* gene (Zhang et al., 2003) have been identified as genes involved in asthma and regulation of total serum IgE, respectively, using positional cloning. Other genes identified as the result of positional cloning in complex diseases include the apolipoprotein gene in late onset familial and sporadic forms of Alzheimer's disease (Corder et al., 1993), the NOD2 gene in Crohn's disease (Hugot JP, 2001), and the calpain 10 gene in type 2 diabetes mellitus (Horikawa et al., 2000).

Genome screens for asthma and related phenotypes have now been completed in various study populations (Table 1.1). Multiple regions of the genome have been observed to be linked to varying phenotypes with differences between cohorts recruited both from similar and different population (Holloway et al., 2003). Differences in study design and sample size may also account for variations between studies. The results reflect the genetic and environmental heterogeneity seen in allergic disorders and highlight the fact that asthma is a heterogeneous disease with many loci contributing to the disease phenotype. They also illustrate the difficulty of

identifying susceptibility genes for complex genetic diseases. The most consistently replicated regions are on chromosomes 2q, 5q, 13q, 6p, and 12q (Hoffjan and Ober, 2002). Up to date information of all the genome screens are available in the Asthma Gene Database (Wjst and Immervoll, 1998).

At the time of writing, *ADAM33* (Van Eerdewegh et al., 2002) and *PHF11* (Zhang et al., 2003) are the only genes that have been identified as the result of positional cloning using a genome-wide scan for allergic disease phenotypes. In the study of Eeerdewegh and co-workers (Van Eerdewegh et al., 2002), a genome-wide scan on 460 Caucasian families identified a locus on 20p13 that was linked to asthma (LOD 2.94) and BHR (LOD 3.93). SSCP analysis and direct sequencing was used to identify one hundred and thiry-five SNPs in twenty-three genes that lay in the 2.5 Mb around the peak of linkage, which were subsequently typed. Fourteen of these SNPs lay within *ADAM33* achieving significance of P=0.005-0.05. *ADAM33* is a member of family of genes which encode a subgroup of zinc dependent metalloprotease. ADAM proteins are membrane-anchored metalloproteases with diverse functions including the shedding of cell surface proteins such as cytokines and cytokine receptors (Mullberg et al., 2000). ADAM33 is expressed in lung fibroblasts and smooth muscle cells, but not in bronchial epithelial cells or leukocytes, suggesting that ADAM33 may play a role in airway remodelling.

In the study of Zhang and co-workers (Zhang et al., 2003) a genome-wide study identified linkage of atopy and related phenotypes to chromosome 13q14. Following construction of a dense SNP map, three SNPs in intron X, intron V, and the 3' UTR of the *PHF11* gene were found to have independent effects on serum total IgE. These variants were also associated to severe clinical asthma. Although the exact role of PHF11 in IgE regulation and asthma is not yet clear, the gene product is thought to be involved in chromatin-mediated transcriptional regulation.

Table 1.1. Genome scans for asthma, atopy, and associated phenotypes

Position	Phenotype	Population	Author/Year
1p21-1p22	Asthma (strict/loose) and Atopy[3]	US Hutterites	(Ober et al., 1998)
1p31	Asthma[4]	France	(Dizier et al., 2000)
1p32	Asthma[2]	US Hispanics	(CSGA, 2001)
1q21	Atopic dermatitis	UK	(Cookson et al., 2001)
1q25	Atopy and asthma[6]	China	(Xu *et al.*, 2002)
2pter	Asthma, BHR, IgE and RAST	Germany	(Wjst et al., 1999)
2p25	Atopy and asthma[6]	China	(Xu et al., 2002)
2q21-q23	Der p specific IgE	US Caucasians	(Hizawa et al., 1998)
2q33	Asthma	US Hispanics	(CSGA, 1997) (, 1997b)
2q	Atopy & IgE	Holland	(Koppelman et al., 2002)
3p24.2-22	Asthma symptoms	US Hutterites	(Ober et al., 1998)
3q21	Atopic dermatitis	Europe	(Lee *et al.*, 2000)
4p15.3	Atopy and asthma[6]	China	(Xu et al., 2002)
4q24-27	Allergic Rhinitis	Denmark	(Haagerup et al., 2001)
4q35	Asthma[1]	Australia	(Daniels et al., 1996)
4q35	Mite sensitive atopic asthma[5]	Japan	(Yokouchi et al., 2000)
5p15	Asthma	US African American	(CSGA 1997)
5q23-31	Asthma	US Caucasians	(CSGA 1997)
5q31-33	Asthma, BHR & symptoms	US Hutterites	(Ober et al., 1998)
*5q31	Asthma[2]	US Total	(CSGA 2001)
5q31	IgE	Holland	(Xu et al., 2000)
5q31-33	Mite sensitive atopic asthma[5]	Japan	(Yokouchi et al., 2000)
6p21	Asthma[2]	US Caucasians	(CSGA 1997) (GSGA 2001/Xu et al., 2001)
6p21	Atopy[3]	US Hutterites	(Ober et al., 2000)
6p21.3	Asthma, IgE, RAST & eosinophils	Germany	(Wjst et al., 1999)
6p22-p21.3	Mite sensitive atopic asthma	Japan	(Yokouchi et al., 2000)

Position	Phenotype	Population	Author/Year
6p	Atopy & IgE	Holland	(Koppelman et al., 2002)
6q16.3 - 25.2	Asthma[1]	Australia	(Daniels et al., 1996)
*8p23	Asthma	US Total	(CSGA, 2001)
8p23-21	Der p specific IgE	US African Americans	(Hizawa et al., 1998)
9p	Asthma, IgE & RAST	Germany	(Wjst et al., 1999)
10p25	Atopy and asthma[6]	China	(CSGA 2001/Xu et al., 2001)
11p13	Asthma[4]	France	(Dizier et al., 2000)
11p15	Asthma	US Caucasian	(CSGA, 1997) (, 1997b)
11q13	Asthma[1]	UK & Australia	(Daniels et al., 1996)
11q13	Asthma[4]	France	(Dizier *et al* 2000),
11q21	Asthma[2]	US African Americans	(CSGA 2001/Xu et al., 2001)
11q	Atopy	Holland	(Koppelman et al., 2002)
12q	Total IgE	Holland	(Xu et al., 2000)
12q13	Asthma	Germany	(Wjst et al., 1999)
12q 15-24.1	Asthma, BHR & symptoms	US Hutterites	Ober et al., 1998
12q14-24.2	Asthma, BHR & IgE	US Caucasians and Hispanics	(CSGA, 2001)
12q21-q23	Mite sensitive atopic asthma, BHR	Japan	(Yokouchi et al., 2000)
12q24	Asthma[4]	France	(Dizier et al., 2000)
13q11	Mite sensitive atopic asthma[5]	Japan	(Yokouchi et al., 2000)
13q12-13	IgE	Holland	(Koppelman et al., 2002)
13q14.1-14.3	Asthma[1]	Australia & UK	(Daniels et al., 1996)
13q14.1-14.3	Mite sensitive atopic asthma, BHR	Japan	(Yokouchi et al., 2000)
13q21.3	Asthma	Caucasian	(CSGA, 1997,) (, 1997b)
13q31	Asthma[4]	France	(Dizier et al., 2000)
14q11-13	Asthma	US Caucasian	(CSGA 1997) (, 1997b)
14q32	IgE and BHR	US Total	(CSGA 1997) (, 1997b) (CSGA 2001/Xu et al., 2001)
*15q13	Asthma[2]	US	(CSGA 2001/Xu et al, 2001)

Position	Phenotype	Population	Author/Year
16p12	Atopy[3]	US Hutterites	(Ober et al., 1998)
16p12	Atopy and asthma[6]	China	(CSGA, 2001)
16q24.1	Asthma[1]	Australia & UK	(Daniels et al., 1996)
17p11.1 – 17q 11.2	Asthma	US Afro-Americans	(CSGA, 1997) (, 1997b)
17q12-21	Asthma[4]	France	(Dizier et al., 2000)
17q25	Atopic dermatitis	UK	(Cookson et al., 2001)
17q25	Eosinophils	Holland	(Koppelman et al., 2002)
19q13	Asthma	US Hutterites	(Ober et al., 1998)
19q13	Asthma[4]	France	(Dizier et al., 2000)
19q13	Asthma	Caucasian	(CSGA 1997) (, 1997b)
19q13.1	Atopy and asthma[6]	China	(CSGA 2001)
20p12	Atopic dermatitis & asthma	UK	(Cookson et al., 2001)
21q21	Asthma	US Hutterites	(Ober et al., 1998)
21q21	Asthma	US Hispanics	(CSGA, 1997) (, 1997b)
22q11.2	Atopy and asthma[6]	China	(CSGA, 2001)
22q	Atopy	Holland	Koppelman *et al* 2002

1) Four intermediate phenotypes associated with asthma

2) Asthma intermediate phenotypes

3) Five intermediate phenotypes associated with atopy

4) Asthma and four intermediate phenotypes

5) House dust mite sensitive asthma

6) Intermediate phenotypes (QTLS) include BHR, FEV1, FVC, Total IgE, Eosinophils, SPT wheal size and specific IgE.

* Significant only after conditioning for another locus (Xu et al., 2001)

Ober et al., 1998: 'loose asthma' phenotype = BHR and/or asthma symptoms
'strict asthma' = Asthma

Ober et al., 2000: cough, wheeze, shortness of breath; 2/3 and BHR = 'strict asthma'
Either symptoms or BHR = 'loose asthma'

(From Holloway J.W, Cakebread JA, Holgate ST: The Genetics of Allergic Disease and Asthma. In Leung DY, Sampson H, Geha R and Szefler SJ (eds): Pediatric Allergy: Principles and Practise, St. Louis, Missouri, Mosby, 2003, pp 23-28.)

Candidate gene approaches in asthma

There are now over two hundred studies that have examined polymorphism in over 100 genes for association with asthma and allergy phenotypes (Holloway et al., 2003). Genetic association studies do not examine familial inheritance patterns, rather, they are case-control studies based on a comparison of unrelated affected and unaffected individuals from a population (Lander and Schork, 1994). An allele A at a gene of interest is said to be associated with the trait if it occurs at a significantly higher frequency among affected compared with control individuals. Statistical analysis is carried out using a 2x2 contingency table.

The knowledge that suggests the candidacy of a gene can come from its pathophysiologic role in the disease process, differential gene expression in disease, homology with other disease genes, functional studies in animal models, or its chromosomal location in a region linked to the disease by linkage studies (Schork, 1997). Caution is needed when interpreting results in association studies, since positive association between an allele and a phenotype can arise for three reasons (Cardon and Palmer, 2003): (i) The allele itself is functional and directly affects the expression of the phenotype. (ii) The allele itself does not cause the trait but is in linkage disequilibrium with a causative allele located nearby. Linkage disequilibrium arises from an increased frequency of particular haplotypes across a population (Silverman and Palmer, 2000). The greater frequency of some haplotypes is due to population admixture, natural selection, genetic drift, or new mutations. Therefore, linkage disequilibrium is most likely to occur in a young, isolated population. (iii) A positive association could also be attributable to chance, selection bias, or population stratification. Population stratification is the most often cited reason of non-replication of genetic association results (Cardon and Palmer, 2003). It arises in mixed populations, when a trait present at a higher frequency in an ethnic group will show positive association with any allele that happen to be more common in that group.

To prevent spurious associations arising from population admixture, a number of steps can be taken. First, association studies should be performed within homogeneous populations (Silverman and Palmer, 2000). Second, protection of stratification can be achieved by establishing a pseudo case-control study, in which

cases are the parental alleles transmitted to the affected offspring and controls are those that were not transmitted. The most popular method that uses family-based controls is the transmission-disequilibrium test (TDT) (Spielman et al., 1993). The TDT uses a trio designed of two parents and one affected child and evaluates the transmission of a marker allele from a heterozygous parent to an affected offspring using the χ^2 tests. Since the control and disease samples in the TDT are obtained from the same individuals, the contribution of stratification is reduced or eliminated (Spielman et al., 1993). A third method that protects against population stratification problems is using genetic markers scattered throughout the genome as controls. As few as 30 markers have been proposed as sufficient to detect population substructure among the cases and controls (Pritchard and Rosenberg, 1999).

To date, there have been many candidate gene studies in asthma, including the cytokine cluster on chromosome 5q (*IL4, IL5, IL9, IL13, CD14, ADRB2* (β_2 adrenergic receptor), the human leukocyte antigen (HLA) region on chromosome 6p (including the *TNF* gene), the high affinity IgE receptor (*FCERB*), the Clara cell secretory protein (*CC16*) on chromosome 11q, and the *IL4RA* gene on chromosome 16p (Hoffjan and Ober, 2002). Overall, results of candidate gene studies vary enormously, and associations found in some populations are not replicated in others. By contrast, genes involved in the IL-4/IL-13 pathway have been associated with asthma and atopy phenotypes in many studies. Because of the key role of this pathway in asthma, the overall consistency of the results, and the relevance to this thesis, these studies are described in more detail below.

_IL4_ polymorphisms

IL-4 plays a central role in IgE dependent inflammatory reactions and is critical for the maturation of T helper cells towards the Th2 phenotype (Izuhara and Shirakawa, 1999). The *IL4* gene is localised within 25 kb on the proximal portion of chromosome 5q31, to which many genome searches have identified strong linkage with asthma and atopy (Shirakawa et al., 2000). To date, five variants have been reported in the promoter region of *IL4*-three of them are very rare (Shirakawa et al., 2000, Hoffjan et al., 2002). The T allele of the -589C>T variant (referred to as -590C>T in this study) in the *IL4* promoter was significantly associated with high serum IgE in a sample of

20 asthmatic and 5 control families (Rosenwasser et al., 1995). In the same study, luciferase reporter assay of the *IL4* promoter transfected into Jurkat cells demonstrated an increased level of transcription associated with the T allele. Electrophoretic mobility shift assays (EMSA) revealed that the T allele oligonucleotide had a higher binding affinity to nuclear transcription factors than the C oligonucleotide, in support to the reporter assay. However, other studies have failed to replicate the original association. In 230 nuclear families from Australia, one hundred and twenty-four unrelated atopic asthmatics and 59 unrelated non-atopic, non-asthmatic controls, only a weak association of this variant with house dust mite specific IgE and wheeze was observed (Walley and Cookson, 1996). A case-control study of two Japanese populations, one of families ascertained through an asthmatic child and one in randomly ascertained population as a control, found no significant difference in the prevalence of the –589 genotypes between the two groups (Noguchi et al., 1998). Moreover, no difference was observed between the distribution of genotypes and total IgE. However, using the TDT a significant association between the T allele and asthma was found in the family cohort. An association of the –589T allele and 'probable asthma' at one year of age was observed in a prospective cohort of high-risk children (Zhu et al., 2000), however, a subsequent study did not find association with childhood atopic asthma (Takabayashi et al., 2000). In another study, an association of TT genotype and lower FEV_1 was found among Caucasian asthmatics (Burchard et al., 1999). Another variant in the *IL4* promoter, –34C>T, was found to be associated with asthma in a Japanese population (Suzuki et al., 2000).

IL4R polymorphisms

IL-4Rα is a functional subunit of both IL-4 and IL-13 receptor systems (Gauchat et al., 1997). IL-4Rα is a type I transmembrane domain spanning protein, consisting of 825 amino acids with a total molecular mass of 140-kDa. The human *IL4R* gene was cloned using cross-species hybridization of cDNA encoding the murine analogue and mapped to 16p12.1(Idzerda et al., 1990). Genetic linkage between atopy and flanking markers to *IL4R* on 16p12 was first described by Deichmann and co-workers (Deichmann et al., 1998), who found significant sharing of maternal alleles in atopic children. The *IL4R* gene is highly polymorphic in humans. To date, 14 common SNPs in the coding region of the gene have been identified with 8 of the polymorphisms

leading to amino acid substitutions (Ober et al., 2000a). The Ile50 allele of the extracellular Ile50Val variant has been associated with atopic asthma and raised total serum IgE in a Japanese population (Mitsuyasu et al., 1998). Functional assays in both human and murine B-cell lines revealed that Ile50 upregulates cellular STAT6 activation and IgE synthesis (Mitsuyasu et al., 1999). However, other studies have found no association of this variant with atopic asthma (Xu et al., 2001), or allergic dermatitis (Tanaka et al., 2001, Oiso et al., 2000), or atopy (Haagerup et al. 2001). In a study of patients with hyper-IgE syndrome and severe atopic dermatitis, the Arg551 allele of the Gln551Arg variant (referred to as Gln576Arg in this study) in the cytoplasmic domain of *IL4R* was found in excess among patients compared to normal controls (Hershey et al., 1997). Functional assays by the same group of investigators showed that the Arg576 allele alters the function of the receptor by decreasing the binding of the negative regulatory molecule, tyrosine phosphatase SHP1, causing enhanced signalling to IL-4. As a result, higher levels of expression of CD23 in PBMCs from heterozygote individuals compared to wild type homozygotes were found after incubation of cells with IL-4. However, Wang and co-workers could not replicate the functional effects of the Arg551 allele (Wang et al., 1999), and subsequent studies failed to show any association of the Gln551Arg variant with either hyper-IgE syndrome (Grimbacher et al., 1998), or asthma/atopy (Patuzzo et al., 2000, Haagerup et al., 2001). The opposite was shown in a German population, Arg551 was found in excess among individuals with lower IgE levels suggesting a protective role of this allele (Kruse et al., 1999). In the same study, the Pro478 allele of the Ser478Pro variant (referred to as Ser503Pro in this study) was found to be associated with lowered serum IgE levels as well. The Ser503Pro variant is located within the insulin-IL-4 receptor motif (I4R), which is crucial for binding and tyrosine phosphorylation of IRS-1 (insulin receptor substrate-1) and IRS-2. In vitro analysis of Gln551Arg and Ser503Pro, showed that both variants independently influence STAT6 binding and lead to lower serum total IgE levels (Kruse et al., 1999).

Due to the highly polymorphic nature of the *IL4R* gene and the conflicting results of association studies, subsequent studies have analysed haplotypes rather than individuals polymorphisms. Ober and co-workers (Ober et al., 2000a) genotyped members of a large Hutterite pedigree (Hutterites are an inbred population in South

Dakota, USA, of European discent), as well as outbred white, black, and Hispanic families for all amino acid changing polymorphisms of *IL4R*. No individual alleles were significantly overtransmitted to atopic offsprings in any of the outbred samples, however, Glu375 and Cys406 were overtransmitted to subjects with atopy and asthma in the Hutteries. In contrast, haplotype analysis showed that several combinations of alleles in the intracellular domain of *IL4R* were highly associated with asthma and atopy in all populations studied. To date, three SNPs (-890T>C, -1914T>C, -3223C>T) and a short tandem repeat $(CAAA)_{5-7}$ have been identified in the *IL4R* promoter. In a German population, the presence of the –3223T allele was significantly associated with decreased soluble IL-4Rα concentrations in the serum of healthy volunteers (Hackstein et al., 2001).

***IL13* polymorphisms**

To date, seven tightly linked polymorphisms in *IL13* have been identified (Graves et al., 2000). A polymorphism in nucleotide +2044, relative to the first nucleotide of the open reading frame, located in exon 4 leads to a predicted amino acid substitution of glutamine for arginine (Arg110Gln). In addition, two polymorphisms are located in the promoter region of *IL13*, one in the third intron and three in the 3' UTR. The Arg130Gln variant is in extremely tight linkage disquilibrium with the three polymorphisms in the 3' UTR and the polymorphism in the third intron. The two promoter polymorphisms are in significant degree of linkage disequilibrium (δ=0.87), and both are in linkage disequilibrium with Arg110Gln. The -1111C>T polymorphism (referred to as –1055C>T in this study) is in conjunction with the most distal NF-AT consensus sequence of the IL-13 promoter (van der Pouw-Kraan et al., 1999). Using a sample of 101 allergic asthmatics and 107 non-allergic control subjects, van der Pouw-Kraan and co-workers observed an increased frequency of homozygous for the 1111T allele in the asthmatic group (13/101 vs. 2/107, odds ratio=8.3, p=0.002) (van der Pouw-Kraan et al., 1999). The same group of investigators demonstrated that the –1111C>T SNP has a functional role. Using electromobility shift assays (EMSA), they showed that the presence of the T allele resulted in increased binding of nuclear proteins. In addition, they demonstrated that subjects homozygous for the T allele displayed a significant decreased relative inhibition of IL-13 production, when T cells were stimulated with anti-CD2,

compared to the CC or CT genotypes. The –1111A>C polymorphism was found to be associated with BHR, skin test responsiveness (≥1 positive skin tests), and asthma, but not with serum total IgE levels, in another Dutch population (Howard et al., 2001). The Arg110Gln polymorphism was associated with asthma in British and Japanese populations (Heinzmann et al., 2000). In the same study, increased frequency of Gln110 was observed among asthmatics in the British population (40% vs. 26.7%, p=0.014, odds ratio=1.83), as well as in the Japanese population (62.5% vs. 43%, p=0.013, odds ratio=1.81). However, no association between Gln110 and serum IgE levels in either populations was observed. In the study of Graves and co-workers Gln110 (referred to as Gln130 in this study) was strongly associated with high serum total IgE levels in 1,399 children from three Caucasian populations; a group from Tuczon Arizona (*P*=0.0023), a group from Munich Germany (*P*=0.0081), and a group from Leipzig in former Eastern Germany (*P*=0.0069) (Graves et al., 2000). In subsequent studies, the Arg110Gln variant was associated with high serum total IgE and atopic dermatitis in a German multicenter atopy study (Liu et al., 2000), and specific IgE, but not asthma, in Hong Kong Chinese children (Leung et al., 2001). However, no association of Arg110Gln with asthma was found in a study of a large Hutterite pedigree (Ober et al., 2000b), and no association of this variant with serum total IgE levels was found in 83 Costa Rican nuclear families (Celedon et al., 2002).

_IL2RG_ polymorphisms

IL-2Rγc is a component of many receptor complexes including the IL-2 receptor complex (Takeshita et al., 1992), IL-4 receptor (Kondo et al., 1993), IL-7 receptor (Noguchi et al., 1993a), IL-9 receptor (Russell et al., 1994), and IL-15 receptor complex (Giri et al., 1994). Mutations of the *IL2RG* gene have been identified in relation to X-linked severe combined immunodeficiency (XSCID), which is characterised by lack of T lymphocytes and nonfuctional B cells (Noguchi et al., 1993b). To date, no genetic variants of *IL2RG* associated with either asthma or atopy have been identified.

***STAT6* polymorphisms**

STAT6 is an essential molecule for functional responses induced by IL-4 and IL-13 (Takeda et al., 1997). The gene encoding human *STAT6* spans 19 kb on 12q13-14, and constists of 23 exons (Patel et al., 1998). Whole genome screens for atopic asthma have shown linkage to the 12q14-12 region (1997b, Ober et al., 1998). A 3' UTR variant, G2964A, in *STAT 6* was found to be associated with mild atopic asthma in a Japanese but not a British population (Gao et al., 2000). In another study, a dinucleotide repeat polymorphism in the first exon of *STAT6* was associated with allergic diseases in a Japanese population (Tamura et al., 2001). A subsequent study has identified an additional 13 non-coding SNPs in *STAT6* (Duetsch et al., 2002). Two intronic SNPs and one SNP in the 3' UTR of *STAT6* showed weak association to serum total IgE levels (P= 0.0200, 0.0260, and 0.0280, respectively), whereas a significant association was found between a SNP in intron 18 and higher serum total IgE levels (P= 0.0070). However, the most promising effect was seen between allele A4 of the GT repeat polymorphism in intron 1 and an increase in eosinophil cell count (P= 0.0010). Based on these results, the authors of the study concluded that the human *STAT6* gene is probably involved in the development of eosinophilia and changes in total IgE levels, rather than directly contributing to the pathogenesis of asthma. To date, no polymorphisms in relation to asthma or allergy have been identified in the *JAK1*, the *JAK3*, or the *TYK2* genes (Shirakawa et al., 2000).

1.11. Interleukin-13

The mouse Interleukin-13 (IL-13) gene was first cloned in 1989 (Brown et al., 1989), whereas its human homologue was cloned independently by two different groups in 1993 (Minty et al., 1993). To isolate the human IL-13 cDNA, the first group used systematic sequencing of a subtracted cDNA library from activated PBMCs (Minty et al., 1993), whereas the second group screened cDNA libraries from T cell clones using a mouse IL-13 cDNA probe (McKenzie et al., 1993a). Human IL-13 consists of 132 amino acids and has a molecular mass of approximately 10-kDa (McKenzie et al., 1993a). The genomic structure of the human IL-13 gene comprises sequence of approximately 4.5 kb, and consists of four exons and three introns (McKenzie et al., 1993b). The IL-13 gene is only 12 kb 5' upstream of the IL-4 gene and is positioned in the same 5' to 3' orientation (Smirnov et al., 1995). The human IL-13 gene is localised on the long arm of chromosome 5 on band q31, in the same cluster of genes encoding IL-3, IL-4, IL-5, IL-9, and GM-CSF. The IL-13 gene shares a common intron-exon structure with GM-CSF, IL-4, and IL-5. The close proximity and similar structure of these genes suggest that they may have evolved from gene duplication events (Zurawski and de Vries, 1994). Although IL-13 and IL-4 show only 25% protein homology, (Chomarat and Banchereau, 1998), IL-13 shares many functional properties with IL-4 as a result of the use of common receptor components (de Vries, 1998).

1.11.1. Cellular sources of IL-13

IL-13 is produced by both CD4+ and CD8+ activated T cells in response to antigen-specific or polyclonal stimuli (Jung et al., 1996), B cells after CD40 cross-linking and stimulation with PMA (Chomarat and Banchereau, 1998), mast cells (Burd et al., 1995), basophils (Li et al., 1996) activated by FcεRI and FcγRII or after IL-3 treatment, and dentridic cells isolated from tonsillar germinal centers (de Saint-Vis et al., 1998). In contrast to IL-4, IL-13 is not produced by eosinophils or neutrophils even after IL-5 or anti- FcεRI stimulation (Li et al., 1996). IL-4, which shares many biological activities with IL-13, is produced mainly by Th0 and Th2-like CD4+ T cells (Mosmann and Coffman, 1989), mast cells (Brown et al., 1987), basophils (Brunner et al., 1993) and eosinophils (Moqbel et al., 1995). IL-13 production by T

cells is induced by a wide range of polyclonal activators including Con A, calcium ionophore, PHA, PMA, and anti-CD3. In contrast, IL-4 production by T cell clones is under more stringent control than that of IL-13 gene, since it is induced by calcium ionophore, but not by PMA, PHA, Con A, or anti-CD3 (de Waal Malefyt et al., 1995). IL-13 is produced rapidly following activation of peripheral blood T cells and T cell clones and the production is sustained over long periods of time, whereas IL-4 production is transient after stimulation (de Waal Malefyt et al., 1995). Thus, significant levels of IL-13 mRNA in T cell clones are induced as early as 1 h following activation, whereas IL-4 protein production can be detected after 2 h. IL-13 mRNA levels peak approximately 2 h after activation, but considerable steady state levels of IL-13 mRNA are still observed after 72 h. In contrast, IL-4 mRNA has different kinetics of production with later and transient induction, such that minimal or undetectable levels of IL-4 mRNA expression can be detected in most T cell clones 24 h after activation (de Waal Malefyt et al., 1995). Thus, IL-13 and IL-4 expression is differentially regulated with the IL-4 gene being under more precise control than the IL-13 gene.

1.11.2 Biological activities of IL-13 and comparison with IL-4

IL-13 has profound effects on various cells including monocytes, B cells, fibroblasts and epithelial cells. IL-13 and IL-4 have similar effects since both cytokines share common receptor components (Zurawski et al., 1993). Both IL-13 and IL-4 have dramatic effects on monocyte function. They inhibit production of pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-8, TNF-α, IL-10, and GM-CSF (Zurawski and de Vries, 1994). In addition, IL-13 and IL-4 inhibit the expression of FcγRI, CD14, the receptor for LPS on monocytes, and suppress the production of toxic nitrogen oxide metabolites. These actions result in an inhibition of antibody-dependent cytotoxicity of monocytes (de Waal Malefyt et al., 1993a). IL-13 and IL-4 also exhibit stimulatory activities on monocytes by inducing the expression of class II major histocompatibility complex (MHC) on monocytes, which may be related to an increased capacity to present antigen (de Waal Malefyt et al., 1993b). In addition, IL-4 and IL-13 induce expression of the low affinity receptor for IgE (CD23) and enhance the expression of several members of the integrin superfamily, including CD11b, CD11c, CD18, and CD29 (Zurawski and de Vries, 1994). These β1 and β2

integrins play an important role in cell-cell interactions and interactions with the cell matrix. IL-13 and IL-4 enhance the expression of CD23 and upregulate the expression of the HLA-DR, DP, and DQ class II MHC antigens on B cells (Defrance et al., 1994).

IL-13 induces B cells to switch to production of IgE and IgG4 (McKenzie et al., 1993a). Previously, IL-4 was the only cytokine known to induce germline immunoglobulin epsilon heavy chain C region mRNA synthesis. IL-13-induction of IgE synthesis is IL-4 independent, since it is not inhibited by neutralising anti-IL-4 antibodies (Defrance et al., 1994, Punnonen et al., 1993). In addition to IL-4 and IL-13, costimulatory signals by activated CD4+ T cells are required for IgE switching (Janeway et al., 1999). Interactions take place between CD40L transiently expressed on activated CD4+ T cells, and CD40 constitutively expressed on B cells. Unlike IL-4, IL-13 does not act on T cells, which even after activation, fail to bind IL-13 and do not express functional IL-13 receptors, although mRNA for the IL-13Rα1 has been detected in activated T cells (Graber et al., 1998). Even though IL-13 does not have direct effects on T cells, it may affect T cell function and Th1 cell differentiation indirectly through its downregulatory effects on production of proinflammatory cytokines, particularly IL-12 (Wills-Karp, 2001).

1.12. Interleukin-13 receptor subunits

To elicit biological responses, both IL-13 and IL-4 must bind to their specific cell surface receptors. Competitive binding studies on human erythroleukemia (TF-1) cells, known to respond to both IL-13 and IL-4, have demonstrated that IL-13 can compete for the binding of IL-4 to its receptors and vice versa (Zurawski et al., 1993). Moreover, antibodies to IL-4Rα could block IL-13 action even though IL-13 could not bind to IL-4Rα. These findings suggested that IL-4 and IL-13 receptor complexes share common components, which were later shown to be the IL-4Rα and the IL-13Rα1 subunits (Miloux et al., 1997). The functional IL-13 receptor complex is a heterodimer composed of the IL-4Rα chain and a 65- to 70-kDa protein termed IL-13Rα1 (Murata et al., 1999). This receptor complex is also utilised as an alternative receptor for IL-4, termed type II IL-4 receptor (Miloux et al., 1997). There is an additional IL-13 binding protein referred to as IL-13Rα2. IL-13Rα2 binds IL-13, but not IL-4, with very high affinity but does not seem to be important for IL-13 signalling. It is currently thought to serve as a decoy receptor (Feng et al., 1998). All three receptor components, IL-13Rα1, IL-13Rα2, and IL-4Rα are members of the hematopoietin receptor superfamily (type I cytokine receptor family) (Daines and Hershey, 2002). Type I cytokine receptor family is defined by several features, including four conserved cysteines in the amino-terminal half of the extracellular domain, an W-S-X-W-S (W: tryptophane, S: serine, X: non-conserved amino acid) motif located in the C-terminal of the extra cellular domain, and proline-rich box regions in the cytoplasmic domain, termed 'box1 motifs', that are important for binding of Janus tyrosine kinases (JAK) (Miloux et al., 1997, Leonard and Lin, 2000). Type I cytokine receptors tend to form heterodimers and have no intrisic kinase activity, but rather, they have constitutively associated JAKs, which result in recruitment of downstream signalling molecules.

1.12.1. Interleukin-13 receptor alpha 1 chain (IL-13Rα1)

IL-13Rα1 cDNA was isolated by searching databases of Expressed Sequence Tags (ESTs). A human mRNA tag was found to be similar to previously characterised murine IL-13Rα1 mRNA and was consequently cloned (Aman et al., 1996). The open reading frame encodes for a 65-kDa protein of 427 amino acids with a 26-amino-acid

signal peptide, a 24-amino-acid transmembrane domain, and a 60 amino acid cytoplasmic domain. IL-13Rα1 exhibits two principal transcripts of 4 and 2 kb in length, presumably reflecting alternative polyadenylation (Aman et al., 1996, Ise et al., 1999). According to data from the human genome map deposited in GeneBank, the human IL-13Rα1 gene maps to chromosome Xq24-23.

Expression of IL-13Rα1

Human IL-13Rα1 mRNA is expressed in multiple tissues (Gauchat et al., 1997). In lymphoid organs, IL-13Rα1 expression was highest in lymph nodes, peripheral blood leukocytes, fetal liver, appendix, and spleen, and lowest in bone marrow and thymus. IL-13Rα1 mRNA levels in nonlymphoid organs were highest in liver, intermediate in skeletal muscle, heart, placenta, lung, and pancreas, and almost undetectable in kidney and brain (Gauchat et al., 1997). Among human cells, IL-13Rα1 is expressed on basophils, B cells eosinophils, mast cells, endothelial cells, fibroblasts, smooth muscle cells, and airway epithelial cells (Wills-Karp, 2001). Human T cells isolated from PBMCs or tonsils did not express IL-13Rα1 on the cell surface by means of flow cytometry even following activation with Con A or several cytokines, which is consistent with the unresponsiveness of T cell to IL-13 (Zurawski et al., 1993). However, IL-13Rα1 mRNA was detected in peripheral blood T cells and IL-13Rα1 protein was detected intracellularly in permeabilized T cells by flow cytometry, with intracellular expression being enhanced in response to IL-13 (Graber et al., 1998). IL-13Rα1 mRNA is also expressed in many human cell lines including the monocytic cell line THP-1, the lymphoblastic cell line the IM-9, the mast cell HMC-1, and the eosinophilic cell line Eol-3 (Gauchat et al., 1997). On human B cells, surface expression of IL-13Rα1 was induced following activation through CD40 ligand, as well as with co-stimulation with IL-4 or IL-13 (Graber et al., 1998). Similarly, IL-13Rα1 mRNA expression on human B cells was upregulated following co-stimulation with anti-human immunoglobulin IgM and anti-CD40 antibodies (Ogata et al., 1998). On eosinophils, surface expression of IL-13Rα1 was downregulated by IL-13 and IL-4, and enhanced in response to IFN-γ, TNF-α, and, to the largest extent, TGF-β, whereas IL-13 and IL-4 downregulated IL-13Rα1 surface expression (Myrtek et al., 2004).

1.12.2. Interleukin-13 receptor alpha 2 chain (IL-13Rα2)

The cDNA for human IL-13Rα2 was cloned in 1996, following screening of cDNA libraries derived from renal cell carcinoma (Caki-1) cells, known to respond to IL-13 (Caput et al., 1996). The cDNA sequence encodes for an approximately 70-kDa membrane protein of 380 amino acids with a 26-amino-acid signal peptide, a 20 amino acid transmebrane domain, and a short 17-amino-acid cytoplasmic domain. Alignment studies revealed that IL-13Rα2 shows 27% amino acid homology with the IL-5Rα chain (51% similarity and 27% identity) and to a lesser extend to the prolactin receptor (Caput et al., 1996). The IL-13Rα2 gene maps to chromosome Xq24 (Guo et al., 1997).

The genomic structure of the *IL13RA2* gene

Two independent groups have recently described the genomic organisation of the human IL-13Rα2 locus. The genomic structure of the *IL13RA2* gene in the human keratinocyte cell line HaCaT consists of four 5' noncoding exons, nine coding exons, and twelve introns spanning approximately 38 kb (David et al., 2003). Using 5' RACE experiments on RNA isolated from the human keratinocyte cell line HaCaT, multiple transcription start sites were identified located between +1 to +43 in exon 1, as well as transcripts resulting from alternative usage of the first four noncoding exons. The longer transcript contained all four noncoding exons, whereas shorter transcripts lacked exon 2, or both exon 2 and exon 3, respectively. A 1.5 kb region upstream of the first exon displayed basal promoter activity and was found to have a proximal and a distal STAT6 binding sites (David et al., 2003). The genomic structure of IL-13Rα2 in the glioma cell line U118 consists of two 5' noncoding exons, nine coding exons, and ten introns spanning at least 10 kb (Wu and Low, 2003). A single transcriptional initiation site for IL-13Rα2 was found to be located 270 bp upstream of the translation initiation codon, whereas a 281 bp 5' flanking sequence containing three TATA boxes and one CCAAT site demonstrated basic promoter activity (Wu and Low, 2003).

IL-13Rα2 expression

IL-13Rα2 has a much more restricted pattern of expression when compared to that of IL-13Rα1. Guo and co-workers (Guo et al., 1997) investigated the expression of IL-13Rα2 in human tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas using Northern blot analysis. Of those eight tissues, low levels of a single 1.6 kb transcript were detected only in placenta. In another study, very low levels of IL-13Rα2 mRNA expression was found in liver, lung, bone marrow, thymus, lymph node, and spleen using Northern blot analysis and prolonged, seven-day, autoradiography exposure (Gauchat et al., 1997). Among various cell lines, IL-13Rα2 transcript was detected in the myeloma cell line U266, the human choriocarcinoma cell line JAR, the human renal carcinoma cell line Caki-1, the keratinocytic cell lines A431 and HaCaT, the promyelomonocytic cell line U937, the B-cell line IM9, and the immature mast cell line HMC-1 (Guo et al., 1997, Caput et al., 1996, Gauchat et al., 1997). High levels of IL-13Rα2 expression have been reported in glioblastomas, Kaposi's sarcomas and cells derived from renal, colon, prostate and ovarian carcinomas (David et al., 2003). Among primary human cells, IL-13Rα2 was found to be expressed in fibroblasts, airway epithelila cells, and airway smooth muscle cells (Wills-Karp, 2004).

Daines and Hershey first reported that IL-13Rα2 is predominantly an intracellular molecule with large intracellular pools and very low surface expression in human cultured monocytes, primary nasal epithelial cells, U937 cells and A549 cells (Daines and Hershey, 2002). In the same study, treatment of primary monocytes and U937 cells with IFN-γ or PMA resulted in a rapid mobilisation of IL-13Rα2 intracellular stores to the cell surface. Subsequent studies showed the presence of intracellular IL-13Rα2 protein in human fibroblasts (Yoshikawa et al., 2003) and human bronchial epithelial cells (Yasunaga et al., 2003). In the keratinocyte cell line HaCaT, IL-4 and IL-13 enhanced IL-13Rα2 mRNA and protein expression, and TNF-α potentiated IL-4/IL-13-induced IL-13Rα2 expression, resulting in further induction of IL-13Rα2 surface expression (David et al., 2002). In human fibroblasts, IL-13Rα2 surface expression was enhanced in response to TNF-α or IL-4 through upregulation of gene expression and mobilisation of intracellular receptors (Yoshikawa et al., 2003). In

human bronchial epithelial cell cultures, IL-4 and IL-13 were found to induce IL-13Rα2 mRNA levels (Yuyama et al., 2002). Similarly, Zheng and co-workers showed that in primary bronchial epithelial cells, IL-13Rα2 mRNA was upregulated following stimulation with IL-4, IL-13, or IFN-γ (Zheng et al., 2003). In another study, although IL-4 and IL-13 induced intracellular expression of IL-13Rα2 protein in human bronchial epithelial cells, they did not enhanced surface IL-13Rα2 expression (Yasunaga et al., 2003).

IL-13Rα2 as a decoy receptor

Despite the high affinity of IL-13 binding with IL-13Rα2 (Kd=50 pM), IL-13Rα2 lacks signalling activity (Feng et al., 1998), since its short cytoplasmic domain of 17 amino acids does not conserve the box-1 region, critical for downstream transduction (Ihle et al., 1995). IL-13Rα2 is currently thought to serve as a dominant negative inhibitor or a decoy receptor for IL-13 (Feng et al., 1998). This hypothesis has been verified by Kawakami and co-workers who found that transfection of Chinese hamster ovary cells (CHO-K1) with IL-13Rα1 and IL-4Rα induced STAT6 activation, whereas co-transfection with the IL-13Rα2 chain abrogated STAT6 phosphorylation (Kawakami et al., 2001). Similarly, overexpression of IL-13Rα2 in renal carcinoma cell lines reduced their ability to respond to IL-13 (Bernard et al., 2001), whereas the pro-B cell line Ba/F3 expressing IL-13Rα2 was unable to proliferate in response to IL-13 (Donaldson et al., 1998). IL-13Rα2 can also inhibit IL-13 activities through other mechanisms. Thus, in the glioblastoma cell line 293T transient expression of IL-13Rα2 inhibited IL-13- and IL-4-mediated STAT6 activation, possibly through the physical interaction of the IL-13Rα2 protein and the IL-4Rα chain (Rahaman et al., 2002). In vivo studies have also highlighted the role of IL-13Rα2 in the inhibition of IL-13-mediated responses. Thus, IL-13Rα2-deficient mice displayed enhanced macrophage development and IgE production (Wood et al., 2003), and Schistosoma mansoni infected IL-13Rα2-deficient mice showed a marked exacerbation in hepatic fibrosis compared to wild-type mice (Chiaramonte et al., 2003).

1.13. The IL-13 receptor complex

The functional redundancy of IL-13 and IL-4 suggested that both cytokines shared receptor components (Zurawski et al., 1993). The first evidence for the existence of a common receptor component for IL-4 and IL-13 was provided by experiments showing that a non-functional IL-4 mutant (Y124D) could block both IL-4 and IL-13 responses (Aversa et al., 1993). Further experiments using antibodies to IL-4Rα also demonstrated inhibition of the activities of both IL-4 and IL-13 (Zurawski et al., 1995, Lin et al., 1995). Subsequent experiments in various cell lines revealed that the human IL-13 receptor (IL-13R) is a heterodimer composed of IL-13Rα1 and IL-4Rα (Miloux et al., 1997) (Fig. 1.1). The IL-13Rα1/IL-4Rα complex can act as an alternative IL-4 receptor, especially in nonhematopoietic cells that lack the common gamma chain (IL-2Rγ) that usually forms a heterodimer with IL-4Rα to bind IL-4 (Andrews et al., 2002). The IL-4Rα cDNA encodes for a 140-kDa protein of 825 amino acids with a 25-amino-acid signal peptide, a 207-amino-acid extracellular domain and a long 569-amino-acid intracellular tail (Galizzi et al., 1990). Kinetic analyses have shown that IL-13Rα1 binds IL-13 with low affinity (Kd=2-10 nM), whereas coexpression of IL-13Rα1 and IL-4Rα yields a high-affinity complex (Kd≈400 pM) (Aman et al., 1996). A sequential mechanism for the binding of IL-13 has been suggested in which IL-13 first binds to IL-13Rα1 and this then recruits IL-4Rα to stabilise the interaction and give a high affinity complex (Andrews et al., 2002).

1.13.3. Signalling through the IL-4/IL-13 receptor complex

Signalling of IL-13 and IL-4 through the IL-4Rα/IL-13Rα1 complex is thought to occur through IL-4Rα (Zurawski et al., 1995), since binding of either IL-13 or IL-4 to IL-4Rα/IL-13Rα1 complex results in activation of signalling intermediates characteristic of IL-4 responses, including phosphorylation of JAK1, IL-4Rα, and insulin receptor substrate 2 (IRS-2) (Wills-Karp, 2001) (Fig 1.2). IL-4Rα contains five conserved tyrosine residues in the cytoplasmic domain, Y497, Y575, Y603, Y631, and Y713, which are all important in signalling through the receptor (Nelms et al., 1999). Y497 is part of the IL-4R motif that is necessary for the recruitment of

IRS-2 to IL-4Rα and is critical for IL-4-dependent proliferation (Nelms et al., 1999). The tyrosines Y575, Y603, and Y631 can act as STAT6 docking sites (Ryan et al., 1996), whereas Y713 is part of an immunotyrosine-based inhibitory motif (ITIM) that is important in the negative regulation of IL-4 and IL-13 responses (Kashiwada et al., 2001). In addition to these conserved domains, IL-4Rα bears a region termed insulin-IL-4 receptor (I4R) motif in amino acids 437-557 of the cytoplasmic domain, which has been shown to be essential for cellular proliferation (Harada et al., 1992).

Neither IL-4Rα nor IL-13Rα1 has endogenous kinase activity; rather the IL-4Rα requires receptor-associated kinases for the initiation of signal transduction. The Janus-family tyrosine kinase (JAK) has been shown to be critical in the initiation of signaling by type I cytokine receptors (Taniguchi, 1995). There are four JAKs: JAK1, JAK2, JAK3, and Tyk2. JAK1, JAK2, and Tyk2 are ubiquitously expressed, whereas JAK3 expression is limited in the hematopoietic cells (Hershey, 2003). Thus, in hematopoietic cells IL-4 binding to IL-4Rα/IL-2Rγ complex results in activation of JAK1 and JAK3, since IL-2Rγ is associated with JAK3 kinase. In contrast, IL-13 or IL-4 binding to IL-4Rα/IL-13Rα1 complex results in activation of JAK1 and Tyk2 in both hematopoietic and nonhematopoietic cells. Activation of JAK1 and Tyk2 results in the tyrosine phosphorylation of the IL-4Rα itself, which is followed by phosphorylation of the cytoplasmic signalling molecules, STAT6 and IRS-1/2 (Takeda et al., 1997).

STAT6 is a member of the STAT family. To date six different members, STAT1-STAT6, have been identified (Takeda et al., 1997). Activation of STATs is ligand specific and is controlled by the interaction of a STAT highly conserved Src homology 2 (SH2) domain with the STAT docking site in the receptor. Interaction of the STAT6 molecule with the IL-4Rα leads to STAT6 phosphorylation, followed by disengagement from the receptor and formation of homodimers through interaction of the STAT6 SH2 domain with the C-terminal phosphotyrosine residue of a second activated STAT6 molecule (Mikita et al., 1996). The dimerized STAT6 complexes translocate to the nucleus where they bind to specific motifs in the promoter of responsive genes including those for MHC class II, CD23, unrearranged (germline) immunoglobulin heavy chain C region ε and γ1 genes, and IL-4Rα chain (Nelms et

al., 1999). IL-4Rα activation also leads to phosphorylation and activation of IRS-1 and IRS-2 (Jiang et al., 2000). IRS-1 is the primary substrate phosphorylated in response to treatment of nonhematopoietic cells with insulin-like growth factor I (IGF-1). IRS-1 and IRS-2 are closely related molecules, important for IL-4 and IL-13-dependent cellular proliferation. The I4R motif between amino acids 437 and 557 of the IL-4Rα is critical for the phosphorylation of IRS-1/2 as shown by mutational analysis (Wang et al., 1998). Two distinct pathways are implicated in signalling downstream of IRS-1 and IRS-2: PI-3-K (phosphoinositide-3-kinase) and Ras/MAPK (Mitogen-Activated Protein Kinase) pathway (Nelms et al., 1999). Activation of PI-3-K leads to phosphorylation of membrane anchored phosphotidylinositols, which subsequently activate a number of downstream kinases including different forms of kinace C (δ, ε, η isoenzymes, and the act kinase C, also known as protein kinase B) that play a key role in cell survival and apoptosis (Nelms et al., 1999). Activation of the Ras/MAPK pathway, initiated by the interaction of IRS-1/2 with the SH2 domain of the adapter Grb2, ultimately results in the phosphorylation and activation of the mitogen activated protein kinases ERK-1 and ERK-2. Activated ERK-1/2 translocates to the nucleus and activates the expression of the transcription factors c-fos, and c-Jun. c-fos and c-Jun form AP-1 transcription factors which regulate the expression of many genes involved in cell growth (Nelms et al., 1999).

A number of negative regulators are also involved in the regulation of signalling through the IL-13Rα1/IL-4Rα complex, including SH-2 containing phosphatases and SOCS (Suppressors of Cytokine Signalling) (Jiang et al., 2000). The SH2-containing phosphatases SHP1 and SHP2, as well as SHIP (SH2-containing inositol-5-phosphatase) have been recognised to be critical modulators of cytokine signalling (Yasukawa et al., 2000). The ITIM motif in the C-terminus of IL-4Rα is thought to serve as a docking site for the SH-2 domains of SHP1/2 and SHIP. The SH-2 domains of SHP1/2 are thought to be critical for linking these phosphatases to phosphorylated receptors and proteins, leading to their dephosphorylation. SHP1 was shown to modulate the activity of JAK1, whereas SHP2 has been shown to constitutively associate with JAK1 and JAK3 and to co-precipitate with IRS-1, Grb2, and the p85 subunit of PI-3-kinase after cytokine stimulation (Yasukawa et al., 2000). In contrast, SHIP is thought to regulate the PI-3-K pathway by dephosphorylating the products of

this enzyme. A second regulatory pathway playing a significant role in the modulation of the JAK-STAT pathway involves a series of closely related SH2-domain proteins whose expression is enhanced in response to cytokine-induced STAT activation. These molecules, termed SOCS (for Suppressors of Cytokine Signalling) are also known as CIS (for Cytokine-Induced SH2), JAB (for JAK binding), and SSI-1 (for STAT-induced STAT-inhibitor) (Jiang et al., 2000). To date, eight members of this family have been cloned, SOCS-1 through 7 and CIS (Hershey, 2003). Studies examining the effect of SOCS-1, SOCS-2, and SOCS-3 expression on IL-4 signalling have shown that over-expression of SOCS-1 resulted in inhibition of IL-4-induced phosphorylation of JAK1 and STAT6 (Hershey, 2003), and downregulation of the IRS-1/2 pathway (Nelms et al., 1999).

Although signalling through the IL-4Rα/IL-13Rα1 complex is thought to occur through the IL-4Rα chain, there is evidence to suggest that IL-13Rα1 may also play a role, since a truncated murine IL-13Rα1 was not able to mediate IL-13-induced responses (Orchansky et al., 1999). In addition, the Tyk2 tyrosine kinase can bind to the box-1 region of IL-13Rα1, and two tyrosine residues in its cytoplasmic domain, Y402 and Y405, were shown to be docking sites for STAT3 *in vitro* and may serve as docking sites for additional signalling molecules (Umeshita-Suyama et al., 2000). However, it is not yet clear whether activation of STAT3 through IL-13 or IL-4 can exert cellular responses, since phosphorylated STAT3 was not found to translocate in the nucleus in one study (Wery-Zennaro et al., 1999). Interestingly, the 60-amino-acid long intracellular domain of human IL-13Rα1 is highly conserved between humans and mice, differing by only 3 amino acids, suggesting a critical role of the intracellular domain of IL-13Rα1 for effecting coupling with the IL-4Rα chain (Hershey, 2003).

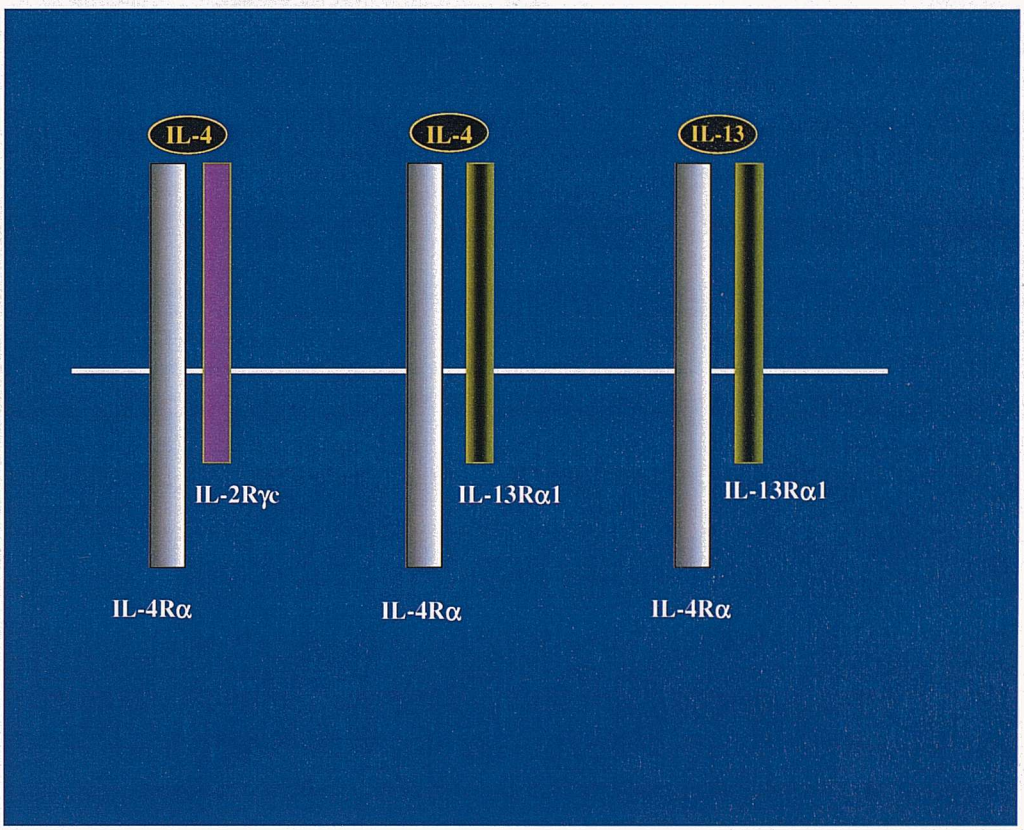

Figure 1.1. Schematic model of the IL-4 and IL-13 receptor complexes. IL-4 binds the complex of IL-4Rα/IL-2Rγc and IL-4Rα/IL-13Rα1. IL-13 binds only the complex of IL-4Rα/IL-13Rα1. (Redrawn from Takeda K et al: STAT6: its role in interleukin 4-mediated biological functions. *J Mol Med* 1997;75:317-26.)

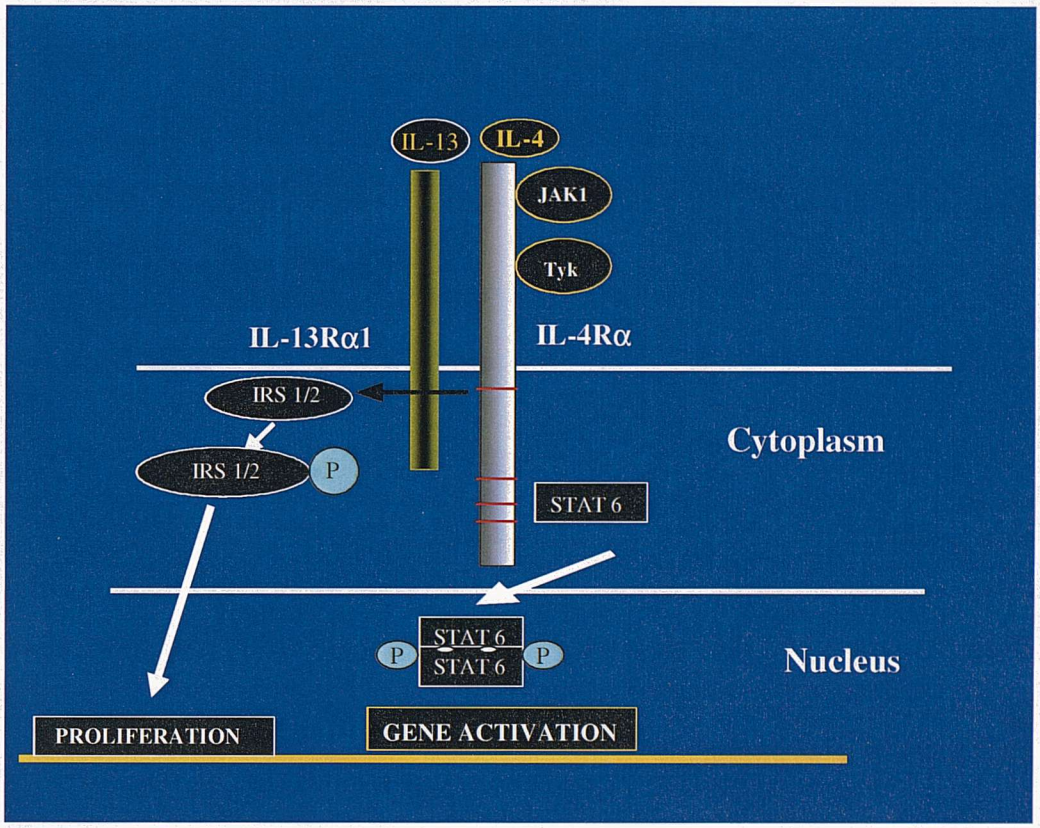

Figure 1.2. Signal transduction through the IL-4Rα/IL-13Rα1 complex. The binding of IL-13 or IL-4 to the IL-4Rα/IL-13Rα1 complex leads to the activation of two cytoplasmic molecules, STAT6 and IRS-1/2. IRS-1/2 is tyrosine phosphorylated by interaction with the I4R motif in the IL-4Rα, triggering other signalling molecules, leading to the activation of genes responsible for cell proliferation. STAT6 tyrosine phosphorylation by JAK1 and Tyk2 results in the formation of a heterodimer, which translocates to the nucleus where it binds to the promoters of IL-4-/IL-13-responsive genes, such as CD23, Ig εpsilon germline transcripts, IL-4Rα, and MHC class II. (Modified from Takeda K et al: STAT6: its role in interleukin 4-mediated biological functions. *J Mol Med* 1997;75:317-26.)

1.14. Clinical aspects of IL-13 receptors in asthma and allergy

The implication of IL-13Rα1 and IL-13Rα2 in asthma and allergy is strongly supported by the known biologic effects of their ligands, IL-4 and IL-13. IL-4 and IL-13 can participate in the pathogenesis of asthma through several mechanisms. Both cytokines promote IgE synthesis by B cells, as well as the accumulation of eosinophils to the airways through induction of vascular cell adhesion molecule ICAM–1 on endothelial cells (Schleimer et al., 1992, Bochner et al., 1995). In addition, IL-13 was shown to induce eotaxin, a potent chemoattractant for eosinophils to the airways (Li et al., 1999), and to augment cholinergically induced contractions of tracheal smooth muscle *in vitro* (Chen, 1999), suggesting the IL-13 can induce BHR by inducing airway eosinophilia and by direct effects on smooth muscle cells. IL-4 and IL-13 are also implicated in remodelling changes through release of TGF-β2, a potent fibrogenic, from bronchial epithelial cells, which may promote myofibroblast transformation of subepithelial mesenchymal cells, a feature associated with thickening of lamina reticularis in asthma (Richter et al., 2001). Inappropriate Th2 responses are critical for the development of asthma and other allergic disorders (Cohn et al., 1999). Although several *in vitro* studies have established that IL-4 is the driving cytokine for Th2 differentiation (Brombacher, 2000), there is evidence to suggest that IL-13 is also an important regulator of Th2 commitment probably through downregulation of IL-12 production by monocytes (McKenzie et al., 1998). Thus, deletion of the IL-13 gene in mice resulted in attenuated development of certain T cell lineages with a consequent reduction of Th2 cell cytokines (McKenzie et al., 1998, Barner et al., 1998).

Further evidence for the role of IL-4 and IL-13 as effector molecules in asthma has been provided by mouse models of experimental asthma using ovalbumin as an allergen. IL-4 plays a critical role in experimental asthma by augmenting Th2 cell responses (Le Gros et al., 1990). However, although antibody-mediated blockade of IL-4 during allergen sensitisation ablates the development of allergic asthma, similar blockade of IL-4 in later stages, that is before or during antigen challenge, inhibits neither allergic inflammation nor BHR (Coyle et al., 1995, Corry et al., 1996).

Moreover, transfer of Th2 cells derived from IL-4 deficient mice is still able to confer BHR. In contrast, blockade of IL-4Rα before antigen provocation in sensitised mice inhibits BHR, eosinophilic inflammation, and mucus hyperplasia (Gavett et al., 1997). Furthermore, STAT6 knockout mice abolish antigen-induced eosinophilic airway inflammation and BHR (Kuperman et al., 1998). These data suggested that IL-13, which also signals through IL-4Rα and activates STAT6, may play a critical role in the development of asthma phenotypes in mouse models of experimental asthma. This hypothesis has been confirmed by two independent studies, which demonstrated that administration of IL-13 induces mucus production, eosinophilic airway inflammation, and BHR in mouse models. In these mouse models, blockade of endogenous IL-13 by the administration of a soluble form of the IL-13Rα2 chain reversed BHR and pulmonary mucous cell metaplasia (Grunig et al., 1998, Wills-Karp et al., 1998). In a transgenic mouse model, targeted pulmonary expression of IL-13 induced many manifestations of the asthma phenotype, such as eosinophilic airway inflammation, mucus cell metaplasia, subepithelial fibrosis, and BHR (Zhu et al., 1999).

Additional evidence for the key role of IL-4 and IL-13 in asthma and atopy was provided by expression studies in diseased tissue demonstrating increased IL-13 and IL-4 production in the lungs of asthmatic and atopic subjects. The expression of IL-4 and IL-13 mRNA in the BAL fluid of asthmatic and rhinitic patients following allergen-challenge with ragweed allergen was increased compared to normal subjects (Huang et al., 1995). In the same study, the cellular source of IL-13 mRNA was identified as being in the mononuclear cell fraction of the allergen-challenged BAL, and an increase in the amounts of IL-5, but not IFN-γ transcript, was also observed suggesting a concomitant up-regulation of Th2 cytokines. Increased number of positive cells per millimeter of basement membrane for both IL-13 and IL-4 mRNA was found in endobronchial biopsies of mild atopic asthmatics compared to normal controls (Kotsimbos et al., 1996). Interestingly, the expression of IL-13 was significantly greater than IL-4, suggesting that in mild atopic asthma IL-13 expression is greater than that of IL-4. After local endobronchial allergen challenge in atopic asthmatic subjects, BAL T cells strongly expressed Th2 cytokines, including IL-4, IL-13, and IL-5 providing strong evidence for the key role of IL-4 and IL-13 in atopic asthma (Bodey et al., 1999). Increased expression of IL-13 mRNA has also been

shown in the bronchial mucosa of patients with asthma regardless of their atopic status, as compared with that in control subjects without asthma (Humbert et al., 1997), suggesting that IL-13 plays a role in the pathogenesis of both atopic and nonatopic asthma. The number of IL-13 positive cells in bronchial biopsies of asthmatic subjects was found to be increased compared to normal control subjects, with a significant decrease in cells expressing IL-13 mRNA after steroid treatment in asthmatic subjects (Naseer et al., 1997). In another study, repeated low-dose allergen provocations of asthmatic patients resulted in a significant increase in mRNA expression of IL-13 mRNA in BAL cells enriched for alveolar macrophages compared with normal subjects (Prieto et al., 2000). IL-13 was also shown to play a significant role in the late asthmatic response, with IL-13 actively secreted during the late asthmatic response in the BAL of mild asthmatic subjects (Kroegel et al., 1996). In the same study, the IL-13 concentration strongly correlated with the eosinophil numbers 18 h post-allergen challenge. The significant role of IL-13 and IL-4 in atopic dermatitis was highlighted in another study, demonstarting that cutaneous allergen challenge in atopic subjects significantly increases the number of cells that were mRNA and protein-positive for both IL-13 and IL-4 (Ying et al., 1997). In the same study, double staining revealed that the majority (>60%) of IL-13 mRNA signals were colocalised to CD3+ T cells. Moreover, the number of mRNA- and protein-positive endothelial cells for IL-13 significantly correlated with VCAM-1 immunoreactivity, suggesting that IL-13 may play an important role in recruitment of inflammatory cells through VCAM-dependent mechanisms (Ying et al., 1997).

1.15. Objectives of this thesis

There is increasing evidence to suggest that IL-13 plays a crucial role in the pathogenesis of asthma by inducing IgE production and by regulating the functions of hematopoietic and airway cells, including smooth muscle cells, fibroblasts and epithelial cells (Hershey, 2003). The *IL13* Arg110Gln variant was found to be associated with asthma (Heinzmann et al., 2000) and raised serum total IgE levels (Graves et al., 2000), whereas the *IL13* -1111A>C polymorphism was found to be associated with asthma, BHR, and skin-test responsiveness (van der pouw-Kraan et al., 1999, Howard et al., 20001), suggesting that *IL13* is an asthma/allergy-susceptibility gene. IL-13 has two cognate receptors, IL-13Rα1 and IL-13Rα2, which are members of the hematopoietin receptor superfamily and share 37% homology at the amino acid level. To elicit biologic effects IL-13 is interacting with a complex receptor system comprised of IL-13Rα1 and IL-4Rα (Murata et al., 1998). IL-13Rα2 binds IL-13 with high affinity, however, it has no signalling function and is thought to act as a decoy receptor playing a major role in the tight regulation of IL-13 responses (Bernard et al., 2001). Despite the plethora of evidence to suggest a crucial role of IL-13 for asthma and allergy, the role of its cognate receptors, IL-13Rα1 and IL-13Rα2, is poorly understood. The primary objectives of this thesis were to examine the influence of polymorphisms in *IL13RA1* and *IL13RA2* on the genetic susceptibility to asthma, to determine the expression of IL-13 receptor components in structural airway cells and to investigate the cellular localisation and regulation of expression of IL-13Rα2 in bronchial epithelial cells and bronchial fibroblasts.

We initially performed mutation screening to investigate the presence of common variants in the coding regions of *IL13RA1* and *IL13RA2*, as well as in the *IL13RA1* promoter. Three approaches were subsequently employed to examine the role of polymorphisms found in the development of asthma and asthma related phenotypes. The first approach used the transmission disequilibrium test (TDT), as a family-based approach to limit potential population substructure, to evaluate evidence for association of the –281T>G and the 1365A>G polymorphisms in *IL13RA1* with asthma and asthma-related traits (Cardon and Palmer, 2003). The second approach used a case-control study

to explore associations of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms with asthma in different study groups in our cohort. The third approach used conventional association studies to investigate correlations between the *IL13RA1* polymorphisms and asthma-related phenotypes.

IL-13 was shown to induce BHR in B- and T-cell-deficient mice (Grunig et al., 1998), and to cause airway fibrosis and mucus metaplasia in transgenic mice with pulmonary-targeted expression of IL-13 (Zhu et al., 1999), suggesting that IL-13 acts directly on bronchial tissue to induce asthmatic phenotypes. Although several studies have examined the expression of IL-4Rα in bronchial tissue and airway cells, the expression of IL-13Rα1 and IL-13Rα2 in the airways has not thoroughly been investigated. We initially evaluated the mRNA expression of IL-13Rα1, IL-13Rα2, and IL-4Rα in hematopoietic cells, primary bronchial epithelial cells, primary airway fibroblasts, and various bronchial epithelial cell lines. Next, we investigated the protein expression of IL-13Rα1, IL-13Rα2, and IL-4Rα by immunostaining of bronchial biopsies, primary bronchial epithelial cells and primary bronchial fibroblasts.

IL-13Rα2 was previously shown to be an intracellular molecule in primary monocytes, primary nasal epithelial cells, airway fibroblasts, and airway epithelial cells. However, its exact cellular localization in airway epithelial cells and airway fibroblasts has not been described. To address this issue, we determined the cellular localization of IL-13Rα2 in primary bronchial epithelial cells and primary bronchial fibroblasts using confocal laser scanning microscopy. Given that IL-13Rα2 binds IL-13 with high affinity, the level of IL-13Rα2 surface expression on airway cells is an important determinant of IL-13 signalling activity in the airways. Previous studies have shown that IL-13Rα2 surface expression is regulated by different cytokines depending on the cell type considered. In the present study, we have analysed the regulation of IL-13Rα2 surface expression on airway epithelial NCI-H292 cells, primary bronchial fibroblasts, and primary bronchial epithelial cells.

CHAPTER 2

Materials and Methods

2.1. Chemicals

Stock solutions of chemicals were stored at room temperature unless otherwise stated. Most solutions were made in deionised water obtained from a RiOs Reverse Osmosis Systems/Milli-Q Ultrapure Water Purification Sytems apparatuses (Millipore, Watford, UK). All chemicals required for general laboratory use were of analytical or molecular biology grade. Chemicals required for specific purposes and their abbreviations are listed below:

Bromophenol Blue, HCL, NaOH Ethidium bromide Bovine serum albumin, L-glutamine TEMED, Trypan blue Osmium tetroxide 4% solution Chloroform, Isopropanol DEPC (Diethyl Pyrocarbonate) Pyridine, Hydroxylamine Hydrochloride Saponin, 2-mercaptoethanol Glycerol, EDTA DMSO, APS, TEMED	Sigma-Aldrich, Poole, UK
dNTPs 100 mM Streptavidin Magnesphere Paramagnetic Particles	Promega, Southampton, UK
15 ml conical tubes 1.5 ml microcentrifuge tube	Alpha Lab, Eastleigh, UK
Potassium bicarbonate, Ammonium chloride Diethylamine, Piperidine solution Ammonium Chloride $MgCl_2$	Fisher Scientific, Loughborough, UK
Urea (Ultrapure Grade) TBE Buffer 10x liquid Tris (Biotechnology Grade)	Amresco, St. Louis, MO, USA

Trizol	Life Technologies, Paisley, UK
Propidium Iodide Nucleic Acid Stain SYTOX Orange Nucleic Acid Stain Alexa Fluor 633 Dye	Molecular Probes Europe, Leiden, The Netherlands
Ethanol (absolute)	Hayman Limited, Essex, UK
Long Ranger 50% polyacrylamide gel	Cambrex Limited, Wokingham, UK
Agarose (Molecular Biology Grade)	Eurogentec Limited, Romsey, UK

2.2. RNA, DNA, culture media, and enzymes

Enzymes, proteins, and DNA were stored at -20°C unless otherwise stated. All enzymes were purchased and used with their respective buffers.

Bronchial epithelial cell basal medium (BEBM) BEGM singlequots: Bovine pituitary extract (BPE) 13 mg/ml Hydrocortisone 0.5 mg/ml hEGF 0.5 μg/ml Epinephrine 0.5 mg/ml Transferrin 10 mg/ml Insulin 5mg/ml Triiodothyronine 6.5 μg/ml Retinoic acid 0.1 μg/ml GA-1000 (Gentamycin sulphate) Amphotericin B	Clonetics, Buckingham, UK
Oligo-dT-(15) primer	Promega, Southampton, UK
Fluorescent dCTP (R110) Gene Scan 2500 ROX Size Standard BigDye Terminator Version 3.0	Applied Biosystems, Warrington, UK
Vistra Green	Amersham Pharmacia Biotech, Little Chalfont, UK
Rnase inhibitor	Ambion, Austin, TX, USA
Labtek II eight-well chamber slides	Fisher Scientific, Loughborough, UK
Primers Oligonucleotides 5' prime biotinylated primers	MWG Biotech, Ebersberg, Germany
IL-4, IL-13, TNF-α, IFN-γ	Pepro Tech, London, UK

Expand DNA polymerase Pwo DNA polymerase 7-deaza-dGTP	Roche Applied Science, East Sussex, UK
RPMI Medium 1640 without glutamine Keratinocyte-Serum Free Medium Foetal Bovine Serum (heat inactivated) Dulbecco's Modified Eagle's Medium (D-MEM), (without pyruvate, with glucose and pyridoxine HCl) Hanks Balanced Salt Solution (HBSS) (without Ca^{2+} or Mg^{2+}) Non Essential Amino Acids (MEM-100mM) (w/o Glut) sodium pyruvate 100mM Gentamycin 50mg/ml	GIBCO Invitrogen Corporation, Paisley, UK
DNA ladders (50 bp, 100 bp)	Life Technologies, Paisley, UK
JumpStart DNA polymerase Taq DNA polymerase BSA (Bovine Serum Albumin) Penicillin/Streptomycin Trypsin/EDTA (10x) PBS tablets (phosphate buffered saline tablets) Protease, Sodium Dodecyl Sulphate Temed, APS, PMA	Sigma-Aldrich, Poole, UK

2.2.1. Molecular biology kits

Buffer kits were purchased with their respective enzymes.

RNeasy Blood Kit QIA Quick PCR Purification kit Omniscripte Reverse Transcriptase	Qiagen, Crawley, UK

2.3. Buffers and solutions

All Buffers were made up in MilliQ H_2O, autoclaved and stored at room temperature unless otherwise noted.

TAE	1.6 M Tris base, 0.8 M Na acetate·$3H_20$, 40 mM EDTA, pH 7.2 (acetic acid)	
TBE	0.045 M Tris-Borate, 0.001 M EDTA	
Gel loading buffers	0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water, or 0.25% bromophenol blue, 40% (w/v) sucrose in water	
2.5x Half Sequencing Buffer	200 mM Tris-HCL (pH: 9), 5mM $MgCl_2$	
1x Annealing Buffer (SP-CCM)	100 mM Tris-HCL 50 mM NaCl 40 mM $MgCl_2$ dH_2O	1 ml 1M 100 µl of 5M 400 µl of 1M 8.5 ml
2x Binding Buffer	10 mM Tris pH 7.5 1mM EDTA 2 M NaCl dH_2O	100 µl of 1M 20 µl of 0.5 M 4 ml of 5M 5.88 ml
TE	10 mM Tris pH 8.0 1mM EDTA dH_2O	100 µl of 1M 20 µl of 0.5M 9.88 ml

5M NaCl	292.2 g of NaCl were dissolved in 800 ml of H_2O and the volume was adjusted to 1 litre with H_2O. The final solution was dispensed to aliquots and autoclaved.
1 M Tris	121.1 g of Tris base were dissolved in 800 ml of H_2O and the pH was adjusted to the desired value by adding concentrated HCL as follows: pH 7.4: 70 ml HCl, pH 7.6: 60 ml HCL, pH 8: 42 ml HCL. The solution was allowed to cool to room temperature before making final adjustment to pH. The volume of the solution was adjusted to 1 litre by adding H_2O. The final solution was dispensed to aliquots and autoclaved.
PBS (Phosphate-buffered saline)	1 tablet of PBS was dissolved in 200ml of sterile H_2O to obtain 0.01M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25^0C.
1M $MgCl_2$	203.3 g of $MgCl_2.6H_2O$ were dissolved in 800 ml of H_2O. The volume was adjusted to 1 litre with H_2O.
0.5 M EDTA	186.1 g of disodium EDTA·$2H_2O$ was added to 800ml of H_2O and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH (about 20 g of NaOH pellets). The final solution was dispensed to aliquots and autoclaved. Comment: the disodium salt of EDTA would not go into solution until the pH of the solution was adjusted to approximately 8.0.

2.4. PCR facility set-up and procedures

2.4.1. Laboratory set-up and pipetting

A range of precautions was taken to minimise the risk of PCR reaction contamination, particularly with "carryover" of PCR products into subsequent PCR reactions. Two separate areas were used for set-up and analysis of PCR products. Area 1 was used exclusively for preparation of master mixes, and for the addition of primers and templates to aliquoted master mix reagents. The PCR machines and gel electrophoresis apparatuses were located in area 2, and PCR products were never brought into area 1. Latex gloves were changed when moving between areas. All PCR reactions were carried out in thin-walled 0.2 ml tubes. To facilitate opening and closing of tubes for addition of PCR reagents, individually capped tubes were used instead of strips. Primers and master mix reagents were stored in 0.6 ml or 1.5 ml micro-centrifuge tubes. All plastic ware was autoclaved before use. All PCR reagents except primers were purchased from Sigma. $MgCl_2$ solution was stored at 4°C after heating and vortexing, while all other reagents were stored at -20°C. dNTPs and buffers were aliquoted to minimise freeze/thaw damage and contamination. Water for PCR reactions was from RiOs Reverse Osmosis Systems/Milli Q Ultrapure Water Purification Sytems apparatus (Millipore, Watfors, UK). It was autoclaved, and aliquoted into sterile 1.5 ml tubes for storage at -20°C.

2.4.2. Reaction set-up

For maximum efficiency and high polymerase fidelity, a "hot start" technique was used. Primers were pipetted onto the walls of the PCR tubes, out of contact with master mix reagents in the base of the tube. Tubes were subsequently heated in the PCR machine block to 80°C, before brief centrifugation and return to the 80°C block. Once all tubes in a reaction run had been centrifuged, the thermal protocol was allowed to proceed. In this way non-specific priming of polymerase reactions at sub-optimal temperatures was prevented. In addition, Jump-Start polymerase (Sigma-Aldrich, Poole, UK) was used to avoid non-specific priming at sub-optimal temperatures.

2.4.3. Primer design

The software programme OLIGO 5.0 (OLIGO Primer Analysis Software, Version 5.0 for Macintosh-National Biosciences, Inc., Plymouth, MN, USA) was used to design PCR primers. This software facilitates design of primer pairs which:

- are highly specific for the target sequence
- do not hybridise to other sites on the template
- form stable duplexes with the template under appropriate conditions
- do not form "hairpins" from internal complementarity
- do not form dimers with themselves
- do not form dimers with each other

OLIGO calculates the hybridisation temperature and secondary structure of an oligonucleotide based on nearest-neighbour thermal stability values, which provides a more accurate measure than other methods. PCR efficiency is optimised since anneal temperatures can be accurately matched. OLIGO also maximises specificity, by identifying potential primers with unstable 3' ends, flanked by highly stable regions, which are unable to prime imperfectly matched sequences. Primer pairs were designed to have melt temperatures as close to 60°C as possible.

2.5. Standard PCR protocols

2.5.1. Reagents

PCRs were carried out using standard Taq DNA polymerase or Jump-Start polymerase protocols, in 25-50 µl volumes. The final concentrations of PCR reaction components were usually those recommended by the manufacturer. The $MgCl_2$ concentration of 2 mM was in the middle of the recommended range, and worked well for most primer pairs. Primers were used at 200 nM in standard PCR, and DNA template between 50 and 200 ng per reaction. For RT-PCR, 1 µg total RNA was used for the downstream PCR reaction. In order to detect contamination, water controls were used in all reactions.

2.5.2. Thermal protocols

All PCR reactions were carried out in either a PTC-225 PCR machine (MJ Research, Boston, MA, USA) or a Gene Amp 9700 PCR machine (Applied Biosystems, Warrington, UK) according to protocols optimised for each primer pair. Generally 35 cycles of amplification were performed with an initial denaturation at 94°C for 5 min. To increase efficiency in some PCR reactions, a 'touch-down' technique was employed. All PCR products were analysed immediately or stored at -20°C.

2.5.3. Electrophoresis and agarose gel separation of DNA

Horizontal electrophoresis was routinely performed using a Horizon 58 electrophoresis tank (Sigma-Aldrich, Poole, UK) for smaller gels or a Kodak MP-1015 unit (Sigma-Aldrich, Poole, UK) for larger gels with a Bio-Rad model 500/200 (Bio-Rad Laboratories, Hemel Hempsted, UK). Agarose was melted in 1x TBE buffer, which was also used as a running buffer. DNA was routinely electrophoresed on 1.5-2% agarose and gels were routinely stained with ethidium bromide (500 ng/ml) for 20 min. Molecular weight markers included a 50 bp ladder or a 100 bp ladder. For genotyping, PCR products were resolved by micro-array diagonal gel electrophoresis (MADGE) (Day and Humphries, 1994), stained with Vistra Green. All gels were visualised by a Fluorimager 595 (Molecular Dynamics, Sunnyvale, CA, USA). DNA was purified from agarose using the QIAquick gel extraction kit according to manufacturer's instructions. Allele-specific PCR products were scored using the Phoretix 1D gel analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

2.5.4. Blood sample collection

All blood samples for RNA and DNA extraction were collected by venopuncture in 10 ml EDTA vacutainers. RNA was extracted immediately for RNA, whereas samples for DNA extraction were stored at -20°C until required. Heparin vacutainers were not used due to the inhibitory effect of heparin on the polymerase chain reaction (Satsangi et al., 1994).

2.6. Extraction of RNA

Total cellular RNA was isolated from peripheral whole blood, U937 cells, and Jurkat cells using the RNeasy Blood Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Total cellular RNA isolation from established primary bronchial epithelial cells, primary bronchial fibroblasts, and bronchial epithelial cell lines was carried out using TRizol. The integrity of the RNA was assessed by electrophoresis of the RNA samples on a 1% ethidium bromide-stained agarose gel and observation of intact 28S and 18S ribosomal bands. The RNA was stored at –80°C until use.

2.6.1. Trizol extraction of total RNA

1. 1 ml of Trizol was added to lyse cells grown in a petri dish. A scraper was used to remove the cells still attached at the bottom of the dish.
2. The cell lysate was then transferred in an Eppendorf tube and passed several times through a pipette.
3. 10 µl of glycogen was added to the homogenate.
4. 200 µl of chloroform (1/5 volume) was subsequently added and sample was homogenised by vortexing for 30 sec.
5. Sample was kept on ice for 15 min.
6. Sample was then centrifuged at 13,000 rpm for 15 min to separate the homogenate into an upper aqueous phase containing exclusively RNA, an intermediate phase containing DNA, and an organic phase containing protein.
7. Upper aqueous phase was transferred into two sterile 500 µl screw cap tubes.
8. An equivalent volume of isopropanol was added to the tubes (200 µl in each tube) and mixed thoroughly by vortexing for 30 sec.
9. Tubes were left at –20°C overnight to allow RNA precipitation.
10. The next day, RNA pellet was recovered by centrifugation (13,000 rpm) at 4°C for 15 min.
11. Isopropanol was removed with a sterile mini-pastette (since the pellet was not visible at that stage, total removal of the isopropanol was avoided).
12. The pellet was washed twice by adding 500 µl of 80% ethanol made in DEPC H_2O to further improve the RNA purity and centrifuged at 13,000rpm at 4°C for 5 min each time.

13. RNA was left to air dry for at least 10 minutes avoiding to dry the pellet completely
14. The pellet was then resuspended in DEPC treated water and dissolved by heating at 60°C for 10 min.

2.7. Reverse transcription

Reverse transcription was performed for 1h at 37°C using 1μg total RNA with 1μM oligo$(dT)_{15}$ as a primer and 4 U Omniscript Reverse Transcriptase (Qiagen, Crawley, UK) in the presence of 0.5 mM dNTPs, 10 U RNase inhibitor (Ambion, Austin, TX), and 1x reverse-transcriptase buffer, in a total volume of 20 μl.

2.8. Preparation of genomic DNA

DNA was extracted from frozen blood with a rapid ethanol precipitation method (Miller et al., 1988). The following reagents were used during the procedure:

(i) Erythrocyte lysis buffer (ELB) x 10	Potassium bicarbonate	5.05 g
Final volume: 500 ml (autoclaved)	Ammonium chloride	41 g
	0.5M ETDA pH 8	1 ml
(ii) Nucleic lysis buffer (NLB),	1.0 M Tris pH 8.0	5 ml
Final volume: 500 ml	5 M sodium chloride	40ml
	0.5 M EDTA pH 8.0	2 ml
(iii) 10% sodium dodecyl sulphate		
(iv) Protease 20 mg/ml		
(v) Saturated sodium chloride		
(approximately 6M)		
(vi) Absolute ethanol		
(vii) 70% ethanol		
(viii) TE (Tris EDTA buffer) 200 ml	1.0m Tris pH 7.5	2 ml
(Autoclaved)	0.5 M EDTA pH 8.0	400 μl

Procedure

10 ml of whole blood samples were defrosted and mixed for at least 1 h on rotary mixer in the cold lab (at 4°C).

A. Erythrocyte lysis

(1) 35 ml cold ELB was added to 10 ml whole blood in a 50 ml conical tube, mixed gently for 30 sec and left on ice for 15 min mixing every 5 min.

(2) Leukocytes/nuclei were spun down at 1500 rpm for 10 min. The supernatant was poured off.

(3) A further 30 ml cold ELB was added, mixed gently for 30 and left on ice for 15 min mixing every 5 min.

(4) Leukocytes/nuclei were spun down at 1500 rpm for 10 min. The supernatant was poured off.

(5) Additional 30 ml of cold ELB was added, mixed gently for 30 sec, and left on ice for 15 min mixing every 5 min. The third wash with ELB ensured the complete lysis of erythrocytes.

(6) Leukocytes/nuclei were spun down at 1,500 rpm for 10 min. The supernatant was poured off. Tubes were kept inverted to drain excess ELB.

B. Overnight protein digestion

-3ml of NLB were added to the tubes and the whole homogenate was transferred into a 15ml conical tubes.

-300 µl of 10% SDS and 200 µl protease were added to the homogenate.

-After gentle mix sample were incubated at 37°C overnight in a shaker incubator.

C. Ethanol precipitation of DNA

This step was carried out in small batches of tubes, e.g. 10-12 samples, as this minimised the length of time the DNA was in solution with a relatively high salt concentration.

(1) 1 ml saturated NaCl was added and samples were vigorously shaked for 15 sec. The precipitated proteins were then spun down at 4,000 rpm for 15 min at room temperature to precipitate protein.

(2) As much as possible of the supernatant (approximately 4-4.5 ml) was transferred into fresh 15 ml conical tubes using the 5 ml Gilson. At that stage, it was sometimes necessary to transfer the supernatant to microtubes and centrifuge at 14,000 rpm for 5 min to get a clear supernatant. Twice the volume (approximately 8-9 ml) of cold absolute ethanol was subsequently added. Tubes were inverted gently until DNA formed a pellet.

(3) DNA pellets were removed using yellow tips, transferred into 1.5 ml microcentrifuge tubes and washed with 1 ml of 70% ethanol.

(4) Samples were pulse spun to maximum speed and down, approximately 10-15 sec. Ethanol was poured off and tubes were end-blotted allowing DNA to nearly dry.

(5) 1 ml of TE buffer was added and DNA was allowed dissolved overnight on the rotary mixer.

2.8.1. Nucleic acid quantitation

RNA and DNA were quantified in a GeneQuant spectrophotometer (Pharmacia Diagnostics, Central Milton Keynes, UK) using 5 µl quartz capillaries and re-suspension buffer (usually MilliQ water) for calibration (50 µg/ml DNA was assumed to have an OD of 1.0 at 260 nm).

2.9. Mutation detection

The Solid Phase Chemical Cleavage of Mismatches (SP-CCM) and Denaturing High Performance Liquid Chromatography (DHPLC) were the methods used for detection of novel polymorphism in the *IL13RA1* and the *IL13RA2* genes (Figure 2.1).

2.9.1. Solid-Phase Chemical Cleavage of Mismatches

Chemical cleavage of mismatches was developed in 1988 as a method for screening cloned pieces of DNA for single base mismatches (Cotton et al., 1988), but in combination with PCR was later used for mutation analysis of genomic DNA (Montandon et al., 1989) and mRNA (Dahl et al., 1989). The basic chemical mismatch detection method entails the amplification of test and wildtype (probe) DNA using internal fluorescent labelling and their annealing to form heteroduplexes. Mismatched C and T residues on either strand are modified by hydroxalamine and osmium tetroxide, respectively, allowing cleavage of DNA with piperidine at the site of mismatched bases. This cleavage is detected by electrophoresis on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Warrington, UK), with the size of the fragments indicating the position of the mismatch and hence the mutation. Chemical cleavage of mismatches was significantly simplified using a solid phase protocol with biotinylated primers and streptavidin coated magnetic beads, avoiding multiple precipitation procedures (Rowley et al., 1995).

A. PCR reaction

The region of interest was amplified with two sets of primers. Targets were amplified with normal unlabelled primers, whereas probes were amplified using 5' biotinylated primers. Both probe and targets were internally labelled using fluorescently labelled dCTP. The reaction volume for targets was 25 µl, while the reaction volume for probe varied depending on the number of screens performed. The reaction mixture for targets contained 2 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2mM dNTPs, 2 µM fluorescently labelled dCTP, 0.2 µM sense and anti-sense oligonucleotide primers, and 25 U/ml JumpStart Taq DNA Polymerase, in a total volume of 25 µl.

B. Preperation of DNA and probes

1. The biotinylated probe was cleaned using QIAquick PCR purification kit (Quigen, Crawley, UK) according to manufacturer's instructions.

2. The product was eluted from column with 50 µl of dH_2O.

3. An equal volume of 50 µl of 2x annealing buffer (200mM Tris-HCl pH 8.0, 100 mM NaCl, 80 mM $MgCl_2$) was added.

At that point, the concentration of the probe was quantified using a Gene-Quant spectrophotometer (Pharmacia Diagnostics, Central Milton Keynes, UK). 5 µl of both the probe and the sample were loaded onto 1% agarose gel to compare concentrations.

C. Heteroduplex formation

5. For heteroduplex formation, 50 ng of the probe, 6 µl of 5x annealing buffer (500 mM Tris-HCl pH 8.0, 250 mM NaCl, 200 mM $MgCl_2$) and an excess of the target were mixed until a final volume of 30 µl.

6. The mix was incubated at 95°C for 5 min, followed by hybridisation steps at 65°C for 60 min, and at 55°C for 60 min. The mix was finally cooled down at 4°C. All reactions were performed in a PCR thermal cycler block.

D. Hydroxylamine and osmium tetroxide reactions

Reactions at this stage were performed in a fume hood and all waste was kept in sealed tubes until discarded in yellow bins.

7. 20 µl of streptavidin-coated magnetic beads (streptavidin magneshere PMP, Promega, Southampton, UK) were washed twice with 1x binding buffer (5 mM Tris pH 7.5, 0.5 mM EDTA, 1 M NaCl) and resuspended in 30 µl 2x binding buffer (10 mM Tris pH 7.5, 1 mM EDTA, 2 M NaCl).

8. The mix (30 µl) was added to the suspended beads (30 µl) followed by incubation at room temperature for 15-30 min.

9. Tubes were placed on a magnet to separate the clumped beads, and the liquid was then discarded.

E1. Hydroxylamine reaction

10a. Beads with bound DNA were gently resuspended in 25 µl of hydroxylamine solution [(1.6 g hydroxalamine- hydrochloride in 5.7 ml water, with pH adjusted to 6.0 with approximately 1.14 ml diethylamine (2.3M)]. The final hydroxalamine concentration was 2.5 M.

11a. Samples were incubated at 37°C for 1-2 h.

E2. Osmium tetroxide reaction

10b. Beads with bound DNA were resuspended in 25 µl of osmium solution (2 µl osmium tetroxide 4%, 0.4 µl pyridine, and 17.6 µl TE).

11b. Samples were incubated at 25°C for 15 min.

F. Genescan

12. The supernatant was removed using a magnet.

13. Beads with bound DNA were washed once with 50 µl TE.

14. TE was removed using a magnet, and tubes left open to dry while making up the loading buffer.

15. 6 µl of the following buffer was added to each sample.

- deionised formamide	3 µl
- piperidine (2M)	1 µl
- ROX 2500 size standard	1 µl
- Loading buffer	1 µl

16. Samples were incubated at 90°C for 30 min in a temperature block and then cooled on ice.

17. 1.5-2.5 µl of sample was loaded on a 12 cm 6% acrylamide gel (6% Long Ranger Gel, 6 mol/l urea, 0.5 x TBE) and run on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Warrington, UK). Initially, the instrument was pre-run under the following settings: 750V, 35mA, 50W, and 51°C for 15 min. Samples were run for 5 hours using the following instrument settings: 750V, 60mA, 200W, and 51°C. Data were analysed using the Genescan software (Applied Biosystems, Warrington, UK).

2.9.2. Denaturing High Performance Liquid Chromatography

Due to its small size, segment I (+38 to +301) of the coding region of the *IL13RA1* gene was screened for polymorphisms using denaturing high performance liquid chromatography (DHPLC). DHPLC is an ion-pair reversed-phase high performance liquid chromatography method. We used the WAVE DNA Fragment Analysis System by Transgenomic (Transgenomic Ltd., Crewe, UK), which uses the DNASep column as the stationary phase. The column is comprised of a polysterene-divinylbenzene copolymer. The mobile phase is comprised of an ion-pairing agent of triethylammonium acetate (TEAA), which mediates binding of DNA to the stationary phase, and acetonitrile (ACN) as an organic agent to achieve subsequent separation of the DNA from the column. A linear gradient of acetonitrile allows separation of fragments based on size and/or presence of heteroduplexes. DHPLC identifies mutations and polymorphisms based on heteroduplex formation between mismatched nucleotides in double stranded PCR amplified DNA. Sequence variation creates a mixed population of heteroduplexes and homoduplexes during reannealing of wild type and mutant DNA. When this mixed population is analysed by HPLC under partially denaturing temperatures, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature. Optimum denaturation temperature was predicted using the WAVEMAKER software (Transgenomic Ltd., Crewe, UK).

2.9.3. Primers and PCR conditions

A 2.2 kb segment of the 5' flanking region of the *IL13RA1* gene containing the *IL13RA1* promoter and the translation initiation codon ATG was amplified by PCR using a single pair of primers. For efficient amplification, the coding region of *IL13RA1* was divided in three segments. The coding region of the *IL13RA2* gene was amplified using two pairs of primers. Primers and PCR conditions used are shown in Table 2.1.

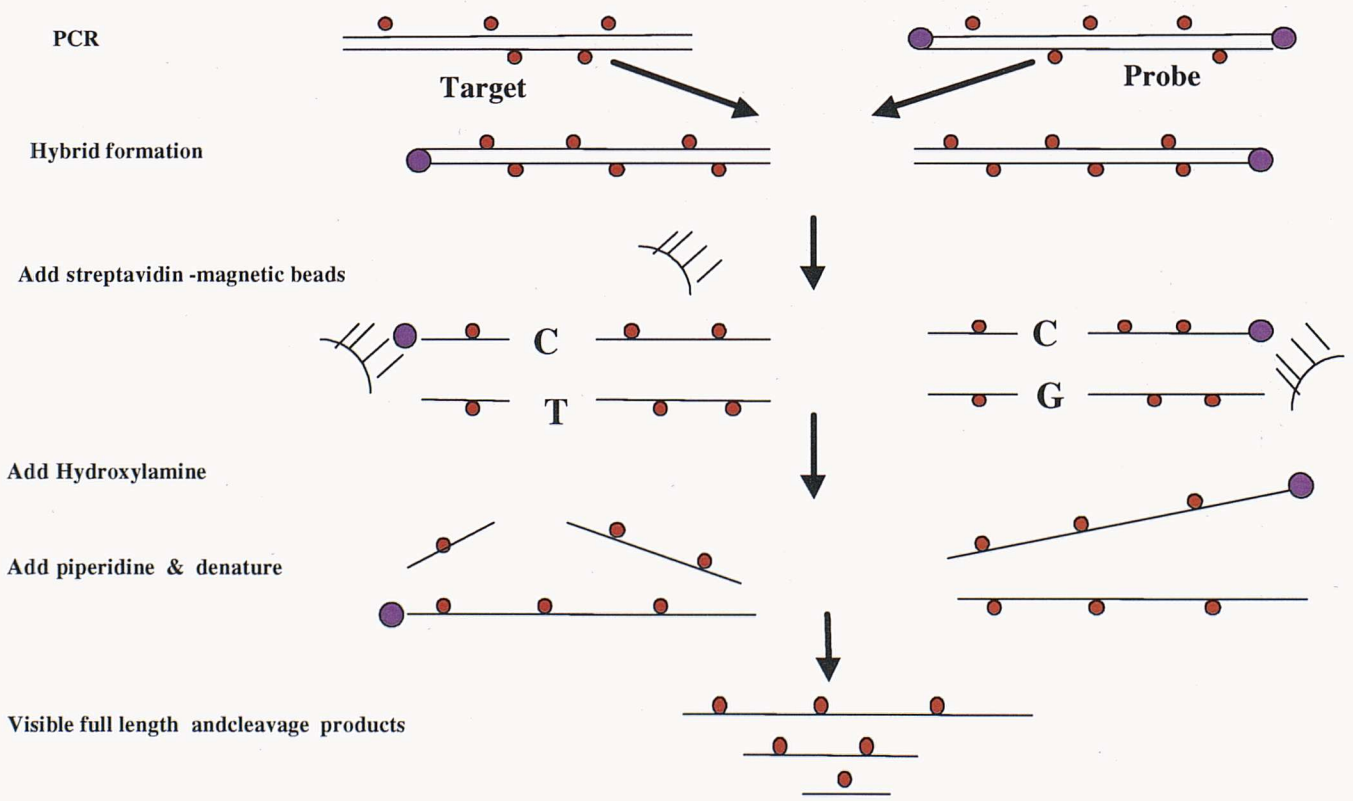

Figure 2.1. Solid Phase Chemical Cleavage of Mismatches. Scheme of solid-phase chemical cleavage of mismatches (SP-CCM) using fluorescent internal label of probe and target strands. Pink colour ovals: label at the 5' end of the primers; small yellow circles: labelled dCTP; arc with prongs: streptavidin-magnetic beads. (Redrawn from Rowley G et al: Ultrarapid mutation detection by multiplex, solid-phase chemical cleavage. *Genomics* 1995;30:574-82.)

Table 2.1. Primers and PCR conditions used for mutation screening and direct sequencing of *IL13RA1* and *IL13RA2*

Primers	Primer sequence	Temp.	Mg^{2+}	Amplicon size
IL13RA1 promoter Accession: AL606485*				
+610 to –1584**	Forward: CTCGCAAGAGCCACCCTTAGACA Reverse: TTGCCCTTATCATTGCCCCTCAG	63°C	1.5 mM	2194 bp
IL13RA1 coding region Accession: Y10659*				
+38 to +301**	Forward: TGTGGGCGCTGCTGCTCT Reverse: TGGGACCCCACTTGCAGACAA	66°C	2 mM	263 bp
+238 to +1347**	Forward: CGGAAACTCGTCGTTCAATAG Reverse: AACAAATGGAGAATGGGAAGA	58°C	2 mM	1109 bp
+889 to +1443**	Forward: TCATGGTCCCTGGTGTTC Reverse: CGGTGCGCGACTCAACATAAA	58°C	1.5 mM	554 bp
IL13RA2 coding region Accession: Y08768*				
-93 to +332**	Forward: CGGATGAAGGCTATTTGAAG Reverse: CCATGGTAAAAGCGTGTGTA	58°C	2 mM	425 bp
+293 to +1217**	Forward: CAAGGGCATTGAAGCGAAGA Reverse: TGAGACTCATATTGAACATTTGG	58°C	2 mM	924 bp

* Reference sequences were retrieved from GenBank

** Position of primers is shown in relation to the ATG start codon

2.10. DNA Sequencing

DNA sequencing was performed by automated sequencing using the ABI PRISM 377 DNA Sequencer (Applied Biosystems, Warrington, UK).

Sample preparation for automated sequencing

DNA was purified from contaminating nucleotides, primers, and DNA polymerase using a QIAquick PCR purification kit (Qiagen, Crawley, UK), and was quantified by spectrophotometry. Approximately 30-90 ng of PCR product was used for cycle sequencing.

A. Sequencing reaction

Each sequencing reaction tube contained the following:

- 8.0 μl BigDye Terminator version 3.0.

Alternatively, 4 μl of BigDye Terminator plus 4 μl of 2.5x half sequence buffer (200 mM Tris-HCl, pH 9, and 5mM $MgCl_2$) was used to reduce cost, while retaining the same high quality of sequencing.

- x μl DNA template (30-90 ng PCR product).
- y μl 1 Primer (3.2 pmol (e.g. 3.2 μl of 1μM)).
- z μl dH_2O.

TOTAL: 20 μl volume.

The following cycle parameters were used:

96°C 10 sec

50°C 5 sec

60°C 4 min

A total of 25 cycles were used, while products were kept at 4°C until purification.

Comment: 1°C/sec ramp time was used for the procedure, since the rapid ramping of some thermal cyclers may have resulted in poor data.

B. Ethanol precipitation

This step removed excess dye terminators and concentrated the product.

- A 1.5 ml tube was prepared with 50 µl ethanol ~ 95%, and 2 µl of 3 M sodium acetate pH 4.6. The sequencing reaction was transferred into that tube and mixed well by vortexing.
- Samples were left at room temperature for 15 min.
- Samples were then centrifuged at maximum speed in a microcentrifuge for 15-30 min at room temperature (tubes were positioned so that the pellet location was easily identified).
- The supernatant was removed carefully and discarded (the pellet may not visible at this stage).
- 250 µl 70% ethanol was added to the pellet, and the samples were mixed briefly by vortexing.
- Samples were centrifuged at maximum speed for 5 min.
-The supernatant was carefully completely removed and discarded.
- Pellets were dried in a hot block with the tube open at 90^0C for 1 minute (pellets could be stored at this stage at -20 ^{o}C until ready to use).
- 5 µl of formamide and 1µl of loading buffer was added to each sample followed by brief vortexing and brief centrifugation.
- Samples were heated at 95^oC for 2 min to allow denaturation of DNA and then immediately placed on ice.
- 1.5-2 µl of samples were loaded on an ABI PRISM 377 DNA sequencer using a 36-well comb. Samples were run using the Sequence Run 36E-1200 module. Sequencing data was analysed using the 'Sequence Analysis' program provided by Applied Biosystems, Warrington, UK.

2.10.1. Primers and PCR conditions

Following mutation detection, polymorphisms of *IL13RA1* and *IL13RA2* were identified by bi-directional sequencing of both positive and negative strands. The same sets of primers used for mutation detection were also used for sequencing (Table 2.1). In addition, the following primers were used as internal primers in the region flanking the *IL13RA1* –281T>G polymorphism: (+155 forward): 5'-CTTTCAGCCGTGACCCTCA-3', (-49 reverse): 5'-CGCCTCCTTCGCTCCCT

CTTC-3', (-663 reverse): 5'-CCTCCCTCATGTTTGGAATG-3' and (-929 reverse): 5'-ATCTCTGCCCACACCTTGATT-3'. Sequencing of the region was performed using the full 2194 bp amplicon as a template (+610 to –1584).

2.11. Promoter database analysis

Allele specific transcriptional factor binding sites were explored using BioInformatics and Molecular Analysis Section (http://bimas.dcrt.nih.gov/molbio/signal), and TFSearch (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html).

2.12. Genotyping

Genotyping assays for the *IL13RA1* –281T>G and 1365A>G polymorphisms were developed based on the tetra-primer ARMS-PCR method (Ye et al., 2001), which adopts certain principles of the tetra-primer PCR method (Ferrie et al., 1992) and the amplification refractory mutation system (Newton et al., 1989) (Figure 2.2). For each assay, four primers are combined in a single tube and detect both alleles of a particular SNP. The region amplified by the two outer primers serves as a control for the quality of PCR amplification and as a template for the subsequent allele specific amplification. Both inner primers encompass a deliberate mismatch at position -2 from the 3'-terminus to enhance allelic specificity. Rules for selecting a nucleotide for the additional mismatch are the same as in classical ARMS (Ye et al., 2001). Thus, a "strong" mismatch (G/A or C/T mismatches) at the 3' terminus of an allelic specific primer would probably require a "weak" second mismatch (C/A or G/T) and vice versa, whereas a "medium" mismatch (A/A, C/C, G/G or T/T) at the 3'-terminus will likely require a "medium" second mismatch. Primers were designed 26 nucleotide or longer to maximise the difference in stability of primers annealed to the target and non-target alleles. Each PCR reaction was carried out in a total volume of 15 µl, containing 25ng of template DNA, 200 µM dNTP, appropriate concentration of $MgCl_2$, 5% DMSO, 0.75 U of Jump Start DNA Polymerase (Sigma-Aldrich, Poole, UK), and the buffer recommended by the supplier.

The inner primer specific for the C allele encompassed a deliberate mismatch at position -2 from the 3'-terminus. The inner primer specific for the A allele did not

encompass any additional mismatches since it was found to be specific for this allele, while additional mismatches reduced the PCR efficiency. For the *IL13RA1* –281T>G SNP, the PCR reaction contained 15 pmol of each inner primer and 3 pmol of each outer primer. For the *IL13RA1* 1365A>G polymorphism, the PCR reaction contained 3 pmol of each outer primer, 15 pmol of the inner primer specific for the G allele, and 30 pmol of the inner primer specific for the A allele in order to increase the PCR efficiency for this particular allele. The PCR cycling conditions for both polymorphisms were: 95°C 5 min; then 10 cycles of 94°C 30 sec, X°C 30 sec (where X was initially 72°C, decreasing 1°C per cycle to 63°C), 72°C 30 sec; then 31 cycles of 94°C 30 sec, 63°C 30 sec, 72°C 30 sec; and finally 72°C 10 min. PCR primers used are shown in Table 2.2. 5 µl of the PCR product was mixed with 2 µl of loading buffer and subjected to horizontal non-denaturing polyacrylamide gel (10%) electrophoresis using the microplate array diagonal gel electrophoresis (MADGE) method (Day and Humphries, 1994, Ye et al., 2001). In brief, an open-faced polyacrylamide gel anchored on a glass plate was used. The gels contain cubical wells, 2 mm^3 for 96-well or 1.5 mm^3 for 384-well gels. The wells are spaced in an 8 x 12 mm, 9 mm pitch format which is directly compatible with PCR microplate transfer, but relative to the line of electrophoresis the 8 x 12 arrays are on the diagonal, e.g. turned by 18.4° to permit longer track lengths. Gels are made using suitably machined plastic plates into which gel mix is poured and air is excluded by direct closure with a sticky silane-coated glass plate, to which the gel will bond. The small gel-bearing glass plate is then prised off for 'dry' or submerged use in horizontal boxes. The MADGE system allowed samples to be loaded and electrophoresed in a 96-well microplate, thus substantially increasing throughput (Figure 2.3). The gel was stained with Vistra Green and scanned using a fluorimager 595 (Molecular Dynamics, Sunnyvale, CA, USA). Semi-automatic computerised data analysis was undertaken combining Phoretix/MADGE software (NonLinear Dynamics, Newcastle upon Tyne, UK) and Microsoft Excel.

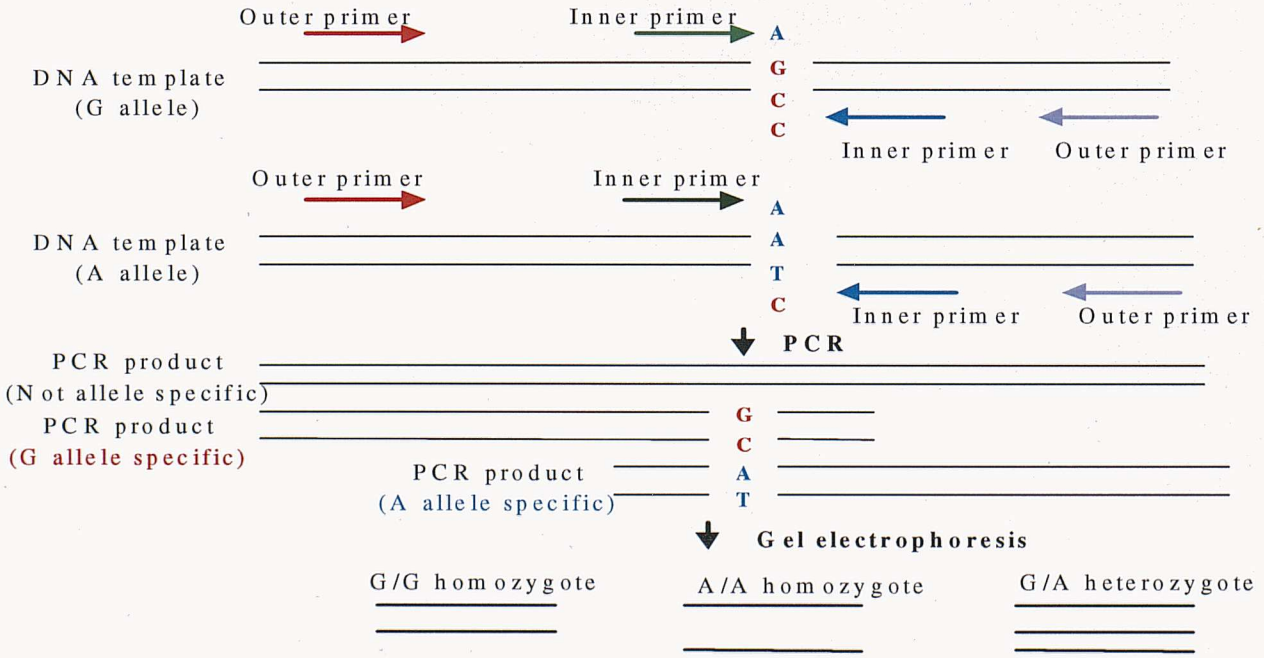

Figure 2.2. Schematic representation of the tetra-primer ARMS-PCR method. The single nucleotide polymorphism used as an example here is a G to A substitution. Two allele-specific amplicons are generated using two pairs of primers, one pair (indicated by red and blue arrows, respectively) producing an amplicon representing the G allele and the other pair (indicated by green and purple arrows, respectively) producing an amplicon representing the A allele. Allele specificity is conferred by a mismatch between the 3'-terminal base of an inner primer and the template. To enhance allelic specificity, a second deliberate mismatch at position -2 from the 3'-terminus is also incorporated in the inner primers. By positioning the two outer primers at different distances from the polymorphic nucleotide, the two allele-specific amplicons differ in length, allowing them to be discriminated by gel electrophoresis. (Redrawn from Ye S et al.: An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res* 2001;29:E88-8.)

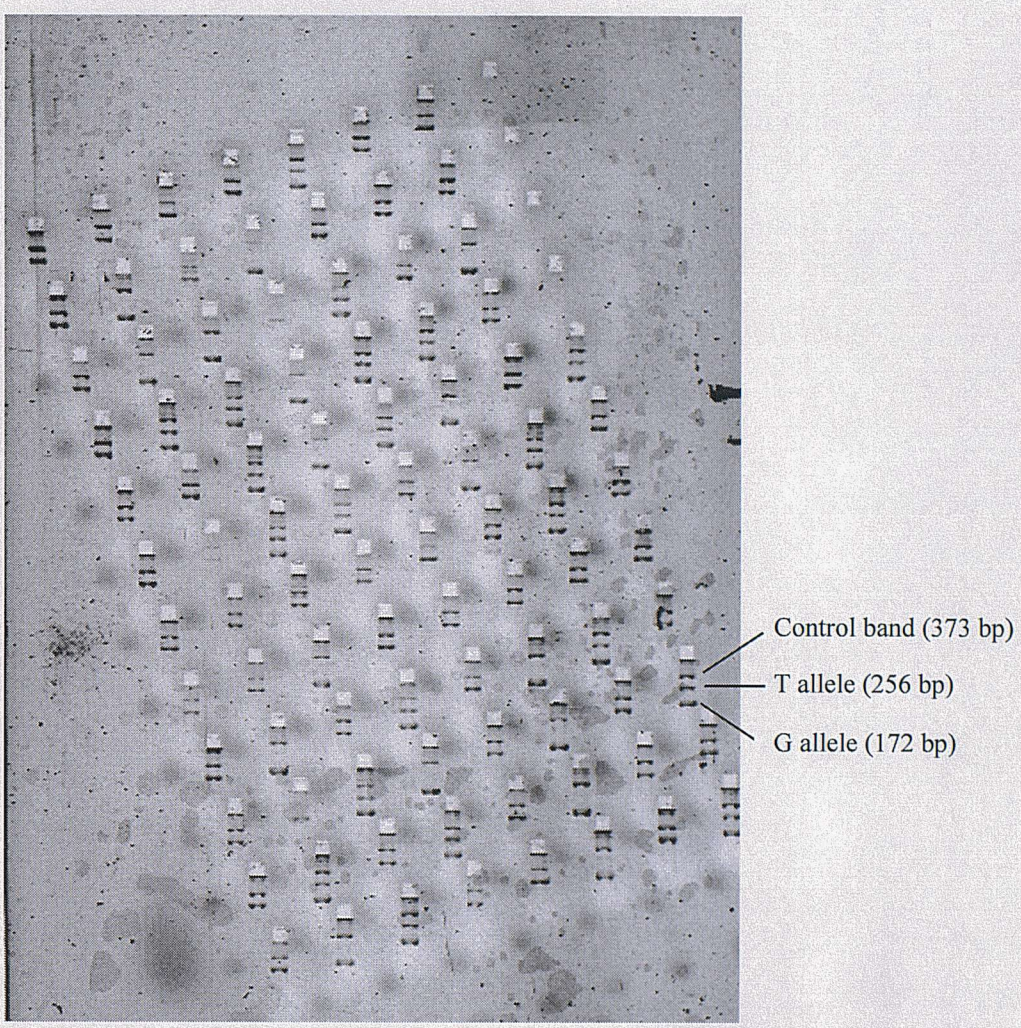

Figure 2.3. Analysis of tetra-primer ARMS-PCR system using the MADGE system. PCR products for the *IL13RA1* −281T>C polymorphism were subjected to MADGE gel electrophoresis. The gel was subsequently stained with Vistra Green and scanned using a fluorimager scanner.

Table 2.2. Primers and PCR conditions for genotyping of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms

Genetic polymorphism	Primer sequence	Mg^{2+}	Amplicon size
IL13RA1 –281T>G	Forward inner primer (G allele):	2 mM	172 bp (G allele)
Accession: AL606485*	5'-CTCCCGCGTCCGGTCTCTGACCG**T**AC-3'**		
	Reverse inner primer (T allele):		256 bp (T allele)
	5'-TGGGCGGCGACGTGGTGGAAGAAGTT**C**TT-3'		
	Forward outer primer:		373 bp (control)
	5'-CCTTCGCTCCCTCTTCCACTTCCCGGCTC-3'		
	Reverse outer primer:		
	5'-CTGTCCTGGTGTCCAGCAGGGCACAGCC-3'		
IL13RA1 1365A>G	Forward inner primer (A allele):	2 mM	226 bp (A allele)
Accession: AL606485*	5'-TCTCCATTTGTTATCTGGGAACTTATTAA-3'		
	Reverse inner primer (G allele):		315 bp (G allele)
	5'-ATGGTGCAGTAGTTTCAGTTTCC**C**TC-3'		
	Forward outer primer:		423 bp (control)
	5'-CATACCCCTACGGTTCCATCCAC-3'		
	Reverse outer primer:		
	5'-CACCACTATCATCACTTTTTGCTTTGTCTTTGTC-3'		

* Reference sequences were retrieved from GenBank

** Deliberate mismatched nucleotides are denoted with a bold letter

2.13. Genetic association studies

2.13.1. Recruitment of families

For association studies of polymorphisms with asthma and asthma-related phenotypes, 341 Caucasian families previously recruited were used. The families were recruited from the Wessex region, UK, from a population of approximately three million people. Recruitment of families took place through local general practitioners (G.P's). The inclusion criteria were that there had to be two affected siblings (age 5-21 years), each with physician's diagnosis of asthma and current medication use (Van Eerdewegh et al., 2002). Medications included regular or intermittent use of inhaled or oral steroids. Three hundred and forty one families comprising 1,508 individuals were recruited over a period of approximately a year and a half. Ethical approval was obtained from the Southampton and SouthWest Hampshire, and the Portsmouth and SouthEast Hampshire Local Research Ethics Committees. An asthmatic sibling cohort was selected (sibling cohort 1), defined by doctor diagnosis, current medication, and questionnaire (mean age = 13.0 years (S.D. 3.4), n = 341) and used to explore association with asthma phenotypes using conventional statistical analysis. An adult cohort composed of doctor diagnosed asthmatic parents (mean age = 40.2 years (S.D. 5.2), n = 189) was used as an additional study group. Non-asthmatic controls with no family history of respiratory disease were recruited from the same area as the main study (mean age = 42.3 years (S.D 10.6), n = 184) (Tables 2.3, 2.4).

Table 2.3. Descriptive characteristics of the population

Phenotype	Parents	Siblings	Sib. 1	Sib. 2	Sib. 3 to 9
Total	681	827	341	341	145
Male	49.9	53.3	55.4	54.0	46.9
Female	50.1	46.7	44.6	46.0	53.1
Mean age	40.5	11.5	13.0	9.9	11.3
Age Range	27.3 to 67.5	5.0 to 22.6	6.1 to 22.6	5.0 to 18.1	5.3 to 20.7

Table 2.4. Clinical characteristics of the population

Phenotype	Parents	Siblings	Sib. 1	Sib. 2	Sib. 3 to 9
Ever had asthma? (%)	28.9	87.9	100	100	100
Doctor diagnosis of asthma (%)	27.8	87.9	100	100	31.0
Currently taking asthma treatment (%)	27.0	88	99.4	100	33.1
FEV1 % predicted	100.8	95.8	94.8	95.6	98.5
FEV1 % Range	29 to 143	42 to 146	44 to 142	42 to 140	60 to 146
Number of positive skin tests	1.3	1.5	1.9	1.6	0.7

2.13.2. Clinical Phenotyping

Clinical phenotyping was based on a case report form and health survey questionnaire completed by each family member on the study day visit. This form included a list of inclusion and exclusion criteria, demographics, medical history, skin prick data, spirometry, challenge dose levels for the bronchial challenge, documentation of laboratory samples taken, and information on medicines taken in the last 12 months. The health survey questionnaire comprised a detailed clinical questionnaire of asthma, eczema, and allergic rhinitis, as well as detailed questions relating to the home environment and early life events. The questionnaire was based on the Medical Research Council (MRC), The American Thoracic Society (ATS) (Ferris, 1978), The International Union Against Tuberculosis and Lung Disease (IUATLD) (Burney and Chinn, 1987) and the International Study of Asthma, and Allergy (ISAAC) questionnaires (Asher et al., 1995).

Phenotypic measurements

Lung function and bronchial provocation testing

Baseline FEV_1 was obtained from pulmonary function testing. Three FEV_1 values within 5% of each other were obtained and the highest FEV_1 was recorded. Airway responsiveness to inhaled methacholine was performed according to the ATS guidelines using a Devilbiss 646 nebuliser in conjunction with a computerised system (KoKo Digidoser) according to the American Thoracic Society (ATS) guidelines for metacholine testing (Crapo et al., 2000). Metacholine dilutions (0.06 mg/ml to 16 mg/ml) were administered with increasing two-fold dilutions until at least 20% reduction from the patient's control (saline) FEV1 was reached.

Skin prick testing

Skin prick testing to 6 common aeroallergens was carried out: mixed grass, mixed trees, cat, dog, *Dermatophagoides pteronyssinus,* and *Alternaria* (Bayer Corporation, Spokane,WA), with a negative (saline) and a positive (histamine) control according to the procedure described by Pepys and co-workers (Pepys and Hutchcroft, 1975, Pepys, 1975).

Total and specific IgE

Total IgE and specific IgE measurements were carried out by IBT laboratory (Kansas, MO, USA) using the Pharmacia CAP System™. Specific IgE was measured for the same allergens as for skin prick testing. An individual was assigned a positive specific IgE value if his/her level was positive (to grass or to tree) or elevated (≥0.35kUA/l) for cat, dog, *Dermatophegoides pteronyssinus*, or *Alternaria* for at least one such measure. Total IgE was adjusted for age by using the number of standard deviations (SD) away from the median for each age group. This value was subsequently transformed using the natural logarithm to improve uniformity of variance. Thus, asthmatic individuals were dichotomised using the following cut-off for elevated serum total IgE levels: age 5-9 yr, >52 kilounits/liter (kU/l); age 10-14 yr, >63 kU/l; age 15-18 yr, >75 kU/l; age≥19 yr, >81kU/l.

2.13.3. Generation of phenotypic scores

Symptom severity score

In order to evaluate symptoms as diagnostic indicators of severity, the questionnaire was sent to an expert panel of experienced professionals in the field of asthma and allergy. This panel included physicians of the Wessex Thoracic Advisory Group and G.P.'s of the Southampton and South West Hampshire Health Authority, as well as physicians of the British Thoracic Society (BTS) and respiratory nurse specialists affiliated to the National Asthma & Respiratory Training Centre. Ten questions on asthma taken from the health survey questionnaire were sent to each member of the panel. The responses were formulated into a symptom severity score and a mean rank for each question was calculated. The reciprocal of the mean rank was taken to enable the "score" of 10 to reflect the most important. The total score was categorised into four levels of severity by dividing the maximum score into four categories and was subsequently used for further analyses. In order to improve the accuracy of clinical phenotyping, each family member was asked to answer questions on a video assisted questionnaire showing exercise-induced wheeze, nocturnal asthma symptoms, and symptoms at rest.

Treatment score

A treatment score for each asthmatic subject in the cohort was developed according to the British Thoracic Society guidelines (1997a). Medications used by subjects in the past 12 months prior to the study, were assigned a value according to BTS guidelines (1997a). Values assigned to each medication were as follows: 1=β_2 agonist (short acting); 2=low dose inhaled steroids (<800 μg), or sodium chromoglycate; 3=high dose inhaled steroids (>800 μg); 4= β_2 agonist (long acting); and 5=oral steroids. Inhaled steroids were converted to beclomethasone dipropionate equivalents (i.e fluticasone propionate dose was multiplied by two as this steroid product is twice as potent as beclomethasone). SPSS version 11 (Chicago, IL, USA) was used to convert the medications into treatment scores.

Atopy

Atopy was defined as either a positive skin prick test (> 3mm) or a raised specific IgE (> 0.35 kUA/l) to one or more common allergens. Serum total IgE was adjusted for age using the number of SD away from the median for each age group. This value was then transformed using the natural logarithm to improve uniformity of variance.

Atopy severity score

An atopy severity score was computed using the method of principal component analysis. The atopy severity was described using four components: (1) the principal component for mean skin wheal size (magnitude of response); (2) the principal component of number of wheals (range of response); (3) the principal component of level of specific IgE response (magnitude of response); and (4) the principal component of number of positive specific IgE responses (range of response).

Asthma severity score

The asthma severity score was developed using the method of first principal component analysis. The following variables were used to derive the asthma severity score: (1) (1/(LS slope+300)x1000; (2) symptom severity score; and (3) treatment score.

BHR

In order to describe bronchial provocation data, the least-squares slope developed by Chinn and colleagues was used and an arbitrary constant of 30 added to avoid negative values (Chinn et al., 1993). The population was categorised into four categories according to the severity of their (1/(LS slope + 30))x1000 value. Table 2.5 demonstrates the conversion of the (1/(LS slope + 30))x1000 values into equivalent PC_{20} values.

Table 2.5. Conversion of (1/(LS slope + 30))x1000 values into equivalent ATS PC_{20}

PC_{20} (mg/ml)	[1/(LS slope + 30)]x1000	Classification
>16	>19	Normal
4-16	10-19	Borderline
1-4	3-10	Mild
<1.0	<3	Moderate/Severe

2.13.4. Transmission disequilibrium test

The transmission disequilibrium test (TDT) was introduced by Spielman and co-workers as a test for linkage between a complex disease and a genetic marker (Spielman et al., 1993). Disease associations in case-control studies can be spurious because of population admixture or mismatches between patients and controls. These problems have led to the use of 'family-based' data instead of the conventional 'population based' data. Although designed as a test of linkage, the TDT is also valid as a test of association in simplex families, even if population structure is present. In this regard, TDT is the optimal test for association between marker and disease overcoming the limitations of conventional case-control studies. In contrast to conventional tests for linkage (e.g., LOD and nonparametric affected-sib-pair methods), which require sibships with multiple offspring, the TDT uses sibships with

a single affected offspring to detect for linkage, provided that disease association with some particular marker is also present.

The TDT considers parents who are heterozygous for an allele and evaluates the frequency with which that allele or its alternate is transmitted to affected offspring (Spielman and Ewens, 1996). When it is used as a test for association, the TDT statistics assumes independent transmissions from parent to affected offspring. However, the alleles transmitted from a heterozygous parent to two affected children cannot be assumed to be independent when testing association. Therefore, one affected child of each family is usually randomly selected, and data from the rest of the children are not used (Martin et al., 1997).

The TDT is carried out using χ^2 on a 2 x 2 table. For example, we consider a marker locus M, with two alleles M_1 and M_2, and obtain genotypes for affected individuals and their parents. For each of the parental genotypes (M_1M_1, M_1M_2, M_2M_2) we determine the number of times that the M_1 or the M_2 allele was transmitted to an affected offspring. These counts can be denoted as follows: a, number of times that M_1M_1 transmits M_1 to affected offspring; b, number of times that M_1M_2 transmits M_1 to affects offspring; c, number of times that M_1M_2 transmits M_2 to affected offspring; d, number of times that M_2M_2 transmits M_2 to affected offspring. The null hypothesis is that the marker and disease are unlinked. The TDT uses data only from those parents who are heterozygous M_1M_2, therefore, only observations b and c are used. The TDT statistic is $(b-c)^2/(b+c)$; it tests for equal numbers of transmissions of M_1 and M_2 from heterozygous parents to affected offspring. If there is linkage between marker and disease, as well as allelic association, b and c will tend to be different in value. The statistical significance of the TDT is tested by χ^2 ('McNemar Test'), or by the exact binomial test; a significant difference provides evidence that the marker is linked to the disease locus.

2.13.5. Linkage disequilibrium

An observed statistical association between an allele and a phenotype may be the result of the allele itself being functional, thus directly affecting the phenotype. However, an observed association could also be due to chance or an artefact

secondary to bias in selection or population stratification. Finally, association can arise if the marker under study is in linkage disequilibrium with another locus that directly affects the expression of the phenotype (Silverman and Palmer, 2000). Linkage disequilibrium (LD) occurs when haplotype combinations of alleles at different loci occur more frequently than would be expected from random association. Haplotypes are a set of related alleles within a particular genetic region on a chromosome. LD refers to the nonindependence of alleles at different sites. For example, suppose that allele at locus 1 and allele at locus 2 are at frequencies π_A and π_B, respectively. If the two loci are independent than the haplotype frequency of AB would be expected to be $\pi_A\pi_B$. If the population frequency of the AB haplotype is either higher or lower than this, implying that particular alleles are tend to be observed together, then the two loci are said to be in LD (Pritchard and Przeworski, 2001). A wide variety of statistics have been proposed for to measure the amount of LD. A particularly popular measure of LD between pairs of biallelic markers is denoted D' (Lewontin, 1964). For example, let us consider two biallelic loci on the same chromosome, with alleles A1 and A2 at the first locus and with alleles B1 and B2 at the second locus. The layout and notation of the 2 x 2 table are given below.

Alleles	B1	B2	Total
A1	n_{11}	n_{12}	Q
A2	n_{21}	n_{22}	1-Q
Total	R	1-R	N

In the table above, Q i s the number of haplotypes bearing the A1 allele, 1-Q is the number of haplotypes bearing the A2 allele, R is the number of haplotypes bearing the B1 allele, and 1-R is the number of haplotypes bearing the B2 allele.

The covariance between the two SNPs is $D= n_{11} n_{21} - n_{21} n_{12}/N^2$, and

$D'= |D|/\text{minimum } ((1-Q) (1-R))$.

Several studies have found that LD extends over very long distances, longer than predicted by standard models, and studies using microsatellites have observed substantial LD between pairs of markers separated by ≥1 cM (Peterson et al., 1995). On the other hand, data from short intergenic regions show less LD than would be

expected. Features behind this discrepancy include recent admixture, local variations in recombination rates and gene conversion (Pritchard and Przeworski, 2001).

2.14. RT-PCR analysis

To investigate mRNA expression of IL-13Rα1, IL-13α2, IL4R, and IL-2Rγ total RNA was initially extracted from whole blood using the RNeasy Blood Kit (Qiagen, Crawley, UK), and from established primary cell cultures and various cell lines using Trizol (Life Technologies, Paisley, UK). RNA integrity was assessed by electrophoresis of the RNA samples on a 1% ethidium bromide-stained agarose gel and observation of intact 28S and 18S ribosomal bands. RT-PCR reaction was carried out as described in section 2.7. Aliquots of PCR products were run on 2% agarose gels and visualised by ethidium promide staining. All amplicons were designed to span introns and were tested to ensure they would not amplify genomic DNA (Table 2.6). The specificity of amplicons was confirmed by dideoxy dye terminator cycle sequencing.

Table 2.6. Primers and PCR conditions for RT-PCR analysis of IL-13Rα1, IL-13Rα2, IL-4Rα, and IL-2Rγ

Primers	Primer sequence	Mg^{2+}	Temp.	Amplicon size
IL13RA1 (+899 to +1443)* Accession: Y10659**	Forward: TCA TGG TCC CTG GTG TTC Reverse: CGG TGC GCG ACT CAA CAT AAA	2 mM	58°C	565 bp
IL13RA2 (+772 to +1278)* Accession: Y08768**	Forward: GGA GCA TAC CTT TGG GAC CT Reverse: TTG GCC ATG ACT GGA AAC TG	2 mM	64°C	426 bp
IL4RA (+420 to +849)* Accession: X52425**	Forward: CTG ACC TGG AGC AAC CCG TAT Reverse: CCG CTT CTC CCA CTG TGA CCC	1.5 mM	58°C	450 bp
IL2RG (+564 to +991)* Accession: NM_000206**	Forward: TAC CGG ACT GAC TGG GAC CAC Reverse: TGG GGG AAT CTC ACT GAC GA	1 mM	58°C	447 bp
APRT (+244 to +542) * Accession: NM_000485**	Forward: GCT GCG TGC TCA TCC GAA AG Reverse: CCT TAA GCG AGG TCA GCT CC	1 mM	56°C	245 bp

* Position of primers is shown in relation to translation initiation codon ATG.

** Reference sequences were retrieved from GeneBank

2.15. Maintenance of cell lines

The lung mucoepidermoid adenocarcinoma cell line NCI-H292 (American Type Culture Collection-ATCC) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. The promyelomonocytic cell line U937 (ATCC) was maintained in RPMI 1640 medium supplemented with 10% FCS, 50 IU/ml penicillin, 50μg/ml streptomycin, and 2mM L-glutamine. The lung adenocarcinoma cell line A549 (ATCC) was grown in D-MEM with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. The T cell leukemia cell line Jurkat (European Collection of Cell Cultures, Salisbury, UK) was cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and 50 μM β-mercaptoethanol. BEAS-2B cells (ATCC), which are SV-40 immortalized cells derived from normal human bronchial epithelium, obtained from autopsies of non-cancerous individuals (Reddel et al., 1988). Cells were cultured in serum-free keratinocyte medium, which was supplemented with 10 ng/ml of epidermal growth factor and 30 μg/ml of bovine pituitary extract. The human bronchial epithelial cell line 16HBE was maintained in DMEM supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. All cell cultures were incubated in a humidified incubator at 37°C, 5% CO_2. Fetal calf serum (FCS) was stored at –20°C prior to use; it was then thawed at room temperature and heat inactivated by incubation at 56°C for 30 min.

2.16. Primary bronchial epithelial cell cultures

Primary bronchial epithelial cells were generously provided by Dr. J. Lordan and Dr. S. Puddicombe (Division of Infection, Inflammation and Repair, University of Southampton). Cells were obtained from central airways during bronchoscopy. Bronchoscopy was performed using a fiberoptic bronchoscope (Olympus FB-20D, Tokyo, Japan) in accordance with standard published guidelines (1991). Bronchial cells were obtained using a standard sterile single-sheathed nylon cytology brush (Olympus BC9C-26101). This was passed by direct vision via the bronchoscope channel into the lower airways, and five to six consecutive brushings were sampled from the bronchial mucosa of the second and third generation bronchi. Cells were detached from the brushes by vigorous agitation in 5ml sterile PBS (~2 brushes/5ml

aliquot). The brush was then rinsed in fresh vial of PBS before being returned for further brushings. When brushing procedure was complete, 5ml RPMI with 10% FBS was added to each 5ml of PBS/cell. Cells were then centrifuged at 150 x g for 5 min ensuring that most cells were pelleted. If not, then cells were re-spun again and any large cell clump was collected with a pipette before removing the supernatant. Cell cultures were established by seeding freshly-brushed cells into culture dishes containing 5ml serum-free bronchial epithelim growth medium (BEGM) hormonally supplemented with 30 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human EGF, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyroxine, 0.5 mg/ml albumin, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 50 ng/ml amphotericin B. For stimulation, cells were cultured in unsupplemented BEGM containing 1% ITS, 100 mg/ml bovine serum albumin, and antibiotics. When confluent, cells were passaged (p1) using trypsin and were allowed to further expand until used for experimentation at passage 2 or 3 (Figure 2.4). Cell phenotype was assessed by immunohistochemical staining of cultures grown on culture chamber slides using a pan-cytokeratin antibody, as well as antibodies specific for cytokeratin 13 and 18. Viability was assessed by exclusion of trypan blue dye and was consistently >90%.

2.17. Primary bronchial fibroblast cultures

Primary bronchial fibroblasts were generously provided by Dr. A. Richter (Division of Infection, Inflammation and Repair, University of Southampton). Fibroblasts were grown as explants from bronchial biopsies obtained from three mild asthmatic adult subjects by bronchoscopy. Bronchial biopsies were minced to pieces of around 1 to 2 mm^3 using sterile scalpel blades. The minced pieces were vigorously washed in PBS and the washing procedure was repeated until the supernatant became clear. The pieces were then cultured in Dulbecco's Modified Eagle's Medium containing 10% (v/v) heat inactivated FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids. Cultures were incubated in a humidified incubator at 37°C, 5% CO_2 for approximately one to two weeks. During that time fibroblasts migrated from each piece and grew to subconfluence, while the remaining pieces removed by gentle pipetting. The

outgrowing fibroblasts from those pieces were harvested from the wells using trypsin-EDTA. Cultures were fed every third day and passaged when reached 70-80% confluency. Cells used for experiments between passages 3 and 8. For stimulation, cells were cultured in serum-free medium (Ultraculture; Biowhitaker, Wokingham, UK) supplemented with penicillin and streptomycin.

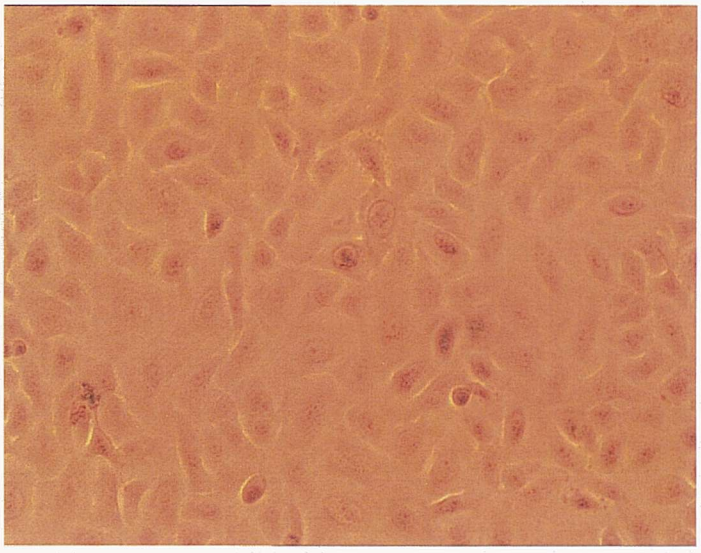

Figure 2.4. Primary bronchial epithelial cells in culture. Brushed primary bronchial epithelial cells were seeded into culture dishes and allowed to further expand until used for experimentation at passage 2 or 3. Cells shown here are at passage 3. Original magnification: x63.

2.18. Immunohistochemistry

GMA-embedded bronchial biopsies obtained from three mild asthmatic subjects were sequentially cut at 2 μm with Supercut Rotary Microtome (Leica Instruments, Heidelberg, Germany), floated onto ammonia water (0.2%), picked up on poly-L-lysine coated glass slides, and allowed to dry at room temperature for 1-4 h. Sections were pre-treated with 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min to inhibit endogenous peroxidases. Non-specific background staining was reduced by blocking with 1% bovine serum albumin and 10% fetal calf serum in TBS. Sections were incubated overnight at appropriate concentrations (anti-IL-13Rα1 1:250, anti-IL-13Rα2 1:200, anti-IL-4Rα 1:100). Optimal antibody titre was initially determined by immunostaining with serial dilution of the specific antibody in GMA embedded tonsil sections, and subsequently confirmed in bronchial biopsies. After washing (3x5mins), biotinylated secondary rabbit anti-mouse antibody (1:600) (Sigma-Aldrich, Poole, UK) was applied for 1 h. After further washes (3 x 5min), staining was performed using the avidin-biotin complex (ABC) method according to the manufacturer's instructions (DakoCytomation, Cambridgeshire, UK). Slides were subsequently developed using liquid 3,3'-diaminobenzidine (Liquid DAB) substrate (DakoCytomation). Sections were counterstained for 1-2 min in Mayer's haematoxylin and coverslipes were mounted onto slides using Moviol gel. All washes, as well as dilutions of primary and secondary antibodies were carried out in 0.05M Tris buffered saline, pH 7.6. Control sections were routinely immunostained in the absence of primary antibody or unrelated isotype-matched IgG.

Immunostaining of cells

Primary bronchial epithelial cells and primary bronchial fibroblasts grown on tissue culture-coated slides were washed in TBS three times, submerged in ice-cold methanol for 15 min and allowed to dry for 30 min. After a TBS wash, cells were pre-treated with 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min to inhibit endogenous peroxidases. Non-specific background staining was reduced by blocking with 1% bovine serum albumin and 10% fetal calf serum in TBS. Sections were incubated with primary antibodies for 1 h at appropriate concentrations (anti-IL-13Rα1 1:250, anti-IL-13Rα2 1:200, anti-IL-4Rα 1:100). Optimal antibody titre was

initially determined by immunostaining with serial dilution of the specific antibody. After washing (3x5 min), biotinylated secondary rabbit anti-mouse antibody (1:600) was applied for 1 h. After further washes (3 x 5min), staining was performed using the avidin-biotin complex (ABC) method according to the manufacturer's instructions (DakoCytomation, Cambridgeshire, UK). Slides were subsequently developed using liquid 3,3'-diaminobenzidine (Liquid DAB) substrate (DakoCytomation). Sections were counterstained for 1-2 min in Mayer's haematoxylin and coverslipes were mounted onto slides using Moviol gel. All washes, as well as dilutions of primary and secondary antibodies were carried out in 0.05M Tris buffered saline, pH 7.6. Control sections were routinely immunostained in the absence of primary antibody or unrelated isotype-matched IgG.

2.19. Confocal laser scanning microscopy

Primary bronchial epithelial cells and primary bronchial fibroblasts grown on tissue culture-coated slides were washed three times in cold PBS with 1% FBS, and subsequently fixed in 2% paraformaldehyde pH 7.4 for 15 min. Following three washes with PBS, cells were incubated with a monoclonal anti-IL-13Rα2 antibody for 1 h in a solution containing PBS, 1% FBS, and 2% goat serum. Following PBS washes, a secondary goat-anti-mouse Alexa Fluor 633 nm antibody was added for 1 h. Coverslipes were washed in PBS and counterstained with the nuclear dye SYTOX Orange (1μl in 1ml PBS) for 2 min. For permeabilized cells, 0.2% saponin was added to the antibody solution and to all subsequent washes. After the final wash slides were covered with a covereslip and the edges sealed with a nail polish. Cells were observed on a Leica SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany).

2.20. Flow cytometry

Primary bronchial epithelial cells and primary bronchial fibroblasts were detached from flasks using trypsin, whereas NCI-H292 cells were liberated from flasks using a non-enzymatic solution. Cells were rinsed with PBS containing 0.1% sodium azide and resuspended in PBS containing 2% FCS in a concentration of 1 x 10^6 cells/ml. A total of 100 µl aliquot of the cell suspension was subsequently incubated for 1h at 4°C with a monoclonal anti-IL-13Rα2 antibody, then washed three times and resuspended in 100 µl PBS containing 2% FCS, and FITC-conjugated anti-mouse IgG secondary antibody in a final concentration of 1:50. After 1h incubation in the dark at 4°C, cells were washed three times with PBS containing 0.1% sodium azide and then processed for analysis using a FACScan flow cytometer (Becton Dickinson Biosciences, Oxford, UK). Data were analysed using WinMDI 2.8 software.

For intracellular staining, cells were fixed in 1% paraformaldehyde and 0.2% saponin. Saponin (0.2%) was also added to the antibody and wash solutions as permeabilization with saponin is reversible. The correct antibody dilutions for FACS were established by titration (Figures 2.5, 2.6). Isotype controls were used to confirm specificity and to enable subtraction of the background staining. Antibodies used in the study are shown in Table 2.7.

2.20.1. Labelling of non-viable cells

To determine the surface expression of IL-13Rα2 on intact cells using flow cytometry we excluded non-viable cells, which can absorb antibodies intracellularly resulting in false-positive staining, using propidium iodide exclusion. Propidium iodide is a high affinity nucleic stain that easily penetrates cells with compromised surface membranes yet will not cross the membrane of live cells, and emits in FL 2 channel which can be separated from the FITC-conjugated secondary antibody, which emits in FL 1 channel. A stock solution of 250 µg/µl propidium iodide in PBS was made up and stored in a clear bottle covered with foil at 4°C. After antibody labelling of intact cells was completed, stock solution in a final concentration of 1:100 was added to the cell suspension (for example, 10 µl of stock solution added in 1ml of cell suspension containing 1 million cells). During analysis data were acquired from the FL2 detector

and brightly-labelled cells were gated out during subsequent off-line analysis (Figure 2.7).

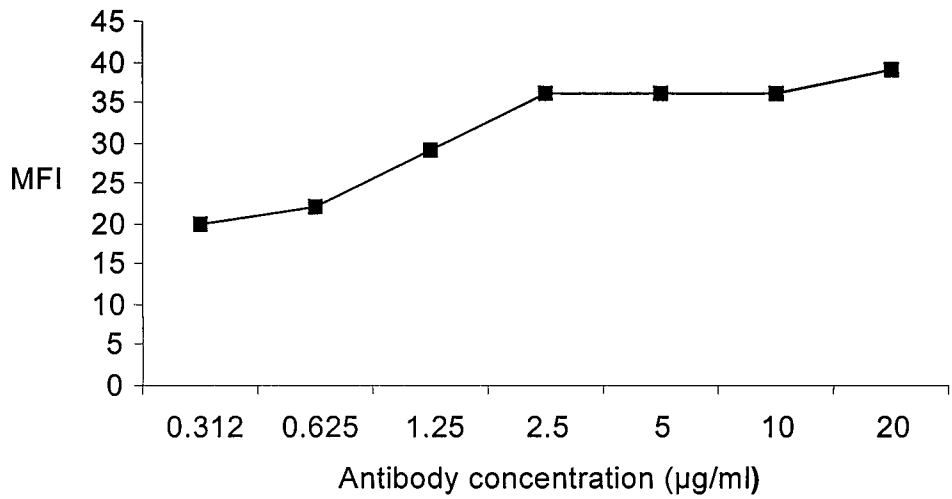

Figure 2.5. Titration of ICAM-1 antibody. NCI-H292 cells were stained with serial 2-fold dilutions of a specific monoclonal anti-IL-13Rα2 antibody followed by a goat anti-mouse FITC-conjugated antibody. The median channel fluorescence (MFI) of cells determined for the IL-13Rα2 and plotted as a function of antibody concentration. Above 10 ng/ml the nonspecific component begins to appear. Below 2.5 ng/ml epitope saturation no longer occurs and there is a steep slope to the titration curve. The titre is determined as the shoulder area where epitope saturation has occurred and nonspecific binding is low. The titre of the antibody used was 2.5 µg/ml (1:400 dilution) in a final volume of 100 µl.

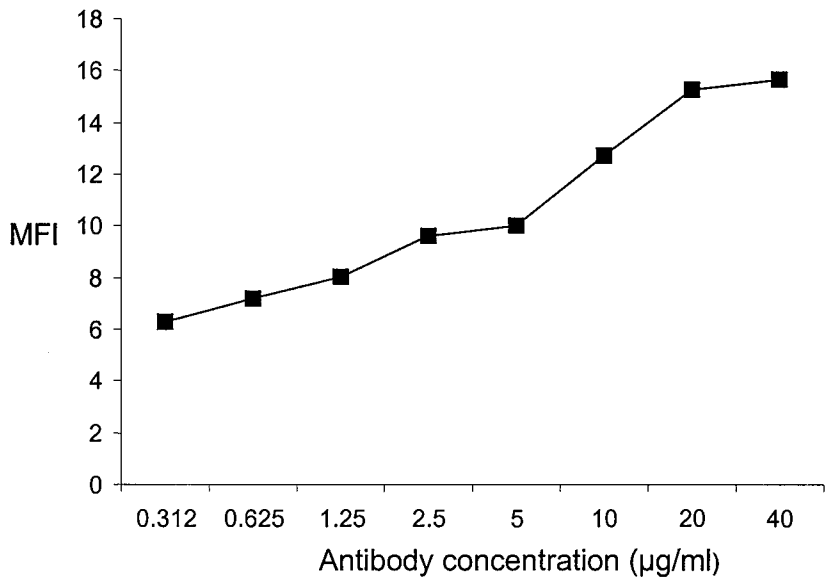

Figure 2.6. Titration of IL-13Rα2 antibody. NCI-H292 cells were initially fixed with 1% paraformaldehyde and subsequently permeabilized with saponin. Cells were subsequently stained with serial 2-fold dilutions of a specific monoclonal anti-IL-13Rα2 followed by goat anti-mouse antibody and analysed by flow cytometry. The graph shows median fluorescence intensity (MFI) at different antibody concentrations. Titration curves demonstrate three parts corresponding to (1) weak staining, (2) correct antibody concentration and specific staining, (3) too high antibody concentration resulting in non-specific staining. The concentration of antibody used was 2.5 µg/ml (1:400 dilution).

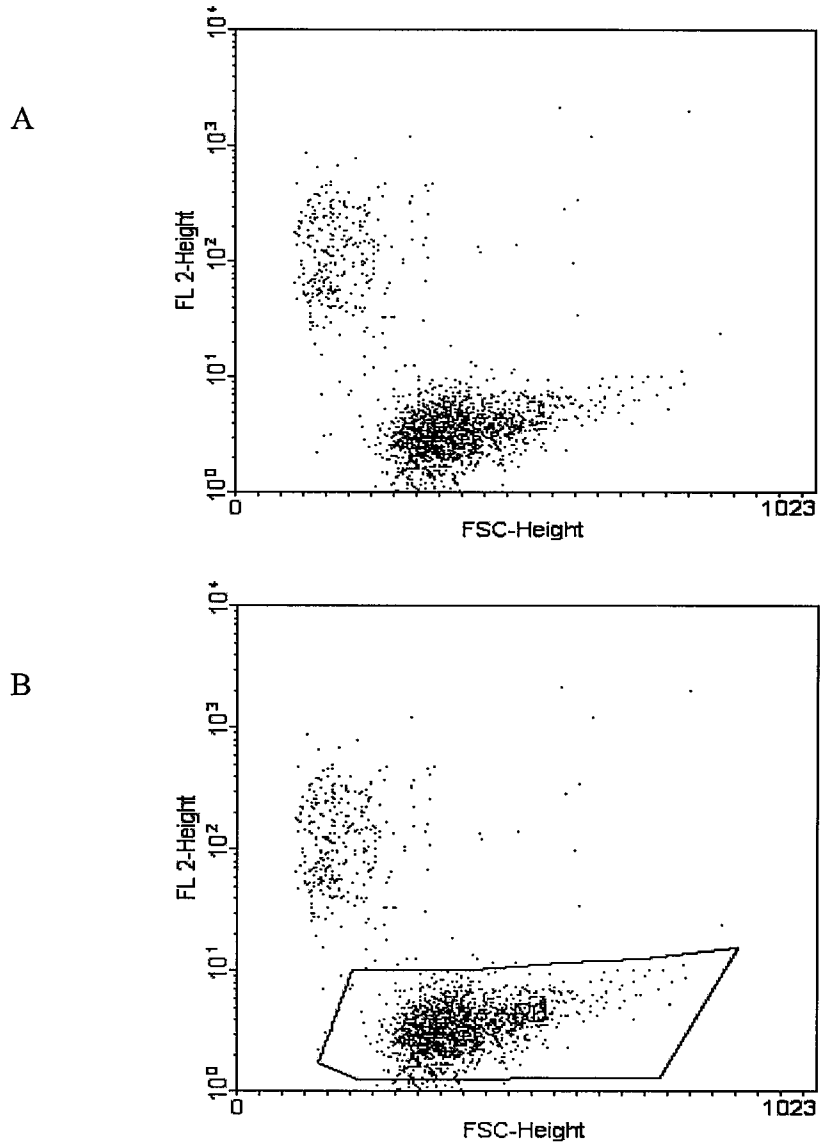

Figure 2.7. Labelling of non-viable NCI-H292 cells during flow cytometry. NCI-H292 cells were stained with a specific monoclonal anti-IL-13Rα2 antibody for IL-13Rα2 surface expression assessment. Before processing of cells for flow cytometric analysis, propidium iodide was added to the cell suspension in a final concentration of 2.5 μg/ml. The vertical scale represents propidium iodide emission in FL 2 channel, whereas the horizontal scale represents the forward scatter that is proportional to the cell size (A, B). The brightly-labelled cells, outside the designated area, were gated out during subsequent analysis as shown in panel B.

Table 2.7. Monoclonal antibodies

Antibody/ Isotype	Antigen	Source	Application	Dilution
UU15 Ascites fluid, mouse IgG2α	IL-13Rα1	Gift- Dr.Izuhara, Japan	Immunostaining	1:200
B-D13 Mouse IgG1	IL-13Rα2	IDS, Boldon, UK	Flow cytometry, immunohistochemistry	1:200
HA58 Mouse IgG1	ICAM-1	Insight Biotechnology Ltd., Wembley, UK	Flow cytometry	1:300
C11 Mouse ascites fluid	Cytokeratins 4, 5, 6, 8, 10, 13, 18	Sigma-Aldrich, Poole, UK	Flow cytometry	1:300
DF1485 Mouse IgG1	HCAM	Autogen Bioclear Ltd, Calne, UK	Flow cytometry	1:300
V9 Mouse ascites fluid	Vimentin	Sigma-Aldrich, Poole, UK	Flow cytometry	1:200
BM-75.2, Mouse ascites fluid	α-smooth muscle cell actin	Sigma-Aldrich, Poole, UK	Flow cytometry	1:400
hSM-V Mouse asctites fluid	Myosin	Sigma-Aldrich, Poole, UK	Flow cytometry	1:100
MAB230 Mouse IgG2α	IL-4Rα	R&D Systems Europe Ltd, Abingdon, UK	Immunohistochemistry	1:100
Goat Anti-mouse IgG-FITC	Mouse IgG	Becton Dickinson UK Ltd, Oxford, UK	Flow cytometry	1:50
Alexa Fluor 633- Goat anti-mouse IgG	Mouse IgG	Molecular Probes Europe BV, Leiden, The Netherlands	Immunohistochemistry	1:400

CHAPTER 3

Mutation screening of the *IL13RA1* and the *IL13RA2* genes

3.1. Overview

At the time of writing, two previous studies have examined the presence of genetic variants in the coding and proximal 3' UTR of the *IL13RA1* gene and found a silent, 1050C>T, variant in the coding region and a non-coding, 1365A>G, SNP in the proximal 3' UTR of *IL13RA1*. No genetic variants in the coding region of the *IL13RA2* gene were found in a single previous study. We performed mutation scanning of the promoter, coding region, and proximal 5' UTR of the *IL13RA1* gene, as well as of the coding region of the *IL13RA2* gene.

Results: We have identified a novel common variant, -281T>G, in the *IL13A1* promoter and we have confirmed the presence of the previously identified *IL13RA1* 1050C>T and 1365A>G SNPs. We found no common variants in the coding region of the *IL13RA2* gene.

IL-13 elicits its biologic effects via its receptor complex, which is composed of the heterodimeric proteins IL-13Rα1 and IL-4Rα (Miloux et al., 1997). The IL-13Rα1/IL-4Rα complex is also utilised by IL-4 as an alternative receptor, especially in non-hematopoietic cells that do not express the common gamma chain (IL-2Rγ) (Murata et al., 1998). IL-13Rα2 is a decoy receptor providing tight regulation of IL-13 by trapping excess IL-13 (Feng et al., 1998). There is strong evidence to suggest that genetic variation of *IL4* and *IL4R* predispose to the development of asthma and allergy (Shirakawa et al., 2000). Previous studies have also shown that the Gln110 variant of the Arg110Gln polymorphism in *IL13* constitutes a risk factor for the development of asthma in adults (Heinzmann et al., 2000) and increased serum total IgE levels in children (Graves et al., 2000). Given that genetic variation in the IL-4/IL-13 axis predisposes to the development of asthma and allergy, we hypothesised that genetic variation in the *IL13RA1* and the *IL13RA2* genes, may also play a critical role in the development of asthma phenotypes or/and predict severity. Ethical approval for the use of human volunteers in this study was obtained from the Southampton and SouthWest Hampshire and Portsmouth and SouthEast Hampshire Local Research Ethics Committees.

At the initiation of this work there were no reported studies on *IL13RA1* and *IL13RA2* genetic variation. We therefore performed mutation analysis to identify common genetic variants in the promoter, coding, and proximal 5' UTR of *IL13RA1*, as well as in the coding region of *IL13RA2*. While this work was carried out, two studies investigating polymorphisms in the coding region of the *IL13RA1* gene and a single study investigating polymorphisms in the coding region of the *IL13RA2* gene have been reported. Ahmed and co-workers (Ahmed et al., 2000) screened the coding region of the *IL13RA1* gene in a Japanese paediatric population and identified a rare C to T non-amino acid altering polymorphism at position 1050 relative to the translation initiation codon ATG, which is situated in the transmembrane domain of the *IL13RA1* gene. Heinzmann and co-workers (Heinzmann et al., 2000) performed a mutation scanning in British and Japanese populations and identified an A to G substitution at position 1365 relative to the translation initiation codon ATG, which is situated in the proximal 3' UTR of the *IL13RA1* gene. Kawakami and co-workers (Kawakami et al., 2000) screened the coding region of the *IL13RA2* gene in human glioblastoma multiforme cell lines and found no polymorphisms.

3.2. Genomic structure of the *IL13RA1* gene

The human cDNA for the *IL13RA1* gene was cloned by Aman and co-workers through screening of human cDNA libraries based on the sequence of the murine *IL13RA1* (Aman et al., 1996). The open reading frame of IL-13Rα1 encodes for a 427 amino acid protein with an apparent molecular weight of 65- to 70-kDa. *IL13RA1* exhibits two principal transcripts of 4.0 and 2.0 kb, reflecting alternative polyadenylation. To the best of our knowledge, at the time of writing, the full genomic structure of the human *IL13RA1* gene has not yet been reported. Based on latest data from the human sequence project deposited at GenBank, the *IL13RA1* gene is composed of eleven exons, spans at least 68 kb, and maps to X_q24-25. Our own comparisons between the published sequence for the human IL-13Rα1 mRNA (Aman et al., 1996) and genomic sequence data from GenBank indicated that the extracellular region of the *IL13RA1* gene is encoded by exons 1-9; the 24 amino acid transmembrane domain is encoded by the middle portion of exon 9; whereas the 60 amino acid transmembrane domain is encoded by the teminal portion of exon 9, exon

10, and the proximal portion of exon 11 (Figure 3.1). The *IL13RA1* promoter has been fully characterised and was found to lack typical TATA box and CCAAT box motifs (Ise et al., 1999). Although TATA-less promoters are often accompanied by several transcriptional initiation sites, only one transcription initiation site was found, 123 bp upstream of the translation initiation codon ATG. Several potential binding sites for transcription factors were observed, including Sp1 binding sites around nucleotides –7 to nucleotide –60 (Ise et al., 1999).

3.3. Genomic structure of the *IL13RA2* gene

The cDNA for the *IL13RA2* gene was cloned by Caput and co-workers following screening of libraries from human renal carcinoma (Caki-1) cells, previously shown to express high binding sites for IL-13 (Caput et al., 1996). The open reading frame of the *IL13RA2* gene encodes a 380 amino acid membrane protein, with a signal peptide of 26 amino acids, a 343-amino-acid extracellular domain, a single membrane-spanning domain of 17 amino acids, and a short cytoplasmic domain of 17 amino acids (Caput et al., 1996). IL13RA2 exhibits a single 1.4 kb transcript and has a short 3' UTR of 103 bases. Fluorescence in situ hybridisation has shown that the *IL13RA2* gene maps to Xq24 (Guo et al., 1997). Two independent groups have recently described the genomic organisation of *IL13RA2* in different cell lines. In the human glioma cell line U118, *IL13RA2* is composed of eleven exons (nine coding ones), and ten introns (Wu and Low, 2003) (Figure 3.2.). The transcriptional start site is located 270 bp upstream of the translation initiation codon ATG, whereas the promoter region is located immediately upstream of the first exon and contains three TATAA and one CCAAT boxes. The open reading frame of *IL13RA2* in U118 cells is encoded by exons 3-11. In the human keratinocyte cell line HaCaT, *IL13RA2* is composed of four 5' non-coding exons, nine coding exons, and twelve introns (David et al., 2003). The *IL13RA2* promoter region in HaCaT cells is located immediately upstream of the first non-coding exon, approximately 10 kb upstream of the translation initiation codon ATG, and was found to lack consensus TATAA or CCAAT boxes, however, two STAT6 binding sites were found. Moreover, studies by 5' RACE provided evidence for the expression of three major transcripts in HaCaT cells resulting from alternative usage of the first non-coding exons (David et al., 2003).

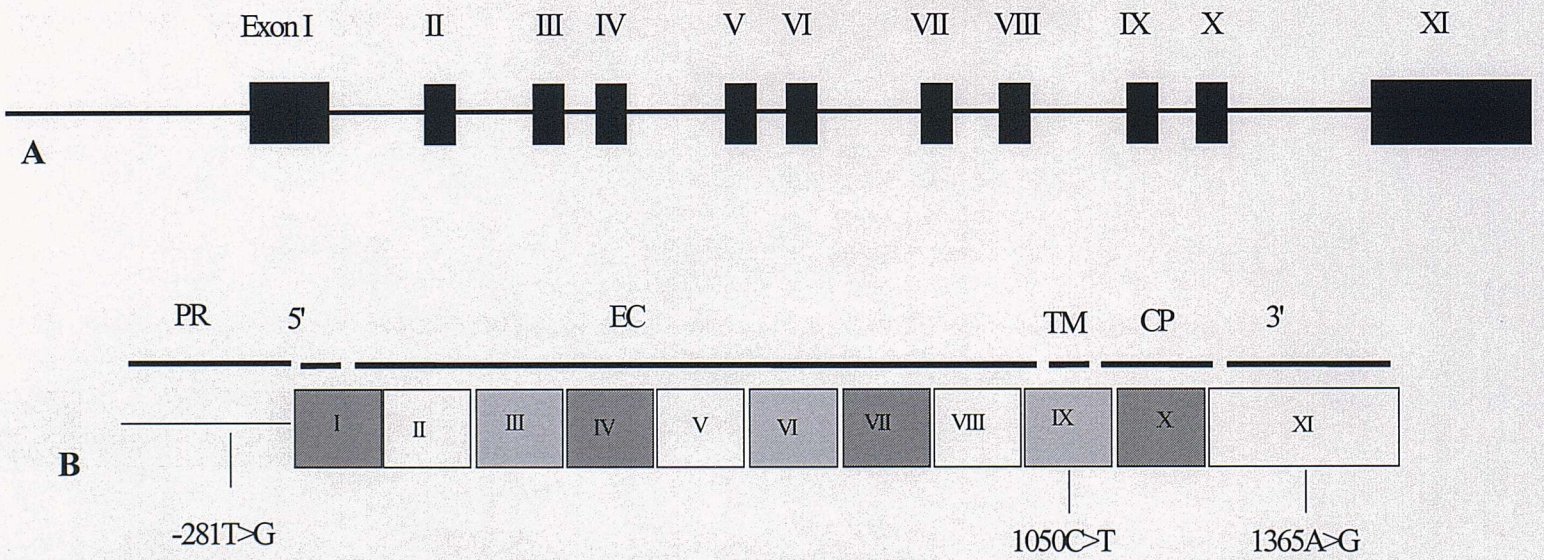

Figure 3.1. Schematic representation of the human *IL13RA1* gene structure. The eleven exons of the *IL13RA1* gene are represented as black boxes in the upper panel (A). The promoter (PR), 5' UTR, extracellular domain (EC), transmembrane domain (TM), cytoplasmic domain (CP), and 3' UTR, as well as the location of the *IL13RA1* polymorphisms are depicted in the lower panel (B).

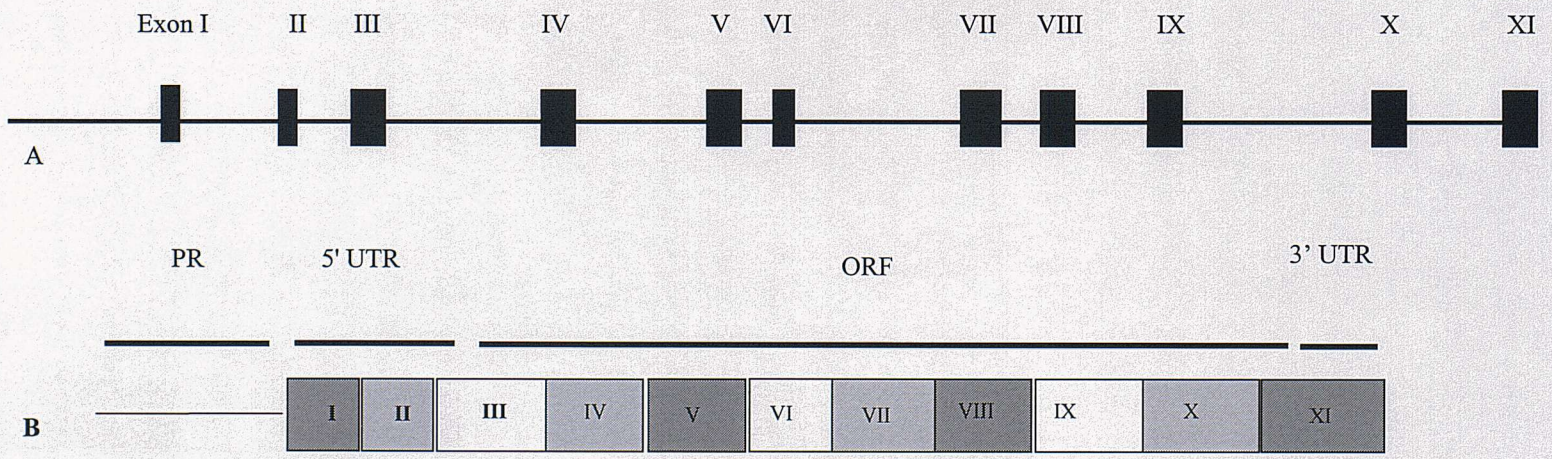

Figure 3.2. Schematic representation of the human *IL13RA2* gene structure in U118 cells. The exon/intron structure of the *IL13RA2* gene comprising eleven exons is shown in the upper panel (A), whereas the promoter region (PR), 5' UTR, open reading frame (ORF), and 3' UTR are depicted in the lower panel (B). (Modified from Wu A and Low WC: Molecular cloning and identification of the interleukin 13 alpa 2 receptor (IL-13Rα2) promoter. *Neuro-Oncology* 2003;5(3):179-87).

3.4. Subjects

3.4.1. Mutation screening of the *IL13RA1* promoter

SNP discovery in the *IL13RA1* promoter was carried out using genomic DNA from whole blood obtained from asthmatic subjects (n=12), and non-asthmatic individuals (n=26). All asthmatics and 6 non-asthmatic subjects were characterised according to symptoms, pulmonary function, and medication use (Table 3.1). Assessment of asthma severity was in accordance with the Global Initiative for Asthma guidelines on the diagnosis and management of asthma (Report, 1995). The mild asthmatics were receiving inhaled β_2-agonists only, while the moderate-severe group was maintained on inhaled corticosteroids, plus or minus long acting β_2 agonists. Subjects were tested for atopy using a panel of common aeroallergens, including house dust mite, cat hair, dog hair, grass pollen, Aspergillus fumigatus, Candida Albicans, and mixed tree allergens. BHR was assessed by histamine inhalation challenge and expressed as PC_{20} (the cumulative dose of histamine required to produce a fall in forced expiratory volume in 1 sec by 20% fall). The rest of the asthmatics were recruited on the basis of self-reported asthma and current use of asthma medication. All asthmatic subjects were non-smokers. Overall, the asthmatic group comprised 8 males (mean age: 30 years, range: 21-42) and 4 females (mean age: 27, range: 21-41). The non-asthmatic group comprised 12 males (mean age: 29.7 years, range: 21-42) and 14 females (mean age 30.5, range: 21-55). A total of 56 X chromosomes were screened for polymorphisms.

3.4.2. Mutation screening of the coding region of the *IL13RA1* gene

For mutation screening of the coding region of *IL13RA1*, total RNA was extracted from whole blood of asthmatic subjects (n=24) and non-asthmatic control individuals (n=23). 12 asthmatic and 6 non-asthmatic subjects were characterised according to symptoms, pulmonary function and medication (Table 3.1). The rest of the asthmatic subjects were recruited on the basis of self-reported asthma. Overall, the asthmatic group (n=24) comprised 11 males (mean age: 30, range: 21-42) and 13 females (mean age: 28, range: 21-41), whereas the non-asthmatic group comprised 11 males (mean age: 27, range: 21-41) and 12 females (mean age 31, range 21-55). A total of 47 X chromosomes were screened for polymorphisms.

3.4.3. Mutation screening of the coding region of the *IL13RA2* gene

Due to low mRNA expression of IL-13Rα2 in white blood cells (see section 5.3.2), mutation screening of the coding region of *IL13RA2*, total RNA was extracted from primary bronchial epithelial cell cultures established from asthmatic subjects (n=19) and non-asthmatic control individuals (n=9) obtained by bronchoscopy. All subjects were characterised according to symptoms, pulmonary function and medication (Table 3.2). Overall, the asthmatic group comprised fifteen males (mean age: 21 years, range: 19-55) and four females (mean age: 24, range: 19-29). The non-asthmatic group comprised seven males (mean age: 23 years, range: 20-33) and two females (29 and 45 years old). A total of 28 X chromosomes were screened for polymorphisms.

Table 3.1. Clinical characteristics of asthmatics and normal subjects used for mutation scanning of *IL13RA1*

Subject	Age	Sex	Atopy	Asthma severity	FEV_1 % Predicted	Histamine PC_{20}
GA1	21	F	None	None	95	>32
GA2	21	M	None	None	>100	>32
GA3	23	F	None	None	>100	>32
GA4	21	F	None	None	>100	>16
GA5	21	F	None	None	80%	>16
GA6	37	F	None	None	>100	>32
GB1	22	M	Yes	Mild	92	0.22
GB2	40	M	Yes	Mild	99.7	0.90
GB3	22	F	Yes	Mild	81	1.37
GB4	21	M	Yes	Mild	85	1.37
GB5	20	M	Yes	Mild	>100	0.32
GB6	23	F	Yes	Mild	67%	8
GB7	22	F	Yes	Mild	90	1.47
GC1	41	F	Yes	Moderate-Severe	78	0.76
GC2	35	M	Yes	Moderate-Severe	73	0.48
GC3	35	M	Yes	Moderate-Severe	77	0.7
GC4	42	M	Yes	Moderate-Severe	>100	2.12
GC5	25	M	Yes	Moderate-Severe	96	1.39

Table 3.2. Clinical characteristics of asthmatics and normal subjects used for mutation scanning of *IL13RA2*

Subject	Age	Sex	Atopy	Asthma severiry	FEV_1 % Predicted	Histamine PC_{20}
A1	26	M	Yes	Mild	89	<8
A2	29	F	Yes	Mild	-	
A3	19	M	Yes	Mild	91	<8
A4	20	M	Yes	Mild	-	<4
A5	23	M	Yes	Mild	-	
A6	21	M	Yes	Mild	68	-
A7	19	M	Yes	Mild	100	15.78
A8	37	M	Yes	Mild	99.7	0.9
A9	27	M	Yes	Mild	103	0.32
A10	19	F	Yes	Mild	94	>32
A11	20	F	Yes	Mild	83	5.7
A12	25	M	Yes	Mild	69	0.07
A13	21	M	Yes	Moder.-Severe	100	1.8
A14	55	M	Yes	Moder.-Severe	68	-
A15	28	M	Yes	Moder.-Severe	74.3	0.5
A16	39	M	Yes	Moder.-Severe	78	0.76
A17	22	M	Yes	Moder.-Severe	92	0.91
A18	28	F	Yes	Moder.-Severe	73	0.03
A19	21	M	Yes	Moder.-Severe	76	0.62
N1	45	F	No	None	100	>16
N2	20	M	No	None	104	>8
N3	21	M	No	None	100	>8
N4	20	M	No	None	110	>8
N5	25	M	No	None	100	>8
N6	33	M	No	None	100	>16
N7	29	F	No	None	100	>8
N8	21	M	No	None	100	>8
N9	22	M	No	None	100	>16

3.5. Results

3.5.1. PCR amplification of the *IL13RA1* promoter

Using genomic DNA, a 2.2 kb fragment of the 5' flanking region of *IL13RA1* containing the core *IL13RA1* promoter was generated by PCR (-1584 to +610 relative to the ATG start codon, GenBank accession: AL606485). The *IL13RA1* promoter has previously been characterised and was found to have high GC content (about 75% GC-rich) (Ise et al., 1999). Due to its high GC content, amplification of the promoter region by means of conventional PCR was challenging. Standard PCR procedures using either normal Taq or Hot Start PCR (Jump-Start, Sigma-Aldrich, Poole, UK) and various combinations of six forward and six reverse primers failed to amplify the region of interest, giving either a smeared band or no PCR product. Successful amplification was achieved by using the proof reading polymerase Pwo (Roche Applied Science, East Sussex, UK), DMSO, and 7-deaza-dGTP into the reaction mixture (Figures 3.3, 3.4, 3.5). DMSO and nucleotide analogs such as 7-deaza-dGTP have previously been shown to improve the PCR amplification by disrupting secondary DNA structures. Our experiments demonstrated that addition of DMSO in a range of 2.5% to 5%, as well as substitution of 37.5% of dGTP by 7-deaza-dGTP in a reaction mixture containing a combination of Taq and Pwo in a ratio of 30:1 were critical for the amplification of the GC-rich *IL13RA1* promoter.

3.5.2. Mutation screening of the *IL13RA1* promoter

Mutation screening of a 2.2 kb segment in the *IL13RA1* promoter was performed using SP-CCM. Heteroduplexes were modified using hydroxylamine, followed by piperidine treatment. Samples were run on an 8% denaturing polyacrylamide gel, since running on a standard 6% gel produced excessive smear due to the high GC-content of the amplicon. Nine out of thirty-eight samples showed cleavage products of 1 kb and 1.2 kb, whereas the full length fragment was 2.2 kb (Figure 3.6). Direct sequencing of all positive samples revealed an T to G nucleotide substitution at position –281 bp relative to the translation initiation codon (Figure 3.7). Although direct sequencing of GC-rich regions produces compression artefacts, replacement of 37.5% of dGTP by 7-deaza-dGTP produced high quality sequencing. The *IL13RA1* –281G allele was relatively abundant in our cohort at a frequency of q=0.35 in males

and q=0.3 in females. The potential functional role of the –281T>G polymorphism was explored using promoter analysis software (see section 2.11). Neither the wild type T allele nor the minor allele C were predicted to contain or to generate, respectively, any known transcriptional binding site.

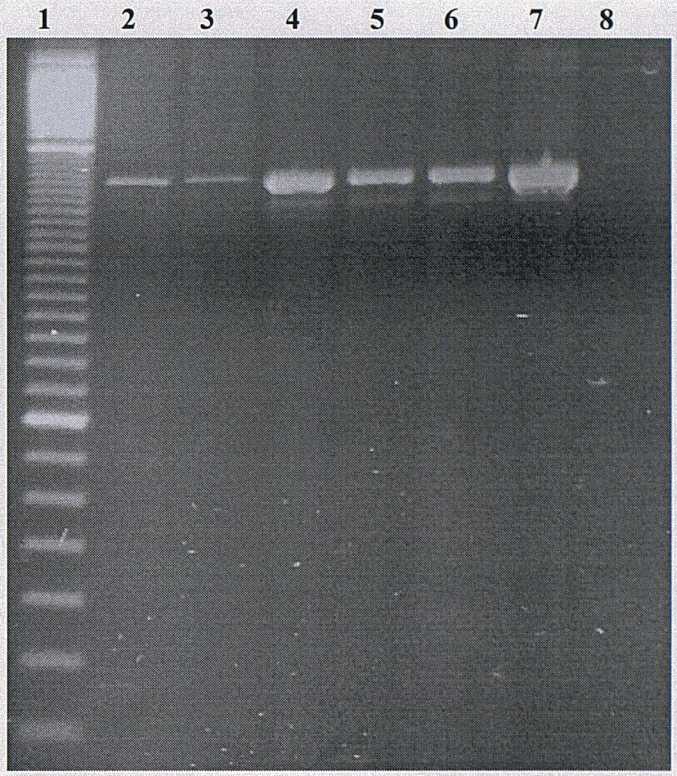

Figure 3.3. Effect of various DNA polymerases on PCR amplification of the *IL13RA1* promoter. A 2.2 kb segment of the 5' flanking region of the *IL13RA1* gene was amplified by PCR. The final reaction mixture contained 0.2 μM of each primer, 2mM $MgCl_2$, 5% DMSO, 0.2 mM dNTPs with 37.5% of dGTP substituted by 7-deaza-dGTP, and different enzymes in a final reaction volume of 50 μl. The PCR protocol consisted of a pre-PCR activation step (95°C, 3 min) followed by 38 cycles of denaturation (95°C, 30 sec), annealing (63°C, 30 sec), and extension (72°C, 2.5 min). Lane 1: DNA marker; lane 2: Taq 0.1 U/μl; lane 3: Jump-start 0.1 U/μl; lane 4; Pfu 0.1 U/μl; lane lane 5: Taq 0.1 U/μl plus Pfu 0.1 U/μl; lane 6: Jump-start 0.1 U/μl plus Pfu 0.1 U/μl; lane 7: Taq 0.1 U/μl plus Pwo 0.0033 U/μl; lane 8: negative control.

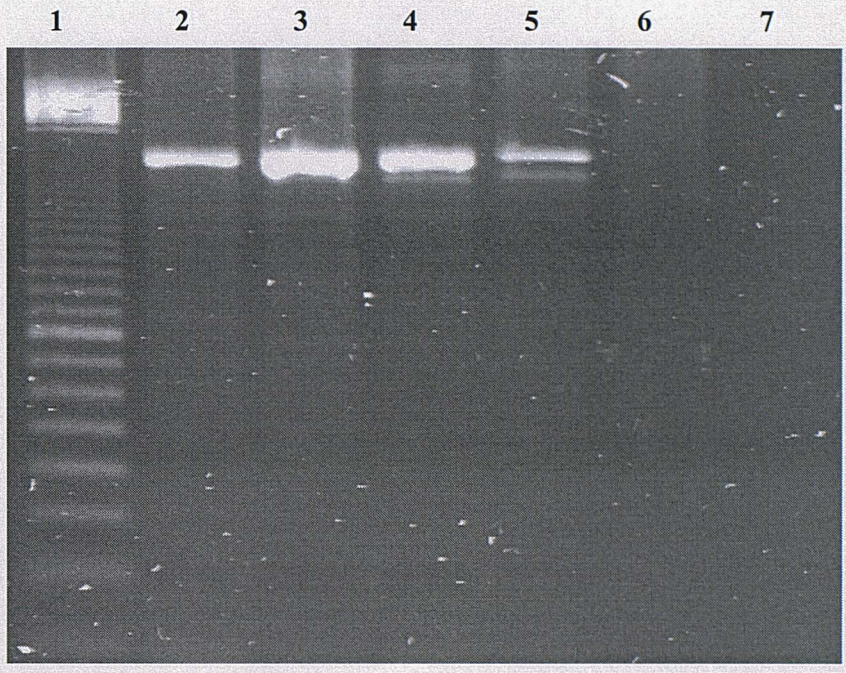

Figure 3.4. Effect of various DMSO concentrations on PCR amplification of the *IL13RA1* promoter. A 2.2 kb segment of the 5' flanking region of the *IL13RA1* gene was amplified by PCR. The final reaction mixture contained 0.2 μM of each primer, 2mM $MgCl_2$, 0.1 U/μl Taq plus 0.0033 U/μl Pwo, 0.2 mM dNTPs with 37.5% of dGTP substituted by 7-deaza-dGTP, and different DMSO concentrations in a final reaction volume of 50 μl. The PCR protocol consisted of a pre-PCR activation step (95°C, 3 min) followed by 38 cycles of denaturation (95°C, 30 sec), annealing (63°C, 30 sec), and extension (72°C, 2.5 min). Lane 1: DNA marker; lane 2: no DMSO; lane 3: 2.5% DMSO; lane 4: 5% DMSO; lane 5: 7.5% DMSO, lane 6: 10% DMSO, lane 7: negative control.

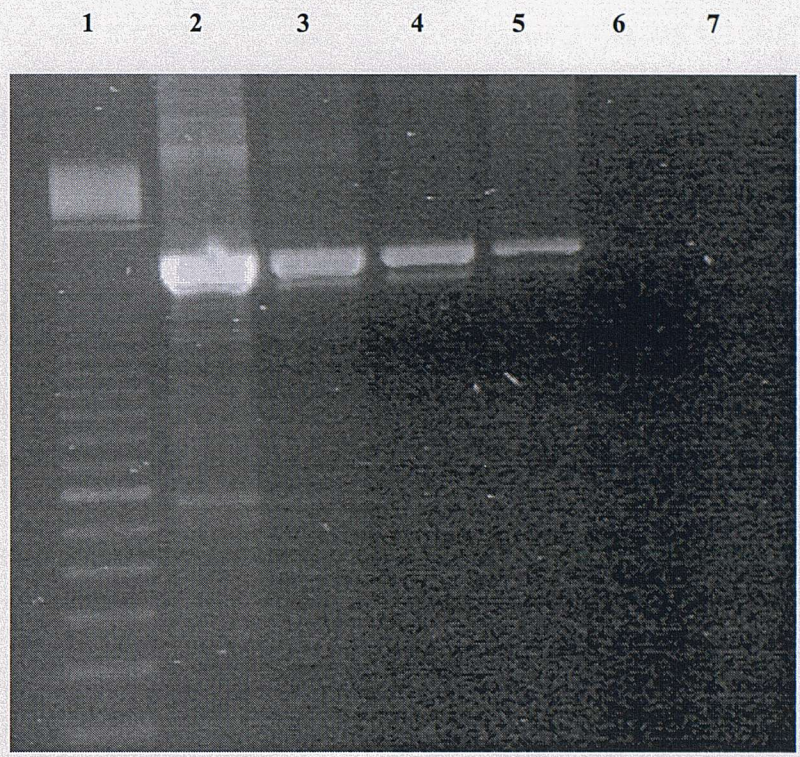

Figure 3.5. Effect of various 7-deaza-dGTP concentrations on PCR amplification of the *IL13RA1* promoter. A 2.2 kb segment of the 5' flanking region of the *IL13RA1* gene was amplified by PCR. The final reaction mixture contained 0.2 μM of each primer, 2mM $MgCl_2$, 0.1 U/μl Taq plus 0.0033 U/μl Pwo, 5% DMSO with different amounts of dGTP substituted by 7-deaza-dGTP in a final reaction volume of 50 μl. The PCR protocol consisted of a pre-PCR activation step (95°C, 3 min) followed by 38 cycles of denaturation (95°C, 30 sec), annealing (63°C, 30 sec), and extension (72°C, 2.5 min). Lane 1: DNA marker; lane 2: no 7-deaza-dGTP; lane 3: 37.5% 7-deaza-dGTP; lane 4: 50% 7-deaza-dGTP; lane 5: 75% 7-deaza-dGTP; lane 6: 100% 7-deaza-dGTP, lane 7: negative control. Note that there is excessive smear and a non-specific band when no 7-deaza-dGTP was included in lane 1. Specific amplification and high PCR yield was achieved with substitution of 37.5% dGTP by 7-deaza-dGTP in lane 3.

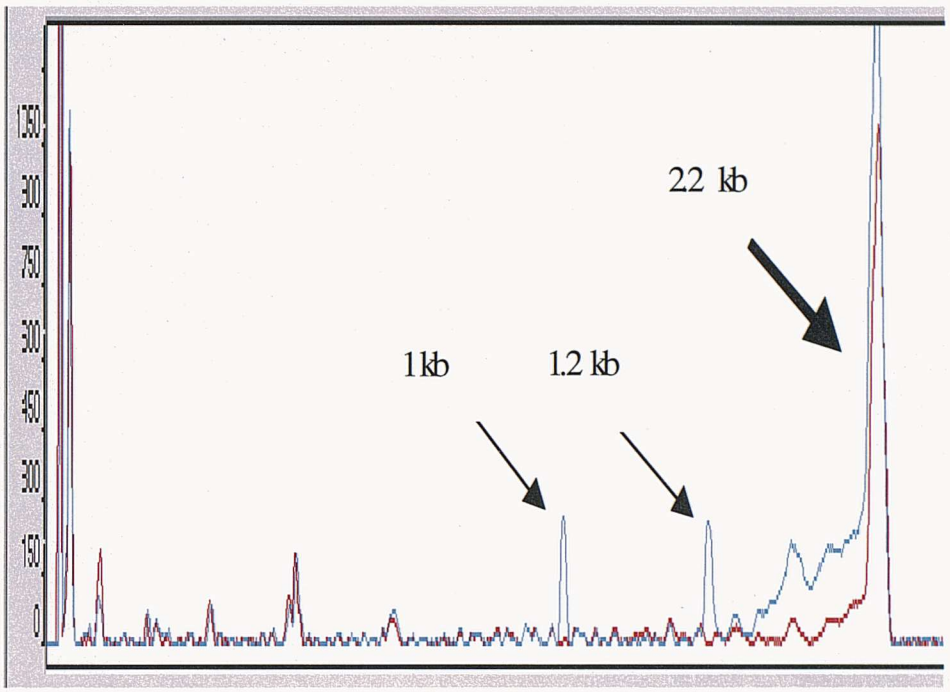

Figure 3.6. Identification of the *IL13RA1* –281T>G polymorphism by SP-CCM.

Heteroduplex DNA was initially modified by hydroxylamine and subsequently incubated with piperidine. Scans and analysis were obtained from an ABI PRISM 377 DNA sequencer. The vertical axis represents fluorescence intensity. Traces demonstrate fragments resolved by molecular weight on the horizontal axis with increasing size from left to right. The two thin arrows represent two fragments produced by cleavage of A/G and C/T mismatched heteroduplexes, whereas the thick arrow represents the full size amplicon.

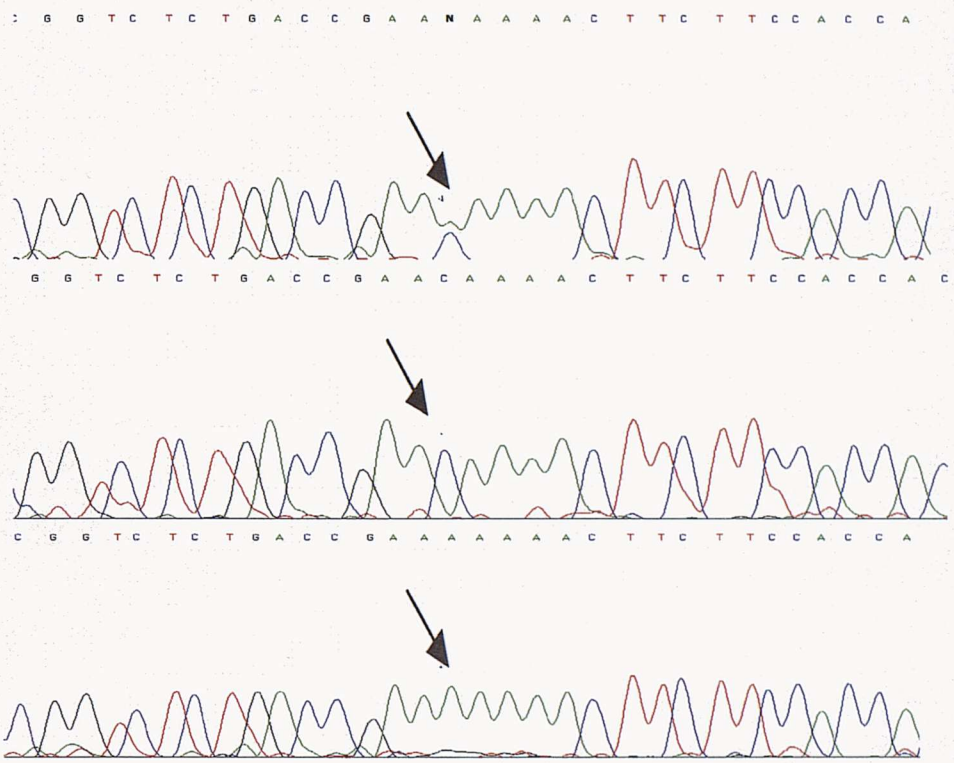

Figure 3.7. Identification of the *IL13RA1* -281T>G polymorphism by sequence analysis. The arrows indicate the *IL13RA1* –281T>G variant in the *IL13RA1* promoter. Figure shows the complementary 3' to 5' strand. From bottom to top: homozygous AA, homozygous CC and heterozygous AC.

3.5.3. Mutation screening of the coding region of *IL13RA1*

Using total mRNA extracted from forty-seven individuals, the coding region and the proximal 3' UTR of the *IL13RA1* gene were screened for the presence of common genetic variants by a combination of SP-CCM and DHPLC. The coding region of *IL13RA1* spans 1281 bp, and PCR amplification of the whole region with a single pair of primers resulted in a weak smeared band on agarose gel, not suitable for downstream applications. To increase PCR yield, the coding region was divided in three segments. Segment I spans from +38 to +301 relative to the translation initiation codon (exon 1 to exon 3), segment II spans from +238 to +1347 (exon 3 to exon 11), and segment III spans from +889 to +1443 (exon 11). Although SP-CCM has successfully been used for mutation analysis of fragments as small as 266 bp (Rowley et al., 1995), there is some concern, that cleavage peaks in such small fragments may not be visible. Therefore, due to its relatively small size of 263 bp, segment I (+38 to +301) was screened using denaturing high pressure liquid chromatography (DHPLC), (WAVE^{Tm} System, Transgenomics Limited, Crewe, UK). We did not identify any polymorphisms in this segment in all samples tested. Mutation scanning of segments II and III was carried out using SP-CCM. Heteroduplexes were treated with hydroxylamine and osmium tetroxide to modify mismatched cytosine and thymidine residues, respectively. Fluorescent reaction products were separated on a denaturing 6% polyacrylamide gel using an ABI PRISM 377 DNA sequencer. Mutation scanning of segment II (+238 to +1347) revealed cleavage products of 300 bp and 252 bp in two samples (Figure 3.8). The cleavage pattern was similar with both hydroxylamine and osmium tetroxide modification. Direct sequencing of samples revealed a C to T nucleotide substitution at position 1050 relative to the translation initiation codon ATG (Figure 3.9). This substitution is located at the third nucleotide of codon 350 and does not result in an amino acid alteration (Figure 3.10)

Mutation scanning of the third segment (+889 to +1443) revealed the presence of an identical cleavage pattern in 12 samples (Figure 3.11). Treatment of heteroduplexes with either hydroxylamine or osmium tetroxide resulted in similar cleavage patterns. Direct sequencing revealed an A to G nucleotide substitution at position 1365 relative to the translation initiation codon ATG (Figure 3.12). The polymorphism is located in the proximal 3' UTR, just 38 bp away from the last nucleotide of the coding region.

The *IL13RA1* 1365G allele was found at a frequency of q=0.15 in males and 0.10 in female.

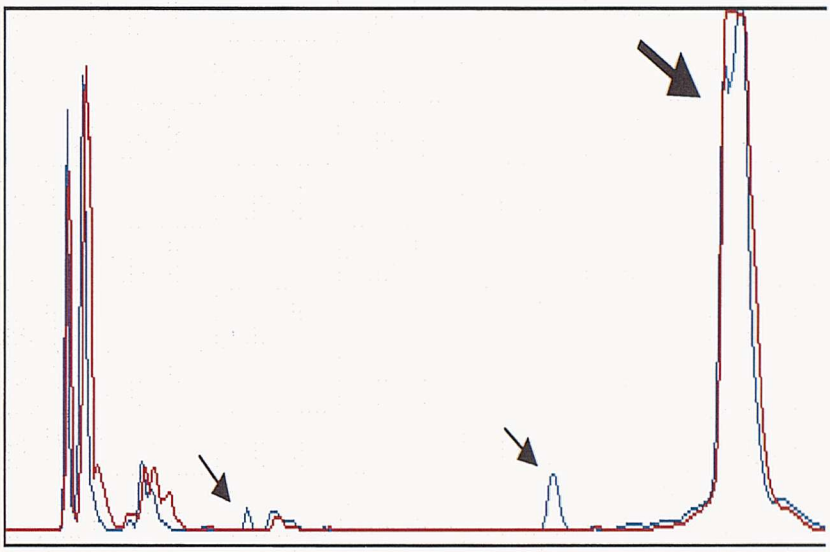

Figure 3.8. Identification of the *IL13RA1* 1050C>T polymorphism by SP-CCM.

The two thin arrows demonstrate the fluorescence traces produced after C/A and T/G mismatched DNA heteroduplexes were cleaved by piperidine followed by resolution of products on a 6% polyacrylamide sequencing gel. The thick arrow represents the fluorescence trace of the full amplicon. Smaller traces on the far left represent short primer-dimer products.

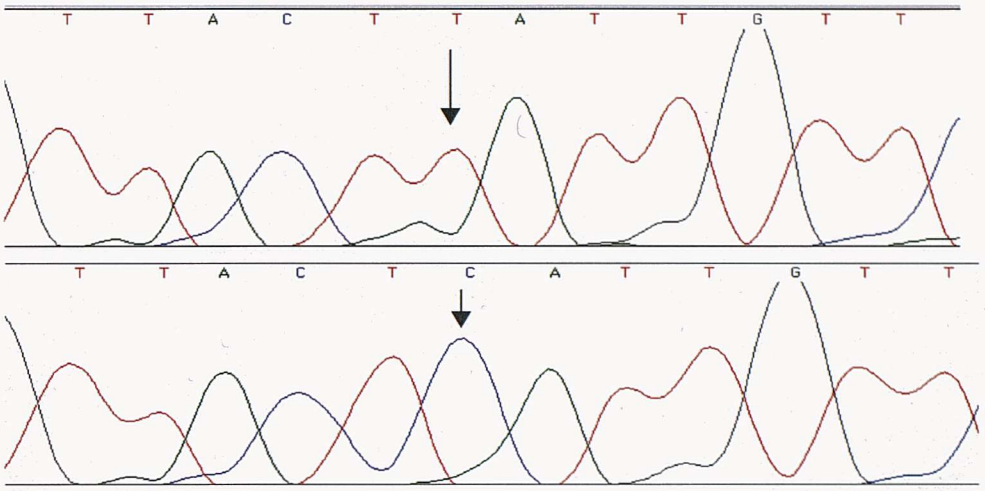

Figure 3.9. Identification of the *IL13RA1* 1050C>T SNP by direct sequencing. Arrows indicate a C-to-T nucleotide substitution found in the coding region of *IL13RA1* 1050 bp downstream of the translation initiation codon ATG. From bottom to top: homozygous CC, homozygous TT.

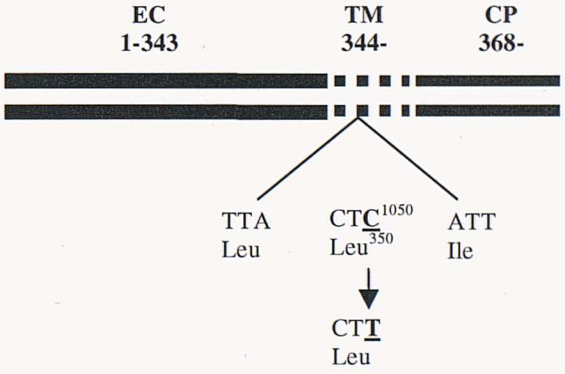

Figure 3.10. Schematic structure of the human IL-13Rα1 transcript and position of the *IL13RA1* 1050C>T polymorphism. The extracellular (EC), transmembrane (TM), and cytoplasmic domains of the *IL13RA1* gene are depicted. Amino acid numbers are shown below these names. The numbers on the amino acid and C nucleotide indicate their position with respect to the ATG start codon. (Redrawn from Ahmed S et al: Novel polymorphism in the coding region of the IL-13 receptor alpha' gene: association study with atopic asthma in the Japanese population. *Exp Clin Immunogenet* 2000;17:18-22.)

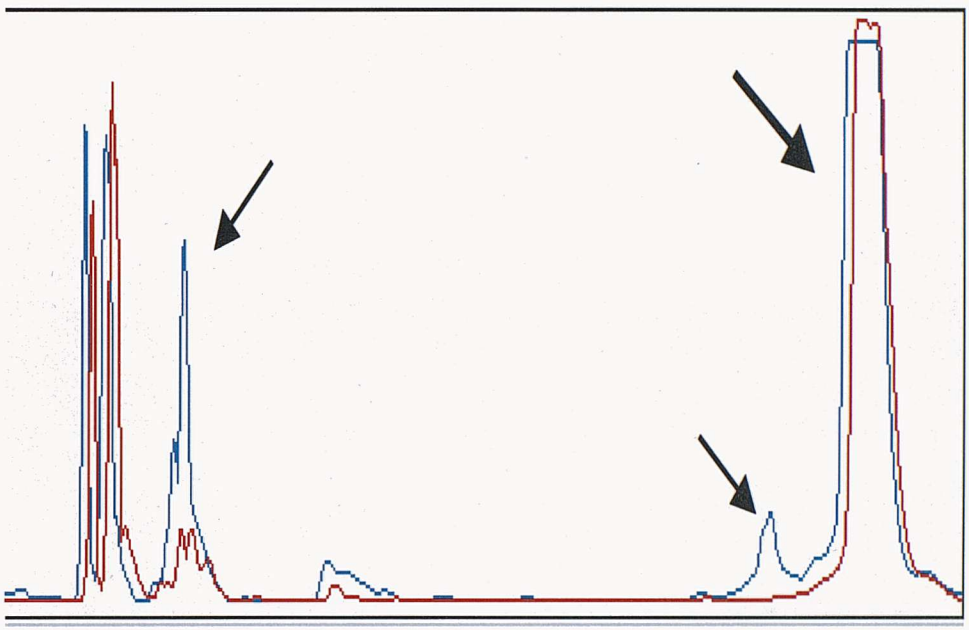

Figure 3.11. Identification of the *IL13RA1* 1365A>G polymorphism by SP-CCM.

Heteroduplex DNA was initially modified by hydroxylamine and was subsequently incubated with piperidine. Traces demonstrate fluorescence intensity after fragments were resolved by molecular weight, as shown on the horizontal axis with increasing size from left to right. Scans and analysis were obtained by ABI PRISM 377 DNA Sequencer. The two thin arrows represent two fragments produced by cleavage of A/C and G/T mismatched DNA heteroduplexes, whereas the thick arrow represents the full size amplicon.

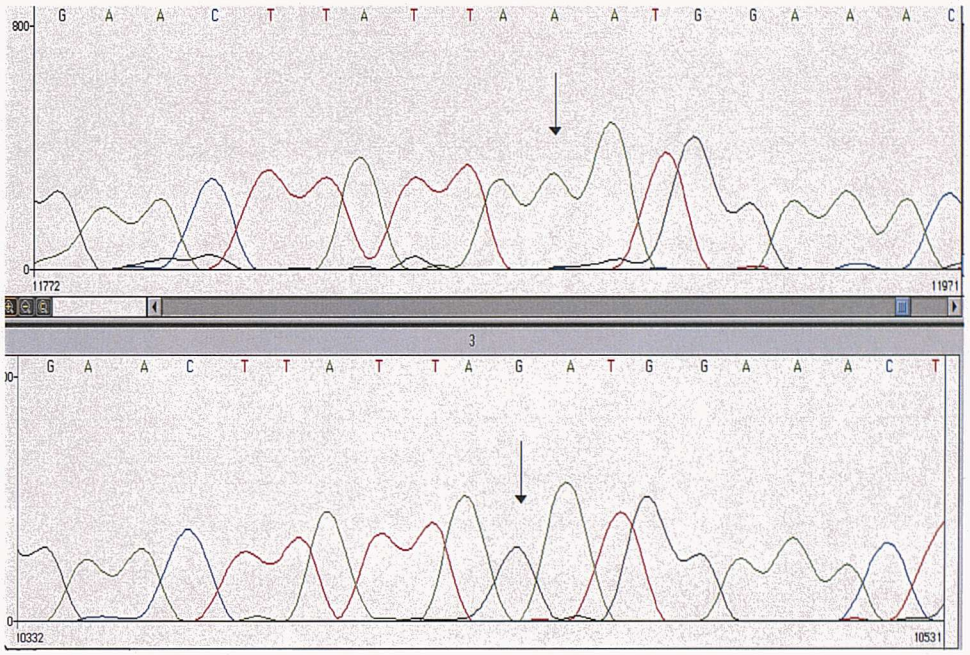

Figure 3.12. Identification of the *IL13RA1* 1365A>G polymorphism by sequence analysis. The arrows indicate an A to G nucleotide substitution in the proximal 3' UTR of the *IL13RA1* gene. From bottom to top: homozygous GG, homozygous AA.

3.6. Mutation screening of the coding region of *IL13RA2*

SNP detection of the coding region of *IL13RA2* was carried out using RNA from primary bronchial epithelial cultures obtained from asthmatic subjects (n=19) and non-asthmatic control individuals (n=9) by fiberoptic bronchoscopy. An extra sample derived from the bronchial epithelial cell line BEAS-2B. A total of 29 X chromosomes were screened for polymorphisms, including the sample from BEAS-2B cells. Whole blood could not serve as a source of RNA, since the expression of IL-13Rα2 in the white blood cells by means of RT-PCR was found to be very weak (see section 5.3.2). The coding region of *IL13RA2* was targeted for SNP discovery based on GenBank reference sequence Y08768. Amplification of the whole coding region of *IL13RA2*, spanning 1140 bp, using a single pair of primers resulted in a weak product as seen on agarose gel, not appropriate for downstream applications. Therefore, the coding region was divided into two segments to increase the PCR yield. Segment I spans from –95 to +313 relative to the ATG start codon, whereas segment II spans from +305 to +1195. Mutation scanning was carried out using SP-CCM, and heteroduplexes were treated with hydroxylamine and osmium tetroxide.

We found no cleavage products in samples derived from either asthmatic subjects or non-asthmatic control individuals. However, a cleavage peak was detected in the sample derived from the BEAS-2B cells. Direct sequencing of the sample revealed a G to A nucleotide substitution at position 1045 relative to the translation initiation codon, compared to the reference sequence (Figure 3.13). This substitution is located in the first nucleotide of codon 349, resulting in an amino acid alteration in which glycine is substituted for serine in the transmembrane domain of the *IL13RA2* gene (Figure 3.14). We reconfirmed the presence of the polymorphism by direct sequencing of genomic DNA extracted from BEAS-2B cells. We subsequently investigated whether this might be a rare polymorphism, by direct sequence analysis of DNA samples derived from 30 non-related adult healthy individuals. The 1045G>A polymorphism was not found in any of the samples analysed.

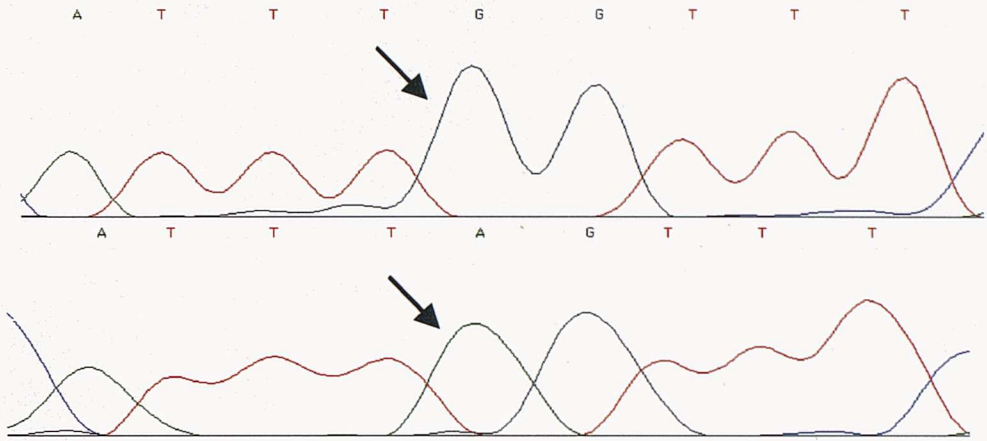

Figure 3.13. Identification of the *IL13RA2* 1045G>A variant in BEAS-2B cells by sequence analysis. The arrows indicate the polymorphic site found in *IL13RA2* cDNA derived from BEAS-2B cells. From bottom to top: allele A, allele G.

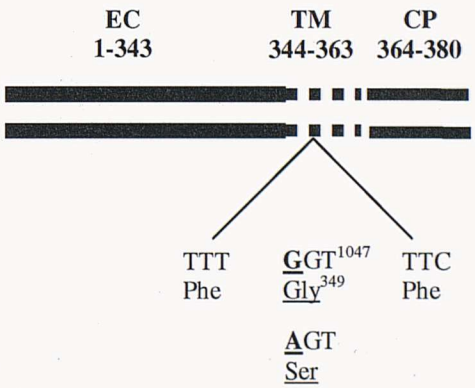

Figure 3.14. Schematic structure of the human *IL13RA2* transcript and position of the 1045G>A polymorphism in BEAS-2B cells. The extracellular (EC), transmembrane (TM), and cytoplasmic domains are depicted. Amino acid numbers are shown below these names. The numbers on the amino acid and T nucleotide indicate their position with respect to the ATG start codon.

3.7. Genotyping assays for the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms

We have developed genotyping assays for the *IL13RA1* -281T>G and the *IL13RA1* 1365A>G polymorphisms using tetra-primer ARMS-PCR. ARMS-PCR adopts certain principles of the tetra-primer PCR method and the amplification refractory mutation system (ARMS) (Ye et al., 2001). For each assay, two primers that are complementary to unique sequences around each polymorphism and two primers that are designed for the allele specific amplification of the –281T>G and the 1365A>G polymorphisms were combined in a single tube. The assay for the –281T>G polymorphism yields a 373 bp control PCR band, a 256 bp band for the T allele, and a 172 bp band for the G allele (Figure 3.15). The assay for the 1365A>G polymorphism yields a 423 bp control PCR band, a 226 bp band for the A allele, and a 315 bp band for the G allele (Figure 3.16). Assays for each polymorphisms were validated by direct sequencing of twelve random samples including wild type homozygous, mutant homozygous, and heterozygous individuals. Results of genotyping were in entire agreement with results of direct sequencing for all samples tested.

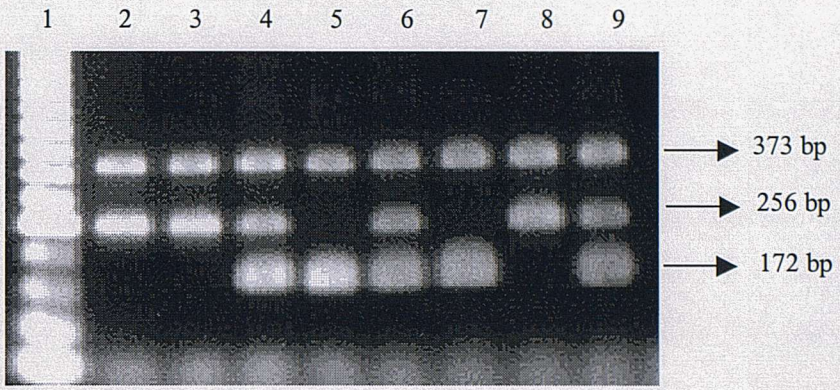

Figure 3 .15. Genotyping o f t he *I L13RA1* – 281T>G p olymorphism u sing t etra-primer ARMS-PCR assay. Electrophoresis gel showing size markers in lane 1. In lanes 2, 3, and 8 the control band (373 bp) and a 256 bp band are visible for homozygotes carrying two T alleles. In lanes 5 and 7, the control band plus a 172 bp band for homozygotes carrying two G alleles are shown. In lanes 4, 6, and 9 the control band plus two bands of 256 bp and 172 bp are visible for TG heterozygotes.

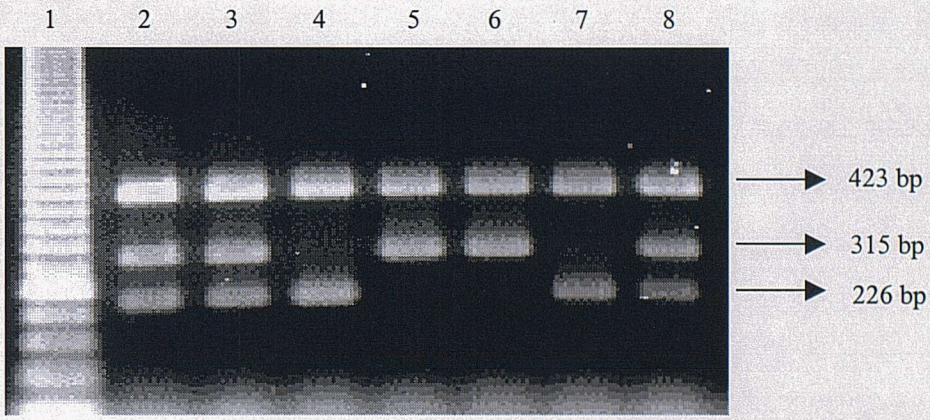

Figure 3.16. Genotyping of the *IL13RA1* 1365A>G polymorphism using tetra-primer ARMS-PCR assay. Electrophoresis gel showing size markers in lane 1. In lanes 2, 3 and 8, the control band (423 bp) plus two bands of 315 bp and 226 bp are visible for AG heterozygotes. In lanes 4 and 7, the control band plus a band of 226 bp for homozygotes carrying two A alleles are shown. In lanes 5 and 6, the control band and a band of 315 bp are visible for GG homozygotes.

3.8. Discussion

3.8.1. Mutation screening

During the past few years there has been an increasing number of novel mutation detection methods. Mutation screening methods routinely used for mutation analysis include the denaturing gradient gel electrophoresis (DGGE) and the single-strand conformation polymorphisms (SSCP). In DGGE, DNA homoduplexes and heteroduplexes are run under denaturant concentration gradient using different concentrations of formamide and urea across an acrylamide gel (Myers et al., 1987). The melting point of each duplex will differ if a mutation is present, therefore, duplexes would be separated in the gel. DGGE has a high sensitivity, however, the method has disadvantages, including the small fragment size that can be scanned (100-500 bp), the costly GC clamps that are sometimes needed, and the time consuming analysis of melting characteristics by computation (Cotton, 1997). SSCP is a popular mutation detection method due to its technical simplicity. The principle of the method entails the different folding of single strand DNA depending on base change, with segments moving at different rates on electrophoresis gel (Orita et al., 1989). However, a drawback of SSCP is the variability in the detection rate depending on fragment size, gel temperature, and gel matrix conditions. Detection rate can improve when several conditions are applied, but this holds true as long as small fragments up to 350 bp are analysed (Hayashi and Yandell, 1993). Sheffield and co-workers found a detection rate of 79% in a study of 64 mutations in three genes (Sheffield et al., 1989). Moreover, SSCP gives no positional information of the polymorphism.

We used denaturing high-performance liquid chromatography (DHPLC) to screen a small segment of the *IL13RA1* coding region. DHPLC compares two or more DNA segments as a mixture of denatured and reannealed PCR amplicons, revealing the presence of a mutation by the differential retention of homo- and heteroduplex DNA on a DNA separation matrix (Underhill et al., 1997). The optimum temperature for the detection of mismatches is determined by means of computation, and maximum detection is achieved when amplicons do not exceed 600bp in length. Drawbacks of DHPLC involve the high cost of the equipment and buffers. Several studies using

DHPLC as a mutation screening method have reported sensitivity and specificity between 95% and 100% (Xiao and Oefner, 2001).

We used SP-CCM as our main mutation detection technique to screen the *IL13RA1* and the *IL13RA2* genes for novel polymorphisms. Chemical cleavage of mismatches, initially described by Cotton and co-workers in 1988 (Cotton et al., 1988), involves treatment of DNA heteroduplexes (following mixing of probe and target DNA) with osmium tetroxide or hydroxylamine, which lead to preferential cleavage at T and C mismatches, respectively, upon subsequent treatment with piperidine. This cleavage is detected by electrophoresis with the size of the fragments indicating the position of the mismatch. The exact polymorphism is subsequently determined by direct sequencing. When the method was first introduced samples were initially radiolabelled for increased sensitivity. The method was later improved by using fluorescent primers in conjunction with analysis on an automatic sequencer (Haris et al., 1994, Verpy et al., 1994). A solid phase protocol using internal labelled of probe and target DNA has significantly simplified the method (Rowley et al., 1995). The solid phase procedure avoids multiple precipitation steps, requires less starting amount of DNA, and significantly decreases labour intensiveness. In addition, fluorescence internal labelling of both probe and target increases the sensitivity and ensures the detection of most mutations by a single chemical treatment, either hydroxylamine or osmium tetroxide, because in most cases one of the two reciprocal heteroduplexes formed by hybridisation of target to probe has a C and the other a T involved in the mismatch. The method has shown to be extremely sensitive with 100% detection power during the characterisation of 400 hemophilia B mutations (Green et al., 1991, Saad et al., 1994). Additional advantages of the method include the potential of screening large segments up to 3 kb, and the precise location of the polymorphisms within the segments analysed (Cotton et al., 1988). The main drawback of the method involves the hazardous nature of the chemicals, especially osmium tetroxide, requiring use of a fume-cupboard.

3.8.2. Genotyping assays

The –281T>G and the 1365A>G polymorphisms in the *IL13RA1* gene were genotyped using tetra-primer ARMS-PCR, combining four primers in a single tube for each assay (Ye et al., 2001). Both inner primers of the tetra-primer ARMS-PCR method encompass an allele-specific mismatch at 3'-terminal base, as well as a deliberate mismatch at position –2 from the 3'-terminus to increase specificity. Additional specificity was added by a touchdown PCR amplification, in which the starting annealing temperature was 72°C and the final one 63°C. In contrast to the standard ARMS method, which involves two separate PCR reactions to amplify the two different alleles of a SNP, the tetra-primer PCR method allows parallel genotyping of both alleles in a single tube. Increased throughput was achieved in our study by combining this method with the microplate array diagonal gel electrophoresis (MADGE) technique, which allowed 96 samples to be loaded and electrophoresed in a 96-well microplate (Day and Humphries, 1994).

The genotyping assays developed in our study were entirely reliable and robust. Firstly, genotype frequencies in our cohorts were in Hardy-Weinberg equilibrium, and secondly, direct sequencing of random samples confirmed the results of genotyping assays. Additional advantages of our genotyping assays include the simplicity and the low cost of the method, since it did not require expensive equipment and did not involve significant post-PCR manipulations (Ye et al., 2001). For example, restriction fragment length polymorphism (RFLP) involves restriction endonuclease digestion of PCR products, whereas allele-specific oligonucleotide (ASO) hybridisation (Wallace et al., 1979) involves tedious optimisations and lengthy blotting procedures. Recent approaches for high throughput SNP typing, including solution-phase hybridisation reactions with fluorescence detection (Taqman^{Tm} and Molecular Beacon assays), primer extension using microarrays, and multiplex detection using ligation methods (Syvanen, 2001), require expensive equipment and costly probes.

3.8.3. PCR amplification of the *IL13RA1* promoter

Amplification of the *IL13RA1* promoter was challenging due to the high GC content of the region. GC-rich regions cause secondary structures, leading to low PCR yield and amplification of undesired non-specific products. A widely used method for improving yield and specificity during PCR involves the addition of organic additives to the reaction mixture, such as dimethylsulfoxide (DMSO), betaine, polyethylene glycol, glycerol, and formamide (Winship, 1989, Bachmann et al., 1990, Pomp and Medrano, 1991, Weissensteiner and Lanchbury, 1996). It has been proposed that organic additives like DMSO enhance PCR by hydrogen bonding to the major and minor grooves of template DNA and destabilising the double-helix (Varadaraj and Skinner, 1994). DMSO is usually used in the range of 2.5-10% in the reaction mixture. Addition of DMSO alone in our PCR mixture, in a range of 2.5 to 5%, improved PCR amplification, however, it did not achieve maximum specificity and gave a smeared product on the agarose gel. Successful amplification of GC-rich regions is also achieved by substitution of the dGTP by the nucleotide analogue 7-deaza-dGTP (Cao et al., 1994). The use of 7-deaza-dGTP disrupts secondary structure in most DNA sequences, since 7-deaza-dGTP forms weaker base pairs with dCTP. In addition to enhancing PCR amplification, 7-deaza-dGTP analogues resolve sequencing gel compressions and improve electrophoretic separation during DNA sequencing. In our experiments, substitution of 37,5% or 50% of the dGTP by 7-deaza-dGTP gave clear bands. Our results are in accordance with a previous study, showing that successful amplification of 10-12 kb GC-rich segments in the factor VIII gene required 25%-50% 7-deaza-dGTP (Liu and Sommer, 1998). Moreover, we found that the addition of a proof reading enzyme was also critical for the PCR amplification. The combination of Taq and Pwo in a ratio of 30:1, and to a lesser extent that of Taq and Pfu in a ratio of 1:1, improved PCR specificity and increased PCR yield.

3.8.4. Mutation scanning of the *IL13RA1* promoter

Mutation scanning of a 2.2 kb region in the 5' flanking region of *IL13RA1* revealed a novel T to G substitution at position –281 with respect to the first nucleotide of the open reading frame. We have used only hydroxylamine for mutation scanning of the *IL13RA1* promoter, and therefore, we may have missed an A to T transversion that is not detected

by hydroxylamine. However, this is a remote possibility given that hydroxylamine alone can detect approximately 95% of all potential mutations (Rowley et al., 1995). The *IL13RA1* –281T>G polymorphism was abundant in our Caucasian cohort, at a frequency of q=0.30 for the minor allele. Promoter polymorphisms in a number of cytokine receptors have been shown to have major effects. A promoter polymorphism in the *IL4RA* gene was found to be associated with lower levels of soluble IL-4Rα (Hackstein et al., 2001), whereas the –159 polymorphism in the *CD14* promoter was associated with higher soluble CD14 levels (Baldini et al., 1999) and raised serum total IgE levels in skin test-positive individuals (Koppelman et al., 2001).

Although promoter analysis software did not predict the presence of any known binding site for either alleles of the *IL13RA1* –281T>G polymorphism, this variant may have a functional role. Further promoter reporter studies are needed to clarify the potential role of the –281T>G SNP on the trancriptional regulation of the *IL13RA1* gene.

3.8.5. Mutation scanning of the coding region of the *IL13RA1* gene

We used SP-CCM as a scanning method to identify polymorphisms in the coding region of *IL13RA1*. Only a small segment (238 bp long), including the terminal portion of exon 1, the whole exon 2 and the proximal portion of exon 3, was screened using DHPLC, due to its small size. We evaluated the polymorphism of *IL13RA1* by examining 47 X chromosomes derived from 47 individuals (twenty-two males and twenty-five females). Due to the X localisation of *IL13RA1*, only a single X chromosome exists in males, whereas in females, the X inactivation process leaves only a single chromosome copy functional (Migeon, 1994). The 47 X chromosomes screened for polymorphisms in the coding region of *IL13RA1* provided adequate power to detect common SNPs with allelic frequency more than 0.05.

Only the silent *IL13RA1* 1050C>T SNP, previously identified in a Japanese population (Ahmed et al., 2000), was found in the coding region of *IL13RA1*. The presence of a single SNP in the 1.4 kb coding region of *IL13RA1* is in accordance with the density of SNPs found in human exons, which is approximately 1 SNP per 1.08 kb, according to the

latest data from the Human Sequencing Consortium (Sachidanandam et al., 2001). The frequency of the 1050C allele was very low in our cohort, in agreement with the study of Ahmed and co-workers who also found a low frequency for the minor allele (q=0.005 in males, and q=0.03 in females) (Ahmed et al., 2000). We have also identified the previously reported *IL13RA1* 1365A>G variant (referred to as 1398A>G in this study) in the proximal 3' UTR, 38 bp away from the last nucleotide of the coding region (Heinzmann et al., 2000). The 1365G allele had a frequency of q=0.15 for both males and females in our cohort.

3.8.6. Mutation scanning of the coding region of the *IL13RA2* gene

No common variants in the coding region of the *IL13RA2* gene were found among 28 X chromosomes analysed. This is in accordance with a previous study, which found no common genetic variants in the coding region of *IL13RA2* in 18 human glioblastoma multiform cell lines (Kawakami et al., 2000). The number of chromosomes analysed for SNP detection in the coding region of *IL13RA2* provided modest power to detect common genetic variants and we may have missed some rare variants. The number of chromosomes analysed for *IL13RA2* SNP detection was lower compared to *IL13RA1*, since samples for *IL13RA2* mutation analysis were obtained by fiberoptic bronchoscopy from volunteers. As a result, the time and expenses needed to recruit individuals had a negative impact on the total number of volunteers.

Only a rare amino-acid altering polymorphism (Gly349Asp) in the coding region of *IL13RA2* was identified in a sample derived from the bronchial epithelial cell line BEAS-2B. We examined 30 healthy individuals (half males) for the presence of this polymorphism using bi-directional sequencing. The Gly349Asp polymorphism was not found in any of the samples, suggesting that this is either a rare polymorphism within the Caucasian population, or alternatively, it may be present only in BEAS-2B cells.

The absence of common genetic variants in the coding region of the *IL13RA2* gene is not surprising. Cargill and co-workers (Cargill et al., 1999) screened the coding and adjacent non-coding regions in 106 genes relevant to cardiovascular, endocrinology and

neuropsychiatry disease by screening an average of 114 chromosomes. Although many genes exhibited considerable variation, for example, 29 SNPs were identified in the coagulation factor V gene (F5), no SNPs were identified in the coding and intronic regions of 13 genes, a rate of approximately 10% of the genes examined.

Summary

- Mutation screening of *IL13RA1* revealed a novel T to G substitution at -281 in the promoter region. The *IL13RA1* –281T>G polymorphism was abundant in our Caucasian cohort, at a frequency of q=0.30.

- Two previously identified silent transitions in the *IL13RA* gene, the 1050C>T SNP in the coding region and the *IL13RA1* 1365A>G variant in the 3' UTR, respectively, were found during mutation analysis.

- In accordance with a previous study, no common genetic variants in the coding region of the *IL13RA2* gene were found.

CHAPTER 4

Genetic association of the –281T>G and the 1365A>G polymorphisms in the *IL13RA1* gene with asthma and asthma-related phenotypes

4.1 Overview

The –281T>G and the 1365A>G polymorphisms in the *IL13RA1* gene can potentially modulate IL-13Rα1 transcription activity and mRNA stability, respectively. A previous study has found association between the 1365A>G polymorphism and raised serum total IgE levels in a British population. We examined the two common genetic variants of *IL13RA1*, -281T>G and 1365A>G, for genetic association with asthma and asthma-related phenotypes.

Results: Using genotype data from 341 asthma-enriched families and 184 non-asthmatic control subjects in conjunction with TDT and case-control analysis, neither –281T>G nor 1365A>G were identified as important determinants for the development of asthma or related phenotypes. Only a borderline association between the *IL13RA1* –281T to 1365A haplotype and raised serum total IgE levels in adult female asthmatics was found.

IL-13 and IL-4 play an important role as effector molecules in asthma through many mechanisms, including induction of IgE synthesis by B cells (Del Prete et al., 1988, Defrance et al., 1994), airway eosinophilia (Bochner et al., 1995), goblet cell metaplasia, mucus hypersecretion (Dabbagh et al., 1999, Zhu et al., 1999), and airway remodelling through release of TGF-β2, a potent fibrogenic, from bronchial epithelial cells (Richter et al., 2001). In mouse models, targeted pulmonary expression of IL-13 in transgenic mouse caused mononuclear and eosinophilic inflammation, mucus secretion, subepithelial fibrosis, eotaxin production, and increase in airways resistance and BHR to metacholine (Zhu et al., 1999). In murine models of experimental asthma, administration of recombinant IL-13 to the airways of non-immunised mice induced pulmonary eosinophilia and BHR, whereas blockade of IL-13 resulted in a complete reversal of allergen-induced BHR (Wills-Karp et al., 1998). In another study, blockade of endogenous IL-13 resulted in significant attenuation of the asthma phenotype in OVA sensitised mice (Grunig, 1998 #396). Genetic variants of IL-13 were found to be strong determinants of serum total IgE levels and asthma. The *IL13* Arg110Gln polymorphism was found to be strongly associated with increased serum total IgE levels among Caucasian children (Graves et al., 2000) and asthma in adult

British and Japanese populations (Heinzmann et al., 2000), whereas the *IL13* –1111C>T polymorphism was found to be associated with asthma (van der Pouw Kraan et al., 1999, Howard et al., 2000), as well as BHR and skin-test responsiveness (Howard et al., 2000).

In view of the detrimental effects of IL-13 on the airways, the association of *IL13* variants with asthma-related phenotypes, as well as the previously reported association of the *IL13RA1* 1365A>G polymorphism with raised serum total IgE levels, we hypothesised that the *IL13RA1* –281T>G and the 1365A>G variants may predispose to the development of asthma and/or predict asthma severity. To test this hypothesis, we evaluated these variants for evidence of association with the diagnosis of asthma and asthma-related phenotypes in a large cohort of 341 asthmatic families and a cohort of 184 non-asthmatic control subjects using two methods: the transmission disequilibrium test (TDT) and case-control analyses.

4.2. Experimental design

4.2.1. SNP genotyping

Genotyping of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G SNPs in our cohorts was carried out using tetra-primer PCR-assays. The development of these assays is described in detail in section 3.7.

4.2.2. Subjects and clinical assessment

We assessed potential associations between the –281T>G and the 1365A>G variants in the *IL13RA1* gene with asthma and asthma-related traits in a large cohort of 341 Caucasian families previously recruited from the Southampton area of the UK (Van Eerdewegh et al., 2002). The families were selected for at least two biological siblings (age 5-21 years) with a current physician diagnosis of asthma who were taking asthma medication on a regular basis. Serum total IgE levels, specific IgE levels for grass, house dust mite, cat, dog, *Alternaria,* and tree allergens were determined by Pharmacia CAP System™. Skin prick testing was also completed for the same common allergens. At least 14 days after any respiratory tract infection, bronchodilator or anti-allergic medication, baseline lung function tests (FEV_1, best of 3 values within 5%) were performed by Vitalograph® dry wedge bellows spirometer.

BHR was measured as the provocation concentration of inhaled methacholine required to reduce FEV_1 by 20% (PC_{20} FEV_1). Non-asthmatic controls with no family history of respiratory disease were recruited from the same Southampton area as the main study through blood donor clinics (mean age = 42.3 years (S.D 10.6), n = 184). The phenotypic characteristics of the study cohorts are shown in Table 4.1

Table 4.1. Phenotypic characteristics of study cohorts

Assessment	Pedigrees (n=1508)	Parents (n=681)	Non-asthmatic parents (n=492)	Asthmatic parents (n=184)	Sibling 1 (n=341)	Sibling 2 (n=338)	Controls (n=184)
Age (mean)	24.6	40.5	40.7	40.2	13.0	9.9	42.3
Age (range)	-	27.3 to 67.5	-	-	6.1 to 22.6	5.0 to 18.1	-
Gender (% male)	51.8	49.9	51.0	47.1	56.9	53.6	47.0
Asthma (%, doctor)	60.1	27.8	0	100	100	100	0
Eczema (%, questionnaire)	45.6	32.7	25.8	50.8	57.8	62.4	0
Hayfever (%, questionnaire)	48.9	46.8	38.0	69.8	64.2	47.0	8.7
Asthma symptom score	15.21	8.21	4.01	19.13	23.47	23.44	ND*
Drug score	1.9	0.65	0.08	2.20	3.40	3.45	ND
FEV_1 (% predicted)	98.05	100.81	103.39	94.12	94.74	95.62	ND
BHR (1/LSlope+30)*1000	19.03	24.33	26.79	17.22	14.55	12.00	ND
log IgE (age corrected)	1.25	0.64	0.49	1.01	1.84	1.93	ND
n	1508	681	492	189	341	338	184

* ND: Not defined

4.2.3. Transmission Disequilibrium Test

The potential association of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G variants with asthma and asthma-related phenotypes was assessed using the Transmission Disequilibrium Test (TDT). This was implemented using STATA 6.0 (Stata Corporation, Texas, USA) and a TDT program written in STATA by David Clayton (MRC Biostatistics Unit, Cambridge, UK). Any test with a *P* value <0.05 was considered significant. Dichotomous variables analysed using TDT were: (i) asthma positive on questionnaire ('Have you ever had asthma?', 'Was this confirmed by a doctor?', and 'Have you used any medicines to treat asthma, or any breathing problems, at any time in the last 12 months?'; (ii) asthma positive on questionnaire, with atopy (defined by raised specific IgE and/or positive skin prick test); (iii) asthma positive on questionnaire, with raised serum total IgE levels (age corrected); (iv) asthma and PC_{20} methacholine <20 mg/ml (severe asthmatics); and (v) asthma and PC_{20} methacholine ≤16mg/ml. Appropriate cut-offs were used to transform the quantitative phenotypes into binary traits. Serum total IgE values were dichotomised using age related cut off points according to United Kingdom National External Quality Service (UK NEQAS), specific IgE≥0.35 kUA/l was classified as positive, and PC_{20} was dichotomised using ATS (American Thoracic Society) criteria (Crapo et al., 2000). Data analysis used the first affected sibling since transmissions to other siblings within the same family are not independent. Given that the TDT utilises data from heterozygous parents, only maternal transmissions were analysed since males are hemizygous at *IL13RA1* (carrying a single copy of the gene), due to the X chromosome location of the *IL13RA1* gene.

4.2.4. Case-control studies

Case-control studies were conducted to evaluate association of the –281T>G and the 1365A>G variants with asthma. Data were analysed in four separate group of cases, and participants in each group were considered to be independent. The group of cases were: asthmatic mothers (n=99), asthmatic fathers (n=85), first affected female siblings (n=222), and affected male siblings (n=214). The control population were healthy Caucasians of the same area of residence, females (n=97) and males (n=85). Genotype frequencies for each group of cases were compared to the control population and analysed using χ^2 in SPSS version 11 (SPSS Inc; Chicago, IL, USA).

4.2.5. Genotype-Phenotype Correlations

We evaluated the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms for association with various asthma-related phenotypes, including: (i) serum total IgE levels (age corrected and $\log_{10}$ transformed to improve normality); (ii) FEV_1% predicted; (iii) slope of FEV_1 response to metacholine (transformed to (1/(least squares slope+30)) to improve normality, avoid negative values and overcome "censored" PC_{20} values); (iv) atopy severity scores; and (v) asthma severity score. Four separate asthmatic groups were studied including fathers with diagnosis of asthma, first male affected siblings, mothers with diagnosis of asthma, and first affected female siblings. An association of the variants with a phenotype was detected when there was statistically significant difference in a phenotypic characteristic between genotype groups. Statistical analysis for male subjects (fathers and first male sibling) was carried out using two sample t-test, since males are hemizygous at *IL13RA1*, that is, they have only a single copy of the gene. Genotype groups in asthmatic female subjects (mothers and first female sibling), were compared by ANOVA or two-sided t-test. When the number of homozygous mutant subjects was low, homozygous mutant and heterozygous subjects were combined and compared to the group of homozygous wild type using two-sided t-test. A P value of <0.05 was considered significant.

4.2.6. Haplotype-Phenotype Correlations

We examined the 2-allele *IL13RA1* haplotypes for association with asthma–related phenotypes in four separate study groups. The groups and variables analysed were identical as for genotype-phenotype correlations. Quantitative traits in the first affected sibling were analysed using ANOVA, whereas t-test was used to calculate differences in asthmatic fathers, since the two haplotypes with lower frequency were combined due to low numbers of subjects. Analysis of phenotypic differences between different haplotypes in asthmatic mothers and first affected siblings was carried out using dichotomous rather than continuous phenotypes, by chi-square analysis. Haplotypes in female individuals were inferred from available genotypes using Arlequin software (version 2 000), which provides a maximum likelihood for haplotype groups (Excoffier and Slatkin, 1995).

4.3. Results

4.3.1. Allele frequencies

Allele frequencies for male and female subjects were evaluated separately due to the fact that male subjects are hemizygous at *IL13RA1* (Table 4.2). Of the 222 first asthmatic female siblings genotyped for –281T>G, 39.2% were homozygous for the wild type T allele, 48.6% were heterozygous for the T and G alleles, and 12.2% were homozygous for the minor G allele. In first asthmatic female siblings, allele frequencies for the 1365A>G variant showed 68.3% homozygous for the wild type A allele, 29.4% heterozygous for the A and G alleles, and 2.3% homozygous for the minor G allele. Allele frequencies in 214 first male affected siblings for the –281T>G polymorphisms showed 66.1% hemizygous for the T allele and 33.9% hemizygous for the G allele. In the same group, allele frequencies for the 1365A>G variant showed 83.5% hemizygous for the A allele and 16.5% hemizygous for the G allele.

Allele frequencies among first asthmatic affected siblings and female controls did not differ significantly from Hardy-Weinberg equilibrium, assessed using chi-squared analysis (Table 4.2). Hardy-Weinberg equilibrium is based on the assumption that gene frequencies and genotype ratios in a randomly breeding population remain constant from generation to generation. Correlation of genotyping data with Hardy-Weinberg equilibrium gave confidence in the accuracy of genotyping techniques. Allele frequencies for male subjects were not assessed for Hardy-Weinberg equilibrium since males are hemizygous at *IL13RA1*. Allele frequencies for the 1365A>G variant in our study were similar to the ones reported in a previous study in a British Caucasian population (Heinzmann et al., 2000).

Linkage disequilibrium between –281T>G and 1365A>G was measured by D' (Lewontin, 1964), and the two variants were found to be tightly linked (D'=0.97). A total of three of the four possible haplotypes were constructed, as a result of the tight linkage disequilibrium between the two variants (Table 4.3).

Table 4.2. Allele frequencies and Hardy-Weinberg equilibrium *P* values for the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms

Assessment	Mothers (n= 99)	Fathers (n= 85)	Female Sibling 1 (n= 222)	Male Sibling 1 (n= 214)	Female Controls (n= 97)	Male Controls (n= 82)
Allele frequency						
-281 T	0.66	0.67	0.63	0.66	0.71	0.62
-281 G	0.33	0.33	0.36	0.33	0.28	0.37
* Hardy-Weinberg *P*	-	-	0.45	-	0.54	-
Allele frequency						
1365 A	0.84	0.83	0.83	0.83	0.85	0.82
1365 G	0.15	0.16	0.17	0.16	0.14	0.17
* Hardy-Weinberg *P*	-	-	0.515	-	0.10	-

*Hardy-Weinberg *P* values were calculated for female siblings and female controls

Table 4.3. Haplotype frequencies in the study cohort

Haplotype	Asthmatic Mothers n	Female Asthmatic Sib. 1 n	Asthmatic Fathers n	Male Asthmatic Sib. 1 n	Female Controls n	Male Controls n
-281T to 1365A	132 (67%)	278 (63.5%)	57 (67%)	161 (66%)	139 (71%)	51 (62%)
-281T to 1365G	34 (17%)	88 (20%)	14 (16.5%)	44 (12%)	27 (14%)	16 (20%)
-281G to 1365A	30 (16%)	72 (16.5%)	14 (16.5%)	40 (16%)	28 (14.5%)	15 (18%)
P value	0.59*	0.10*	0.80+	0.84+	-	-

*versus female controls, + versus male controls

4.3.2. TDT analyses

To investigate the hypothesis that genetic variation in the *IL13RA1* gene may be important to the development of asthma, we assessed evidence of association of the –281T>G and the 1365A>G variants with asthma and asthma-related phenotypes using TDT. No alleles of either the –281T>G or the 1365A>G SNPs were found to be preferentially transmitted from heterozygous mothers to first affected siblings. The TDT was also performed in siblings who had both asthma and the presence of atopy, raised total IgE, $PC_{20} \leq 16$ mg/ml, or $PC_{20} < 4$ mg/ml. Again, we found no evidence of association of either the –281T>G or the 1365A>G polymorphisms with asthma-related traits (Table 4.4). To assess haplotype association due to the combined interaction of the –281T>G and the 1365A>G variants, we compared the estimated number of observed 2-allele haplotype transmissions to the expected number, as derived from the estimated parental haplotypes, using the Arlequin program. We found no significant association between the *IL13RA1* 2-allele haplotypes and asthma-related phenotypes (Table 4.5).

4.3.3. Case-control analyses

We further evaluated genetic association of the *IL13RA1* variants and asthma by carrying out case-control studies. Four case-control analyses were performed in the following study groups: asthmatic fathers, first affected male siblings, asthmatic mothers, and first affected female siblings, versus normal controls. Allelic distribution of genotypes of both –281T>G and 1365A>G did not differ significantly between asthmatic subjects and normal controls as shown in Tables 4.6 and 4.7. Statistical analysis was performed using chi-square. To assess haplotype association, we compared the distribution of *IL13RA1* 2-allele haplotypes between asthmatic subjects and normal controls. We found no association of 2-allele haplotypes with the diagnosis of asthma in any of the study groups (Table 4.3).

4.3.4. Genotype-phenotype correlations

We evaluated potential correlations of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G SNPs with asthma-associated traits in four asthmatic groups, including asthmatic fathers, first affected male siblings, asthmatic mothers, and first affected female siblings, using t-test and ANOVA. No significant associations were observed

between the phenotypes studied and different genotypes of the –281T>G and the 1365A>G polymorphisms in any of the study groups (Tables 4.8-4.11). There was only a trend towards a difference in serum total IgE levels in asthmatic mothers with different genotypes of the 1365A>G variant. The mean log IgE for asthmatic mothers with AA genotype was 0.89 kU/l vs. 0.64 kU/l for subjects carrying AG & GG genotypes, however, this difference did not reach statistical significance (P=0.27, t-test).

4.3.5. Haplotype-phenotype correlations

We investigated potential associations of *IL13RA1* 2-allele haplotypes and various asthma-related phenotypes, including serum total IgE, FEV_1% predicted, slope of FEV_1 response to metacholine, symptom score, and atopy severity score in asthmatic parents and first affected siblings. Statistical analysis was carried out using t-test for the male study groups and χ^2 for the female study groups, after transformation of continues phenotypes into categorical ones using appropriate cut off points.

In the group of asthmatic mothers and first affected female siblings, no significant association between *IL13RA1* 2-allele haplotypes and asthma-related phenotypes was found (Tables 4.12-4.14). However, there was a borderline association between the –281T to 1365A haplotype and raised serum total IgE levels in asthmatic mothers (odds ratio versus G-G haplotype=2.8, odds ratio versus G-A haplotype=1.07, P=0.049) (Table 4.12). We found no evidence for association between *IL13RA1* 2-allele haplotypes and asthma-associated phenotypes in asthmatic fathers and first affected male siblings (Tables 4.14, 4.15).

Table 4.4. Allelic association of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G variants with asthma and asthma-related phenotypes by TDT analysis

Variable	n	Variant	Observed	Expected	*P* value
Asthma diagnosis					
-281	328	T	66	71	0.401
		G	76	71	
+1365	333	A	43	44.5	0.750
		G	46	44.5	
Atopy + asthma					
-281	314	T	60	62	0.719
		G	64	62	
+1365	287	A	37	39	0.650
		G	41	39	
Raised IgE + asthma					
-281	292	T	63	65	0.725
		G	67	65	
+1365	294	A	38	39.5	0.735
		G	41	39.5	
$PC_{20} < 4$ + asthma					
-281	236	T	45	49.5	0.365
		G	54	49.5	
+1365	238	A	25	31	0.127
		G	37	31	
$PC_{20} <= 16$ + asthma					
-281	289	T	56	60	0.465
		G	64	60	
+1365	292	A	34	38	0.358
		G	42	38	

Table 4.5. Genetic association of *IL13RA1* 2-allele haplotypes with asthma and asthma-related phenotypes by TDT analysis

Variable	n	Haplotype -281 to 1365	Observed	Expected	*P* value
Asthma diagnosis	331	T-A G-A G-G	68 47 44	71.5 43.5 43	0.56 0.46 0.83
atopy + asthma	286	T-A G-A G-G	61 39 39	62 38.5 37.5	0.86 0.91 0.73
Raised IgE + asthma	293	T-A G-A G-G	64 41 39	65 40 38	0.86 0.82 0.82
PC_{20} <4 + asthma	237	T-A G-A G-G	44 30 35	48.5 31.5 29	0.36 0.70 0.11
PC_{20} ≤16 + asthma	290	T-A G-A G-G	57 37 41	60 38 36.5	0.58 0.90 0.30

Table 4.6. *IL13RA1* –281T>G genotype frequencies in asthmatic groups and controls

Group	Genotype	n	*P* value
Asthmatic mothers	TT TG GG	46 40 13	0.58*
1st Affected female sibling	TT TG GG	87 108 27	0.08*
Female controls	TT TG GG	51 37 9	-
Asthmatic fathers	T G	57 28	0.62+
1st Affected male sibling	T G	164 84	0.61+
Male controls	T G	70 15	-

* versus female controls

+ versus male controls

Table 4.7. *IL13RA1* 1365A>G genotype frequencies in asthmatic groups and controls

Group	Genotype	n	*P* value
Asthmatic mothers	AA AG GG	74 18 6	0.78*
1st Affected female sibling	AA AG GG	151 65 5	0.20*
Female controls	AA AG GG	73 20 4	-
Asthmatic fathers	A G	71 14	1.0+
1st Affected male sibling	A G	208 41	0.99+
Male controls	A G	70 15	-

* versus female controls using χ^2

+ versus male controls using t-test

Table 4.8. Genotype-phenotype correlations in asthmatic mothers using t-test

Variant	Variable	Genotype	n	Mean	*P* value
-281T>G	Log IgE	TT TG & GG	45 53	0.79 0.86	0.70
	FEV_1%	TT TG & GG	46 53	95.6 98.5	0.40
	PC_{20} slope	TT TG & GG	42 48	16.28 18.94	0.30
	Atopy severity score	TT TG & GG	44 53	1.04 1.03	0.61
	Asthma severity score	TT TG & GG	45 40	3.76 3.88	0.52
1365A>G	Log IgE	AA AG & GG	73 24	0.89 0.64	0.27
	FEV_1%	AA AG & GG	74 24	96.69 98.67	0.62
	PC_{20} slope	AA AG & GG	66 23	17.67 17.81	0.96
	Atopy severity score	AA AG & GG	72 24	1.05 0.95	0.41
	Asthma severity score	AA AG & GG	63 21	3.85 3.78	0.77

Table 4.9. Genotype-phenotype correlations in first affected female siblings using t-test

Variant	Variable	Genotype	n	Mean	*P* value
-281T>G	Log IgE	TT TG & GG	86 135	1.28 1.28	0.10
	FEV_1%	TT TG & GG	87 135	95.08 93.64	0.48
	PC_{20} slope	TT TG & GG	85 126	14.78 13.50	0.44
	Atopy severity score	TT TG & GG	85 134	1.08 1.18	0.47
	Asthma severity score	TT TG & GG	80 117	4.23 4.23	0.94
1365A>G	Log IgE	AA AG & GG	150 70	1.67 1.89	0.22
	FEV_1%	AA AG & GG	151 70	94.79 92.60	0.30
	PC_{20} slope	AA AG & GG	144 66	14.36 13.17	0.50
	Atopy severity score	AA AG & GG	148 70	1.10 1.28	0.24
	Asthma severity score	AA AG & GG	135 61	4.25 4.21	0.72

Table 4.10. Genotype-phenotype correlations in asthmatic fathers using t-test

Variant	Variable	Genotype	n	Mean	*P* value
-281T>G	Log IgE	T G	57 28	1.25 1.07	0.42
	FEV_1%	T G	57 28	90.05 94.11	0.27
	PC_{20} slope	T G	48 27	16.25 16.86	0.80
	Atopy severity score	T G	57 28	1.46 1.24	0.30
	Asthma severity score	T G	47 26	3.76 3.78	0.57
1365A>G	Log IgE	A G	71 14	1.19 1.21	0.94
	FEV_1%	A G	71 14	91.37 91.50	0.97
	PC_{20} slope	A G	62 13	16.12 18.14	0.52
	Atopy severity score	A G	71 14	1.44 1.11	0.21
	Asthma severity score	A G	61 12	3.80 3.80	0.98

Table 4.11. Genotype-phenotype correlations in first affected male siblings using t-test

Variant	Variable	Genotype	n	Mean	*P* value
-281T>G	Log IgE	T G	164 84	1.96 1.89	0.65
	FEV_1%	T G	164 84	95.43 94.42	0.61
	PC_{20} slope	T G	150 80	13.26 14.60	0.37
	Atopy severity score	T G	164 84	1.35 1.18	0.22
	Asthma severity score	T G	140 76	4.33 4.33	0.94
1365A>G	Log IgE	A G	208 41	1.94 1.86	0.73
	FEV_1%	A G	208 41	95.54 94.71	0.75
	PC_{20} slope	A G	191 40	13.98 13.68	0.87
	Atopy severity score	A G	208 41	1.30 1.22	0.64
	Asthma severity score	A G	178 39	4.33 4.33	0.97

Table 4.12. Association of *IL13RA1* 2-allele haplotypes with serum total IgE levels and FEV_1% predicted in first affected female siblings and asthmatic mothers using χ^2

Variable	n	Haplotype -281 to +1365	Haplotype No. Within Normal Range	Haplotype No. Above Normal Range	*P* value
Female sibling by serum total IgE	205	T-A G-A G-G	77 (70%) 18 (16%) 15 (14%)	199 (66%) 57 (19%) 44 (15%)	0.768
Asthmatic mothers by serum total IgE	97	T-A G-A G-G	59 (61%) 16 (17%) 21 (22%)	71 (72%) 18 (18%) 9 (10%)	0.049
Female sibling by FEV_1% predicted	219	T-A G-A G-G	87 (67%) 24 (18%) 19 (15%)	191 (62%) 64 (21%) 53 (17%)	0.832
Asthmatic Mothers by FEV_1% predicted	98	T-A G-A G-G	61 (65%) 17 (18%) 16 (17%)	71 (70%) 17 (16%) 14 (14%)	0.753

Table 4.13. Association of *IL13RA1* 2-allele haplotypes with FEV_1 slope and atopy severity score in first affected female siblings and asthmatic mothers using χ^2

Variable	n	Haplotype -281 to +1365	Haplotype number per category				
			Category 1	Category 2	Category 3	Category 4	*P* value
Female sibling by FEV_1 slope	208	T-A G-A G-G	103(67%) 26(17%) 25(16%)	32(64%) 12(24%) 6(12%)	54(60%) 20(22%) 16(18%)	77(63%) 25(20%) 20(17%)	0.87
Female sibling by atopy severity	216	T-A G-A G-G	86(68%) 25(20%) 15(12%)	90(60%) 31(20%) 31(20%)	83(64%) 26(20%) 21(16%)	14(58%) 5(21%) 5(21%)	0.64
Asthmatic mothers by FEV_1 slope	89	T-A G-A G-G	73(65%) 22(20%) 17(15%)	46(70%) 8(12%) 12(18%)	-	-	0.42
Asthmatic mothers by atopy severity	96	T-A G-A G-G	33(66%) 9(18%) 8(16%)	44(63%) 12(17%) 14(20%)	51(71%) 13(18%) 8(11%)	-	0.70

Table 4.14. Association of *IL13RA1* 2-allele haplotypes with asthma severity score in first affected female siblings and asthmatic mothers using χ^2

Variable	n	Haplotype -281 to +1365	Haplotype numbers per category			*P* value
			Category 1	Category 2	Category 3	
Female sibling by asthma severity score	193	T-A G-A G-G	38 (68%) 6 (11%) 12 (21%)	132 (62%) 47 (22%) 33 (16%)	79 (67%) 22 (19%) 17 (14%)	0.83
Asthmatic mothers by asthma severity score	84	T-A G-A G-G	42 (64%) 15 (23%) 9 (13%)	48 (71%) 6 (9%) 14 (20%)	23 (68%) 8 (24%) 3 (9%)	0.12

Table 4.15. Correlation between *IL13RA1* 2-allele haplotypes and various phenotypes in first affected male siblings using ANOVA

Variable	Haplotype -281 to +1365	n	Mean	*P* value
Total IgE	T-A G-A G-G	161 44 40	12.9 13.3 10.5	0.600
FEV_1 % predicted	T-A G-A G-G	161 44 40	95.4 94.8 93.9	0.856
FEV_1 slope	T-A G-A G-G	147 41 39	13.4 15.7 13.4	0.442
Atopy severity score	T-A G-A G-G	161 44 40	1.36 1.16 1.21	0.432
Asthma severity score	T-A G-A G-G	137 38 38	4.34 4.30 4.35	0.956

Table 4.16. Correlation between *IL13RA1* 2-allele haplotypes and asthma-related phenotypes in asthmatic fathers using t-test

Variable	Haplotype -281 to +1365	n	Mean	*P* value
Serum total IgE	T-A G-A & G-G	57 28	3.3 5.4	0.160
FEV_1 % predicted	T-A G-A & G-G	57 28	90 94.1	0.200
Slope of FEV_1	T-A G-A & G-G	48 27	16.25 16.86	0.808
Atopy severity score	T-A G-A & G-G	57 28	1.46 1.24	0.300
Asthma severity score	T-A G-A & G-G	47 26	3.76 3.87	0.571

4.3.6. Power calculations

We examined the hypothesis that the lack of association of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms with asthma and asthma-related phenotypes was due to insufficient power. The power of the TDT study to detect an important difference was calculated using a formula, which assumes that the recombination fraction $\theta=0$ and there is no linkage disequilibrium (Risch and Merikangas, 1996). The fact that the only informative transmissions were those from mothers, due to the X chromosome localisation of *IL13RA1,* was taken into account by doubling the number of families given by the calculations. The formula is $N= (Z_{\alpha}-\sigma Z_{1-\beta})^2/2\mu^2$, where $Z_{\alpha}= 3.72$ (corresponding to $\alpha= 10^{-4}$), $Z_{1-\beta}= -0.84$ (corresponding to $1-\beta= 0.80$), $\mu=2Y-1$, $\sigma^2= 4Y(1-Y)$, $Y= 1+w/2+w$, $w= pq(\gamma-1)^2/(p\gamma+q)^2$, p is the frequency of allele A, q is the frequency of allele a and γ is the genotypic risk ratio. The power of the case-control study to detect a statistically significant difference was calculated using a statistical programme (PS Program, Version 2.0, 1996, Vanderbilt University School of Medicine, Nashville, Tennessee, USA), which takes into account the alpha level (0.05), sample size, odds ratio, and polymorphism frequency in controls. For the *IL13RA1* –281T>G variant, TDT power calculations showed that the number of families necessary to obtain 80% power (the probability of rejecting the null hypothesis when it is false), at a significance level of 0.05 and genotypic risk ratio of 1.5 is around 316 families (trios), whereas for a genotypic risk ratio of 2 around 106 families would be required. For the *IL13RA1* 1365A>G polymorphism, TDT power calculations showed that the number of families necessary to obtain 80% power is around 474 families for a genotypic risk ratio of 1.5, whereas for a genotypic risk ratio of 2, 152 families would be required.

In the case-control study, in the comparison of asthmatic mothers (n=188 alleles) and female controls (n=188 alleles) for association of –281T>G with asthma, assuming a two-tailed alpha of 0.05 and a baseline frequency of 0.35 for the minor allele, this sample had a power of 67% to detect an odds ratio of 2 and above, and a power of 28% to detect an association with odds ratio of 1.5% and above, given a frequency of the TG plus the GG genotypes combined of 48% (Table 4.6). Comparing asthmatic mothers and female controls for association of the 1365A>G polymorphism with asthma, the power was 50% for an odds ratio of 2 and 20% for an odds ratio of 1.5,

given a frequency of the AG plus the GG genotypes combined of 32% (Table 4.7). In the comparison of first affected female siblings (n=444 alleles) and female controls (n=188 alleles) for association of the –281T>G SNP with asthma, the sample had a power of 80% to detect an odds ratio of 2 and a power of 35% to detect an odds ratio of 1.5, given a frequency of the TG plus the GG genotypes combined of 60% (Table 4.6). Comparing first affected female siblings and female donors for association of 1365A>G with asthma, the power was 65% to detect an association with an odds ratio of 2, and 24% to detect an odds ratio of 1.5% (Table 4.7). In the comparison of asthmatic fathers (n=85 alleles) and male donors (n=85 alleles) for association of the –281T>G polymorphism with asthma, this sample had a power of 60% to detect an odds ratio of 2 and a power of 25% to detect on odds ratio of 1.5 (Table 4.6). Comparing asthmatic fathers and male controls for association of the 1365A>G variant with asthma, the sample had a power of 46% for an odds ratio of 2 and a power of 18% for an odds ratio of 1.5 (Table 4.7). In the comparison of first affected male siblings (n=248 alleles) and male donors (n=85 alleles) for association of the –281T>G SNP with asthma, this sample had a power of 77% to detect an association with an odds ratio of 2 and a power of 34% to detect on odds ratio of 1.5 (Table 4.6). Comparing first affected male siblings and controls for association of the 1365A>G polymorphism with asthma, the sample had a power of 58% for an odds ratio of 2 and a power of 22% for an odds ratio of 1.5 (Table 4.7).

In the haplotype case-control analyses using TDT, comparing asthmatic mothers (n=196 haplotypes) and female controls (n=194 haplotypes) for association of the *IL13RA1* 2-allele haplotypes with asthma, the sample had a power of 92% to detect an association of an odds ratio of 2 and above, and a power of 49% for an odds ratio of 1.5 and above, given a frequency of the G-A plus the G-G haplotypes combined of 33% (Table 4.3). In the comparison of first affected female siblings (n= 438 haplotypes) and female controls (n=194 haplotypes), the sample had a power of 98% to detect an odds ratio of 2 and above, and a power of 63% to detect an odds ratio of 1.5 and above, given a frequency of the G-A plus the G-G haplotypes combined of 36.5%. In the comparison of asthmatic fathers (n=85 haplotypes) and male controls (n=82 haplotypes), the sample had a power of 60% to detect an odds ratio of 2 and a power of just 25% to detect an odds ratio of 1.5, given a frequency of the G-A plus

the G-G haplotypes combined of 33%. In the comparison of first affected male asthmatics (n=245 haplotypes) and male controls (n=82 haplotypes), the sample had a power of 72% to detect an odds ratio of 2 and a power of just 30% to detect an odds ratio of 1.5, given a frequency of the G-A and the G-G haplotypes combined of 28.5%.

4.4. Discussion

This study represents the first comprehensive evaluation of the *IL13RA1* gene as an asthma-susceptibility locus. *IL13RA1* is a rational asthma-susceptibility gene, given that its ligands, IL-13 and IL-4, play a central role in the pathophysiology of asthma. We initially identified the common genetic variation of the 5' flanking and coding regions of the *IL13RA1* gene, and assessed the extent of linkage disequilibrium between the two common variants found, -281T>G and 1365A>G. We subsequently used a comprehensive approach to clarify the role the -281T>G and the 1365A>G polymorphisms, which can potentially modulate the *IL13RA1* gene transcription activity and mRNA stability, respectively, in the development of asthma and atopy in a large, extensively phenotyped cohort ascertained on a basis of asthma. We used the TDT as a family-based approach to protect against stratification, and we extended our investigation by conducting case-control studies between asthma groups in our cohort and a control group, as well as genotype-phenotype association studies of individual study groups.

4.4.1. Linkage disequilibrium between the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms

The two common *IL13RA1* polymorphisms, -281T>G and 1365A>G, were found to be in tight linkage disequilibrium (D'=0.97). In general, higher levels of LD are expected on the X chromosome, since recombination between X chromosomes occurs only in females (Pritchard and Przeworski, 2001). Despite the tight linkage disequilibrium between the two SNPs in the *IL13RA1* gene in our study, the degree of linkage disequilibrium between certain alleles on the same gene is variable. Thus, certain pairs of alleles on the same gene exhibit substantial linkage disequilibrium, whereas others demonstrate reduced levels of linkage disequilibrium. Moreover, the degree of linkage disequilibrium between alleles shows significant variations among different ethnic groups (Pritchard and Przeworski, 2001). For example, in the *IL4RA* gene strong linkage disequilibrium was found between E375A and C406R, as well as C406R and Q551R among a white population, but not a black population (Ober et al., 2000a). Data from various studies show that there does not appear to be an excess of linkage disequilibrium at short physical distances, such the one that spans an individual gene (Drysdale et al., 2000).

Thus, polymorphisms at short physical distance reveal less likage disequilibrium than would be expected. For example, many of the thirteen SNPs of the β_2 adrenergic receptor gene were found to be in strong disequilibrium in Caucasian populations, however, some pairs of close alleles had reduced levels of linkage disequilibrium relatively to more distantly spaced pairs of SNPs (Drysdale et al., 2000).

4.4.2. Association of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G variants with asthma-related traits by TDT analysis

We found no evidence to support a significant association of the –281T>G and the 1365A>G polymorphisms in the *IL13RA1* gene with asthma using TDT. Furthermore, there was no evidence for association of the –281T>G and the 1365A>G polymorphisms with asthma-related traits (asthma and atopy, asthma and raised serum total IgE levels, asthma and PC_{20} metacholine < 4, mg/ml, asthma and PC_{20} metacholine ≤ 16 mg/ml). We also evaluated the possibility of haplotype association, comparing the observed numbers of 2-allele haplotype transmissions, from mothers to first affected siblings, to the estimated ones using the TDT. Again, there was no evidence for association of any marker haplotype with asthma or asthma-related phenotypes. Our study had enough power to detect an important association between the –281T>G SNP and asthma using the TDT, provided a significance level of 0.05 and a genotypic risk ratio of 1.5. Thus, according to our power calculations, the number of families required to achieve 80% power for a genotypic risk ratio of 1.5 was 316, while the number of families used in our study was 342. In contrast, our study did not have enough power to detect a significant association between 1365A>G and asthma using TDT, since 474 families would be required to achieve 80% power at a significance level of 0.05 and a genotypic risk ratio of 1.5.

4.4.3. Association of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G SNPs with asthma in case-control studies

We further examined potential associations between genetic variants of *IL13RA1* and asthma, by conducting case-control studies. We compared the genotype and haplotype frequencies between four asthmatic groups in our cohort (asthmatic mothers, asthmatic

fathers, first affected male siblings, and first affected female siblings) and a control non-asthmatic population. The additional haplotype analysis performed, improved the likelihood of detecting potential association between *IL13RA1* 2-allele haplotypes and asthma-related traits. This is because the combination of allelic variants within a gene can potentially create phenotypic effects distinct from those produced by individual SNPs alone (Raby et al., 2002). Because males are hemizygous at *IL13RA1*, male and female subjects were compared separately.

We found no evidence for a significant association of *IL13RA1* single SNPs or 2-allele haplotypes with asthma. However, our case-control study had potential limitations that might have prevented us from observing a true association of the genetic variants with asthma. According to our power calculations, the highest power for the –281T>G polymorphism was in the group of first affected female siblings with a power of 80% to detect an OR of 2 and a power of 35% to detect an OR of 1.5. For 1365A>G, the maximum power obtained was in the group of first affected female siblings, with a power of 65% to detect an OR of 2 and a power of 25% to detect on OR of 1.5. Thus, power calculations showed that the power to detect an association with asthma for a genotypic odds ratio of 1.5 was particularly low in our study for both the –281T>G and the 1365A>G polymorphisms. In contrast, the haplotype case-control study had a high power to detect an important association between *IL13RA1* 2-allele haplotypes and asthma for an OR of 2, but had only moderate power (25%-63% power in different study groups) to detect an OR of 1.5.

Our findings are in accordance with the only previous study that has examined association between asthma and the 1365A>G SNP, and found no evidence for association of the 1365A>G variant with asthma both in a British and a Japanese population (Heinzmann et al., 2000). To date, no study has examined associations of the –281T>G polymorphisms and asthma-related traits.

4.4.4. Correlations of the –281T>G and the 1365A>G polymorphisms with asthma-related phenotypes

We assessed phenotypic differences between carriers of different *IL13RA1* genotypes and 2-allele haplotypes. Phenotypes studied included: serum total IgE, FEV_1% predicted, slope of FEV_1 response to metacholine, atopy severity score, and asthma severity score. We found no significant association between *IL13RA1* genetic variation and any of the phenotypes examined, apart from a borderline association between the *IL13RA1* –281T to 1365A haplotype and raised serum total IgE levels in adult female asthmatics. However, the *P* value was not adjusted for multiple comparisons and the borderline association found may represent a type I error. Moreover, no association between the T-A haplotype and higher serum total IgE levels in other study groups was found. Previous association studies in the *IL13* gene have shown that the association between *IL13* variants and raised serum total IgE levels is stronger among skin test-negative than among skin test-positive subjects (Graves et al., 2000). Based on that study, we extended our haplotype analysis dividing each of the groups of asthmatic and non-asthmatic parents into skin prick-negative and skin prick-positive. We investigated each of these subgroups for potential association between the –281T to 1365A haplotype and raised serum total IgE levels and found no association. Our results indicate that the *IL13RA1* –281T to 1365A haplotype might confer susceptibility for raised serum total IgE levels only in adult female asthmatics, independent of their atopic status.

In a previous case-control study the 1365A allele was found to be associated with raised serum total IgE in males, but not in females, in a British population (Heinzmann et al., 2000). However, no association between the 1365A allele and higher serum total IgE levels was found in a Japanese population in the same study. The calculated odds ratio was 3.39 (P=0.015) in males and 1.10 (P=0.680) in females. The case-control group included 150 young adult subjects with clinical asthma and atopy, and 150 healthy controls, all from the Oxford region. We were unable to find an association between the 1365A>G variant alone and raised serum total IgE levels in any of the study groups in our cohort. This could be due to the fact that the 1365A>G SNP might be a marker for other functional polymorphisms in *IL13RA1* with different degrees of linkage

disequilibrium between the 1365A>G polymorphism and the functional *IL13RA1* SNPs in the two study cohorts. Moreover, there might have been differences in the asthma severity between the two study cohorts.

The *IL13RA1* 1050C>T polymorphism was not evaluated for potential association with asthma and asthma-related phenotypes, because of the low frequency of the minor allele ($q \approx 0.04$), which would result in insufficient power to detect true associations with asthma and asthma-related traits. Ahmed and co-workers (Ahmed et al., 2000) first described the *IL13RA1* 1050C>T polymorphism in a Japanese population and found a similar low frequency of the minor allele. They subsequently conducted a low powered case-control association study in a paediatric population, and found no significant association between the 1050C>T polymorphism and atopic asthma.

Summary

- The two common *IL13RA1* genetic variants, -281T>G and 1365A>G, are in tight linkage disequilibrium.

- A borderline association between the *IL13RA1* –281T to 1365A haplotype and raised serum total IgE levels in adult female asthmatics was identified.

- No evidence for other associations of the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms with asthma or asthma-related phenotypes was found.

CHAPTER 5

Expression of the IL-13 receptor subunits in the airways

5.1. Overview

There has been increasing evidence to suggest that IL-13 not only plays a pivotal role in the chronic inflammation in the asthmatic airway, but can also act directly on structural airway cells. Based on these observations, we have evaluated the expression of IL-13 receptor subunits in airway cells and bronchial biopsies using RT-PCR analysis, immunohistochemistry, flow cytometry, and confocal laser scanning microscopy.

Results: RT-PCR analysis and immunostaining in primary bronchial epithelial cells and primary bronchial fibroblasts established the expression of IL-13Rα1, IL-13Rα2, and IL-4Rα at both the mRNA and protein levels. Immunohistochemical analysis of IL-13Rα1, IL-13Rα2, and IL-4Rα in bronchial biopsies showed that the bronchial epithelium was the main site of immunoreactivity for all receptor components. Flow cytometry and confocal laser scanning microscopy revealed the presence of a large intracellular pool of IL-13Rα2 in primary bronchial epithelial cells and primary bronchial fibroblasts.

At the initiation of these studies, while it was clear that IL-13 signalling in the airways was crucial to asthma pathophysiology, it was not known whether these effects were direct on airway structural cells or indirect through the effect of IL-13 on inflammatory cells recruited into the airways. Studies in mouse models of asthma have uniformly confirmed the central role of IL-13 in airway inflammation through regulation of recruitment, homing, and activation of inflammatory cells (Grunig et al., 1998, Wills-Karp et al., 1998). *In vitro* studies have also shown that IL-13 can act directly on airway cells. Thus, incubation of primary human bronchial epithelial cells with IL-13 increased the proportion of mucous secreting cells and decreased ciliary beat frequency through interference with the apical binding protein ezrin (Laoukili et al., 2001a), and stimulation of human bronchial epithelial cells with IL-13 caused TGF-β2 (Richter et al., 2001) and IL-8 (Lordan et al., 2002) release. IL-13 has also direct effects on cultured human airway smooth muscle cells *in vitro*, inducing STAT6 and ERK/MAP activation (Laporte et al., 2001), as well as proliferation of myofibroblasts via a STAT6 dependent process (Ingram et al., 2003).

5.2. Experimental design

We hypothesised that all IL-13 receptor subunits are expressed in the airways, and we therefore aimed to determined the mRNA expression of IL-13Rα1, IL-13Rα2, IL-4Rα, and IL-2Rγ in primary bronchial epithelial cells, primary bronchial fibroblasts and various bronchial epithelial and haematopoietic cell lines using RT-PCR analysis. Protein expression of IL-13Rα1, IL-13Rα2, and IL-4Rα in cultured primary bronchial epithelial cells, cultured primary bronchial fibroblasts, and bronchial biopsies was assessed by immunostaining. In addition, protein expression and cellular localisation of IL-13Rα2 in primary bronchial epithelial cells and primary bronchial fibroblasts was further analysed using flow cytometry and confocal laser scanning microscopy, due to suitability of the monoclonal anti-IL-13Rα2 antibody for these applications. Protein expression of IL-2Rγ was not evaluated due to the lack of a suitable antibody. Primary bronchial epithelial cells and primary bronchial fibroblasts were obtained from three mild asthmatic subjects by fiberoptic bronchoscopy. Primary bronchial epithelial cells were established by seeding brushed bronchial epithelial cells into cultured dishes containing appropriate medium as described in section 2.15, whereas primary bronchial epithelial cultures were established by placing submucosal biopsies in petri dishes as described in section 2.16.

5.3. Results

5.3.1. IL-13Rα1 expression in airway cells

Using RT-PCR analysis we confirmed that specific IL-13Rα1 transcript is expressed in unstimulated primary bronchial epithelial cells, primary bronchial fibroblasts, and white blood cells. Specific IL-13Rα1 transcript is also expressed in human bronchial epithelial cell lines 16-HBE, A549, and NCI-H292, as well as in the promyelomonocytic cell line U937 and the T leukemia cell line Jurkat (Figure 5.1). No bands were observed during negative control experiments using RT minus products, genomic DNA, and milli Q water. DNA sequence of the generated amplicons perfectly matched the predicted sequence. APRT was used as a positive control and was consistently expressed in all experiments.

We also evaluated IL-13Rα1 protein expression in cultured primary bronchial epithelial cells and primary bronchial fibroblasts using a specific monoclonal anti-IL-13Rα1 antibody. Bronchial epithelial cells were brightly stained, whereas bronchial fibroblasts showed a weaker staining pattern (Figures 5.2, 5.3). No staining was observed in cells stained with an isotype-matched control antibody. We also assessed IL-13Rα1 immunoreactivity *in vivo*, by staining GMA embedded bronchial biopsies obtained from three mild asthmatic subjects by fiberoptic bronchoscopy. When we analysed the staining pattern we observed that the columnar cells of the bronchial mucosa was the major site of IL-13Rα1 immunoreactivity (Figure 5.4). In contrast, the basal layer of the epithelium and smooth muscle cells were only weakly stained. There was also positive staining among infiltrating inflammatory cells in the submucosa, in keeping with the expression of IL-13Rα1 mRNA in haematopoietic cells.

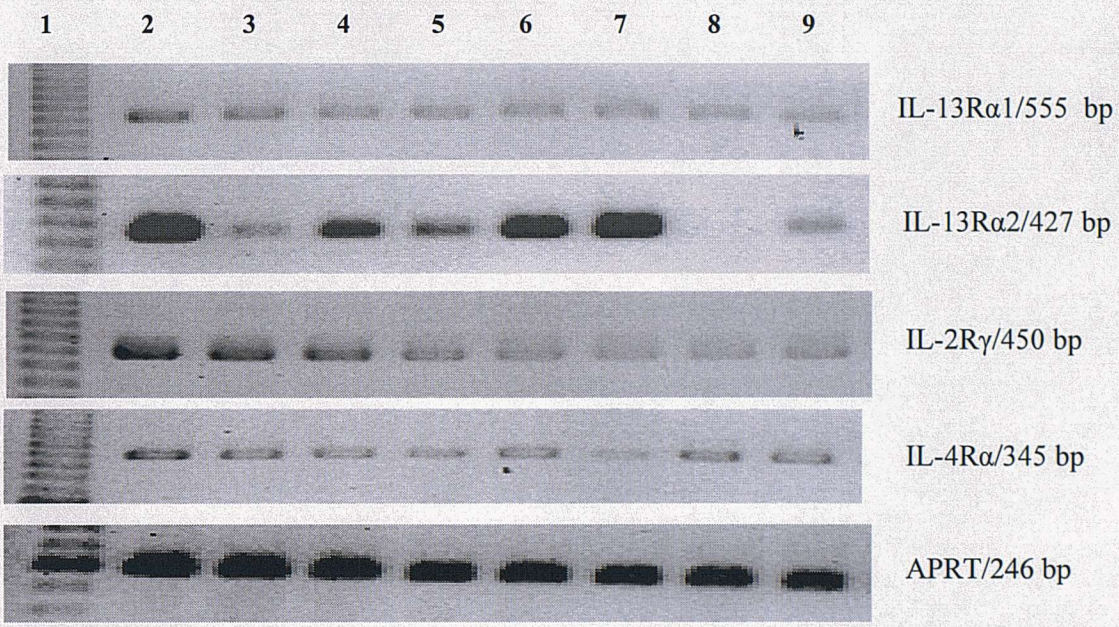

Figure 5.1. mRNA expression of IL-13 and IL-4 receptor components. mRNA expression of IL-13Rα1, IL-13Rα2, IL-2Rγ, IL-4Rα, and APRT was investigated by RT-PCR in primary bronchial epithelial cells (lane 2), U937 cells (lane 3), 16-HBE cells (lane 4), A549 cells (lane 5), NCI-H292 cells (lane 6), primary bronchial fibroblasts (lane 7), Jurkat cells (lane 8), and white blood cells (lane 9). The panel shows that all receptor components are expressed in various cell types, apart from lack of IL-13Rα2 in Jurkat cells (lane 8), and weak IL-13Rα2 expression in U937 cells (lane 3) and white blood cells (lane 9). RNA for fibroblasts, bronchial epithelial cells, and white blood cells derived from an asthmatic subject. APRT was used as an internal positive control. Representative data from four separate experiments are shown.

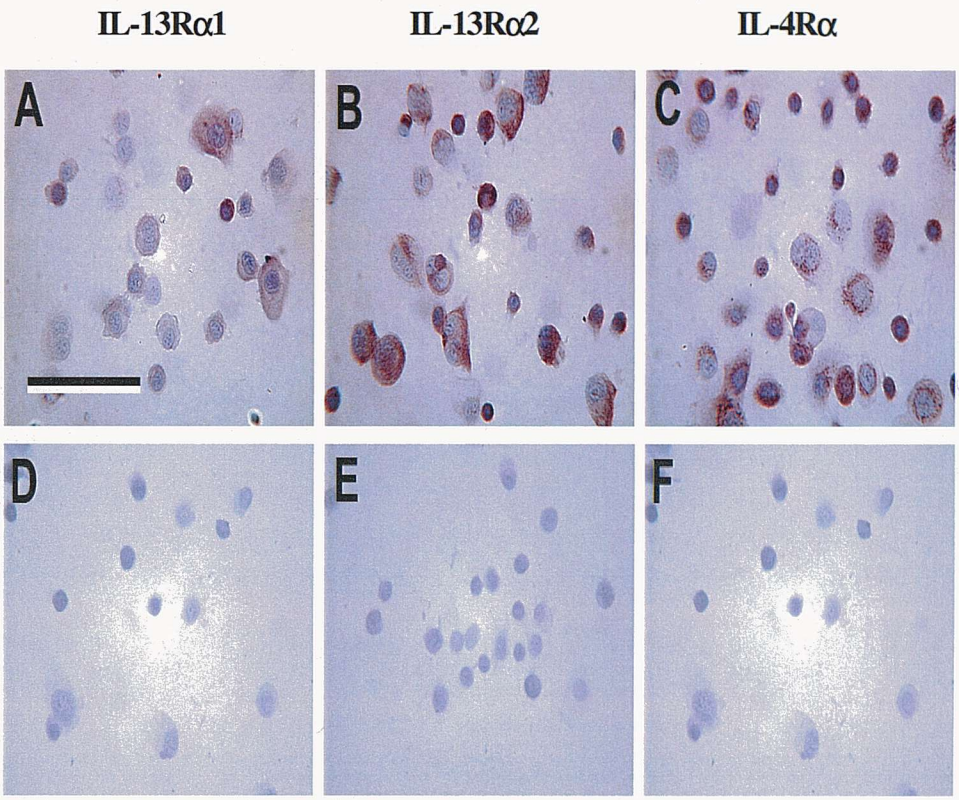

Figure 5.2. Protein expression of IL-13Rα1, IL-13Rα2, and IL-4Rα in primary bronchial epithelial cells. Primary bronchial epithelial cells were obtained from a mild asthmatic subject by fiberoptic bronchoscopy. Cells were seeded into culture dishes and were allowed to expand. They were subsequently transferred to chamber slides at passage 2 and stained with specific monoclonal antibodies for IL-13Rα1 (A), IL-13Rα2 (B), IL-4Rα (C), or with appropriate isotype-matched control antibodies (D-F). Representative data of triplicate experiments are depicted. Original magnification: x40.

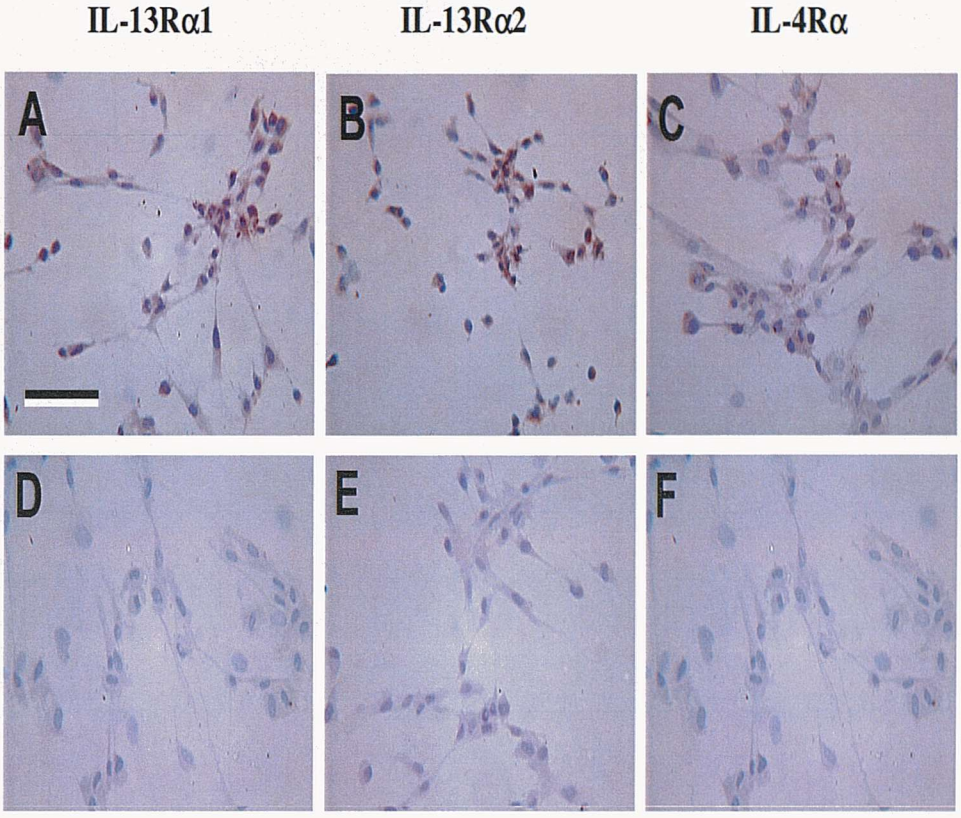

Figure 5.3. Protein expression of IL-13Rα1, IL-13Rα2, and IL-4Rα in primary bronchial fibroblasts. Submucosal biopsies derived from an asthmatic subject were seeded into culture dishes using appropriate culture medium. Cultures were allowed to grow until fibroblasts migrated from the tissue and proliferated on the base of the dish. Cells were transferred to chamber slides at passage 5 and stained with specific monoclonal antibodies for IL-13Rα1 (A), IL-13Rα2 (B), IL-4Rα (C), or appropriate isotype-matched control antibodies (D-F). Representative data of triplicate experiments are shown. Original magnification: x40.

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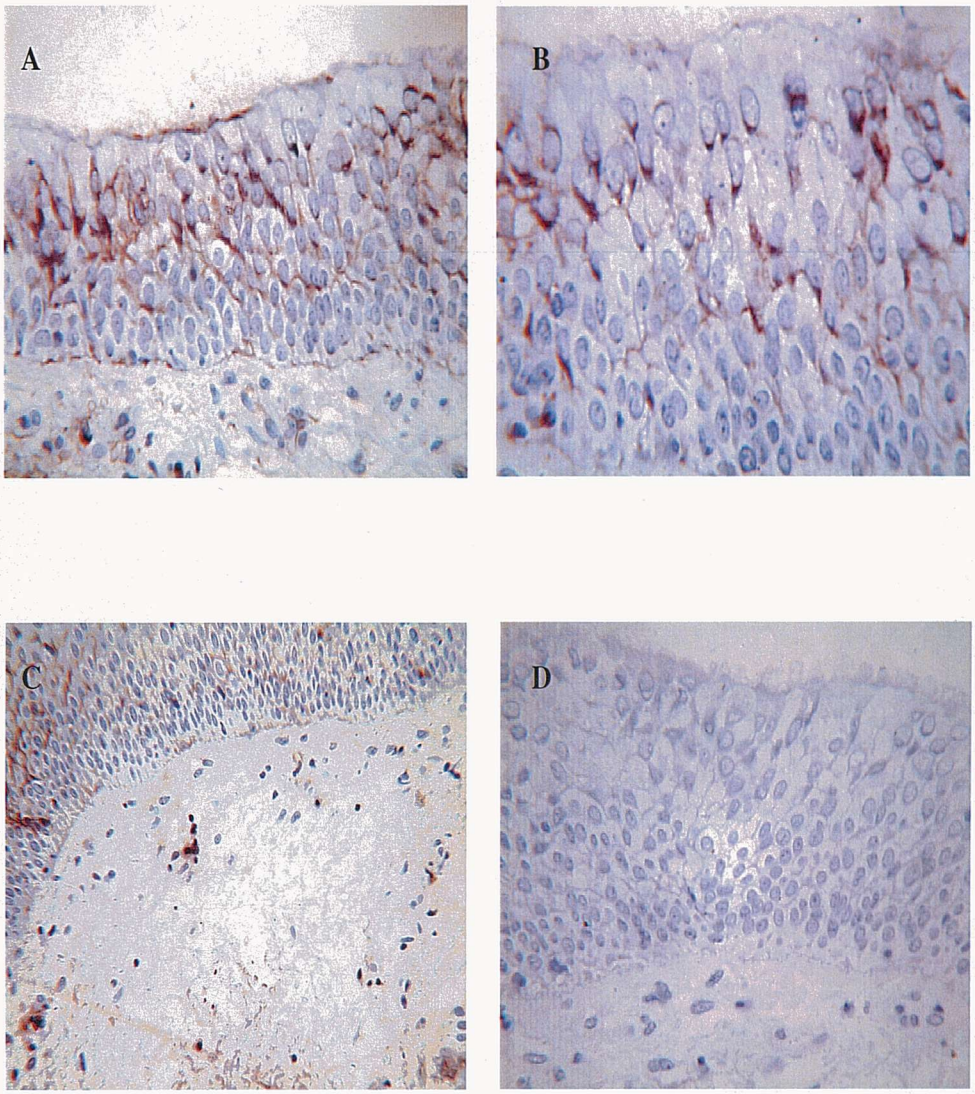


Figure 5.4. Immunoreactivity of IL-13Rα1 in the asthmatic bronchial mucosa. Bronchial biopsies obtained from a mild asthmatic subject by fiberoptic bronchoscopy were sectioned and stained for the presence of IL-13Rα1. The panels show immunohistochemical staining of IL-13Rα1 (A, B, C) and a control section using an isotype-matched control antibody (D). The columnar epithelial cells are intensely stained, whereas basal cells show a weaker staining pattern (A, B). A number of infiltrating cells in the submucosa demonstrate IL-13Rα1 immunoreactivity (C). Sections are representative of three separate experiments. Original magnification: x40 (A, C, D), or x63 (B).

5.3.2. IL-13Rα2 mRNA expression in airway cells

The baseline expression of IL-13Rα2 mRNA in primary bronchial epithelial cells, primary bronchial fibroblasts, and white blood cells was evaluated using RT-PCR analysis. IL-13Rα2 mRNA expression was also assessed in the bronchial epithelial cell lines NCI-H292, 16-HBE, and A549, as well as in U937 cells and Jurkat cells. RT-PCR analysis showed that IL-13Rα2 mRNA is expressed in primary bronchial epithelial cells, primary bronchial fibroblasts and all bronchial epithelial cell lines tested. IL-13Rα2 specific transcript was weakly expressed in U937 cells and white blood cells, whereas no IL-13Rα2 mRNA expression was detected in Jurkat cells. (Figure 5.1).

5.3.3. IL-13Rα2 protein expression

Having established that IL-13Rα2 mRNA is present in primary bronchial epithelial cells and primary bronchial fibroblasts, we assessed whether IL-13Rα2 protein is also expressed in these cells by immunostaining. Cells obtained from three mild asthmatic subjects by fiberoptic bronchoscopy were allowed to grow on chambers slides and fixed in anhydrous acetone before staining with a specific monoclonal anti-IL-13Rα2 antibody. Immunostaining revealed that IL-13Rα2 protein expression is strong in primary bronchial epithelial cells and moderate in primary bronchial fibroblasts (Figure 5.2, 5.3). Next, we evaluated IL-13Rα2 immunoreactivity in the asthmatic bronchial mucosa by staining bronchial biopsies derived from three mild asthmatic subjects. The staining pattern revealed that the columnar cells of the bronchial epithelium were strongly stained, whereas the basal cells demonstrated a weak staining pattern. No positive staining of subepithelial fibroblasts or smooth muscle cells was seen. Moreover, there was no staining of infiltrating inflammatory cells in the submucosa, in keeping with lack of IL-13Rα2 mRNA expression in haematopoietic cells (Figure 5.5).

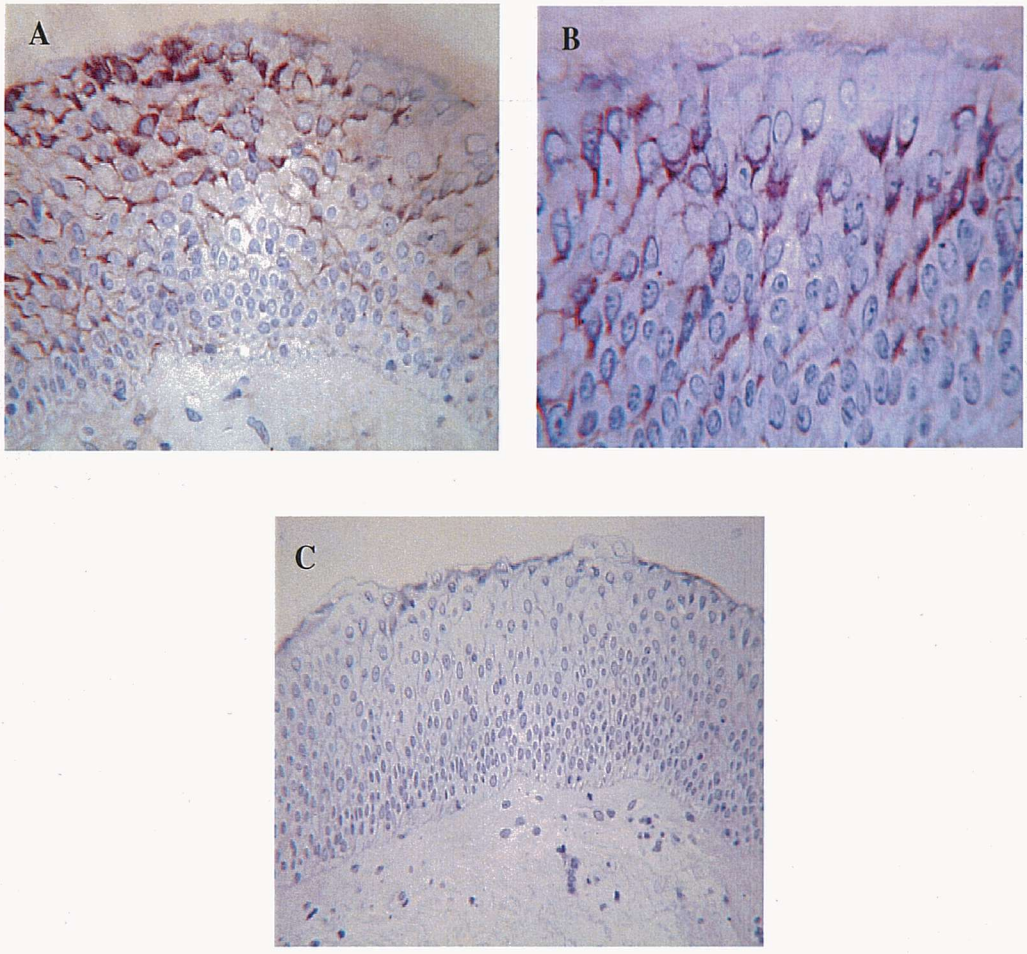


Figure 5.5. Immunoreactivity of IL-13Rα2 in the asthmatic bronchial mucosa. Bronchial biopsies derived from an asthmatic subject were sectioned and stained for the presence of IL-13Rα2. The columnar epithelial cells are intensely stained, whereas basal cells demonstrate a weaker staining pattern (A, B). No staining is seen in a control section using an isotype-matched control antibody (C). Representative sections of triplicate experiments are depicted. Original magnification: x40 (A, C), or x63 (B).

5.3.4. Analysis of IL-13Rα2 expression in airway cells by flow cytometry

Having established that IL-13Rα2 specific transcript is expressed in primary bronchial epithelial cells, primary bronchial fibroblasts, various bronchial epithelial cell lines, and U937 cells, we also evaluated surface IL-13Rα2 expression on these cells by flow cytometry. We used a specific monoclonal anti-human IL-13Rα2 antibody that recognises epitopes of the extracellular domain of the protein (clone B-D13, IDS, Boldon, UK). To our surprise, no surface IL-13Rα2 expression was detected in all cell types in more than five independent experiments, with the fluorescence intensity of cells incubated with the anti-IL-13Rα2 antibody being identical to that of cells incubated with the isotype-matched control antibody (Figure 5.6). To explain the absence of IL-13Rα2 surface expression we examined the possibility of receptor cleavage from cell surface as a result of trypsin-EDTA enzymatic dissociation of cells from flasks. Therefore, we analysed cells using flow cytometry following non-enzymatic dissociation from flasks (Cell Dissociation Solution (1x) Non-enzymatic, Sigma-Aldrich), and again found no surface IL-13Rα2 expression on either bronchial epithelial cells or bronchial fibroblasts. To further investigate the possibility of receptor cleavage following dissociation of cells from flasks, we examined the surface expression of two adhesion molecules, ICAM-1 and CD44, known to be expressed on NCI-H292 bronchial epithelial cells. Flow cytometry demonstrated that both ICAM-1 and CD44 are highly expressed on NCI-H292 cells, following either trypsin-EDTA or non-enzymatic dissociation from flasks (Figure 5.7). Additional experiments using flow cytometry confirmed that ICAM-1 and CD44 are also expressed on the surface of primary bronchial epithelial cells and primary bronchial fibroblasts, following either trypsin-EDTA or non-enzymatic dissociation of cells from flasks (data not shown). Further support for the absence of IL-13Rα2 on the surface of bronchial epithelial cells and bronchial fibroblasts was provided by the fact that U937 cells, grown in suspension, showed no surface IL-13Rα2 by flow cytometry.

Next, we investigated the hypothesis that IL-13Rα2 is located intracellularly in bronchial epithelial cells and bronchial fibroblasts, since we had previously shown

that these cells express IL-13Rα2 mRNA and IL-13Rα2 protein using RT-PCR analysis and immunostaining, respectively. We therefore stained permeabilized primary bronchial epithelial cells and primary bronchial fibroblasts and analysed by flow cytometry. Strikingly, permeabilized cells showed a large intracellular expression of IL-13Rα2 suggesting that IL-13Rα2 is an intracellular rather than a membrane protein in these cells. Similarly, NCI-H292, A549, and U937 cells demonstrated intracellular expression of IL-13Rα2 (Figure 5.8).

Next, we examined the hypothesis that the intracellular expression of IL-13Rα2 shown by flow cytometry might be due to non-specific binding of the antibody to cytoplasmic proteins. We had previously shown by RT-PCR analysis that Jurkat cells do not express IL-13Rα2 mRNA, therefore, are not expected to express IL-13Rα2 protein as well. Analysis of intact and permeabilized Jurkat cells by flow cytometry, using the same specific monoclonal anti-IL-13Rα2 antibody, revealed that Jurkat cells do not express either membrane or intracellular IL-13Rα2 protein (Figure 5.9), providing strong evidence that binding of the IL-13Rα2 antibody in the intracellular compartment is specific.

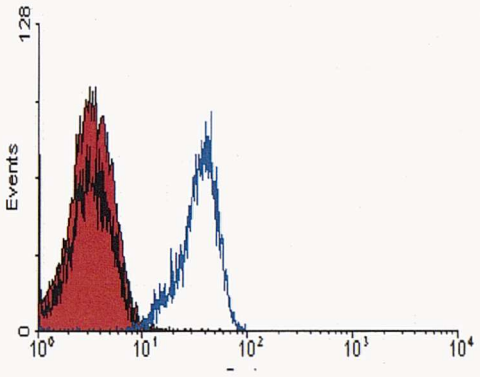


Figure 5.6. IL-13Rα2 is located intracellularly in primary bronchial epithelial cells. Primary bronchial epithelial cells were either left intact or permeabelized with saponin. They were then stained with a specific monoclonal anti-IL-13Rα2 antibody and analysed by flow cytometry. The red filled histogram refers to the background staining, the open histogram with black line represents IL-13Rα2 surface staining on intact cells, and the open histogram with blue line represents IL-13Rα2 staining in permeabilized cells. Representative histogram from eight separate experiments is shown.

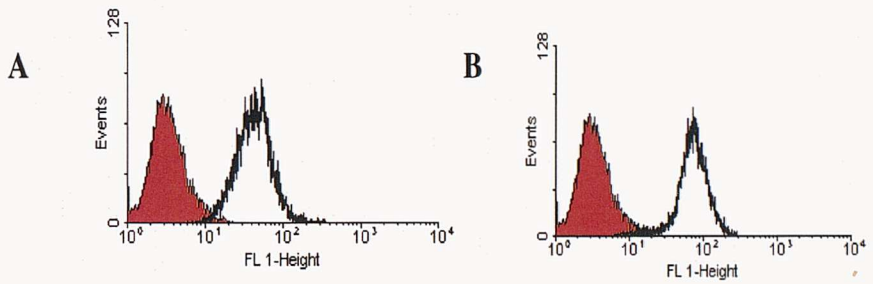


Figure 5.7. Expression of ICAM-1 and CD44 on NCI-H292 cells. Unstimulated cells were stained with either a specific monoclonal anti-ICAM-1 antibody (A), or a specific monoclonal anti-CD44 antibody (B) and analysed by flow cytometry. Fluorescence intensity is represented by open histograms with black line; red filled histograms refer to the background staining of isotype-matched controls. Representative histograms of triplicate experiments are shown.

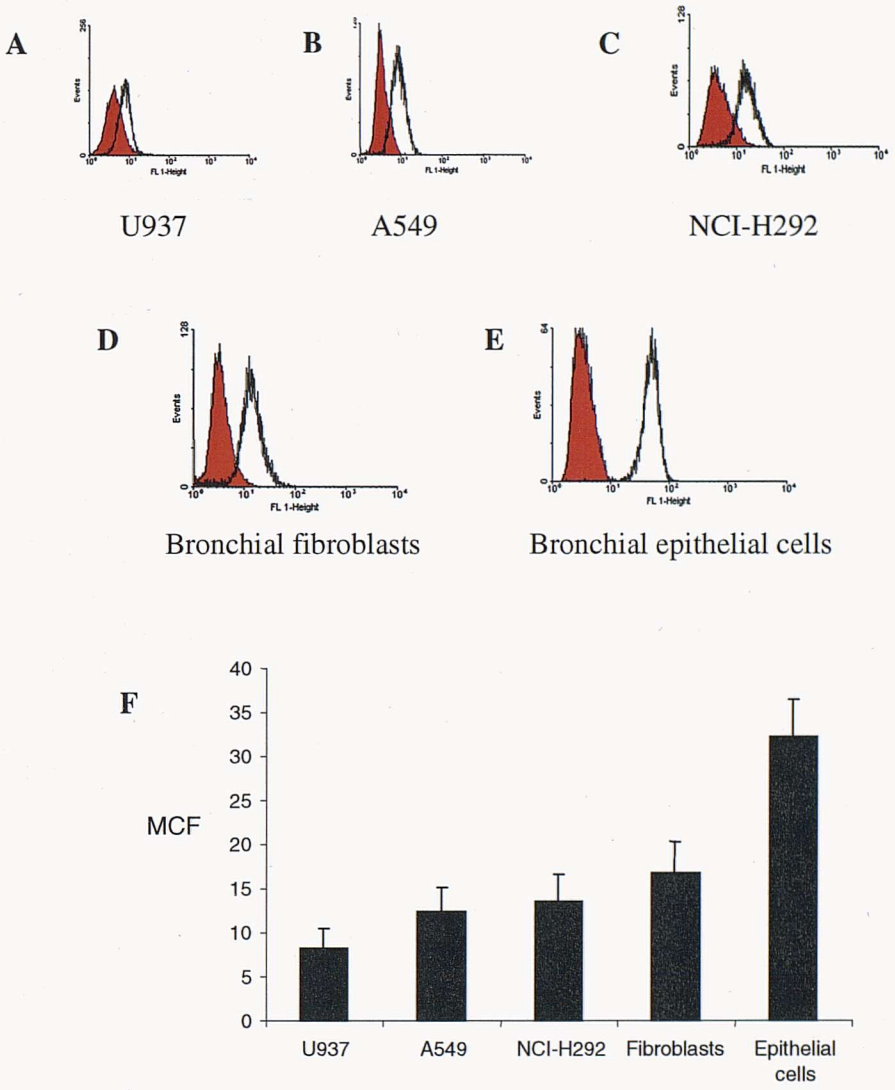
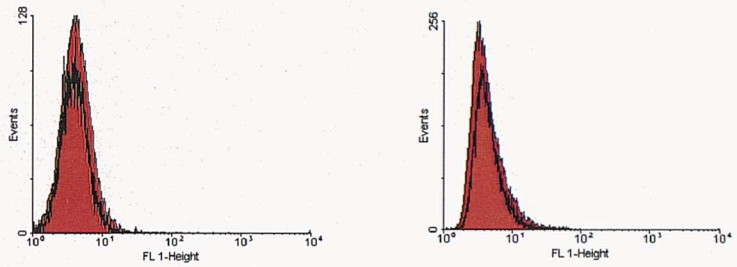


Figure 5.8. Intracellular stores of IL-13Rα2 in different cell types. A549 cells (A), NCI-H292 cells (B), U937 cells (C), primary lung fibroblasts (D), and primary bronchial epithelial cells (E) were permeabilized with saponin and stained with a specific monoclonal anti-IL-13Rα2 antibody. Representative histograms are shown in panels A-E. Three separate experiments were quantitated, and the averages and standard deviations are shown in panel F, which demonstrates the median channel fluorescence (MCF) in permeabilized cells when stained for IL-13Rα2.



Intact cells Permeabilized cells

Figure 5.9. Specificity of the anti-IL-13Rα2 antibody. Jurkat cells were either left intact (A) or permeabilized (B). They were then stained with a specific monoclonal IL-13Rα2 antibody and analysed by flow cytometry. Fluorescence intensity is represented by open histograms with black line; red filled histograms refer to the background staining of isotype-matched control. There is no staining either on intact or in permeabilized cells. Representative histograms from four separate experiments are shown.

5.3.5. Cellular localisation of IL-13Rα2 in primary bronchial epithelial cells and primary bronchial fibroblasts

Having established that IL-13Rα2 is an intracellular molecule, we aimed to identify its exact cellular location. We used confocal laser scanning microscopy in both non-permeabilized and saponin-permeabilized primary bronchial epithelial cells and primary bronchial fibroblasts. Confocal laser scanning microscopy permits examination of "cross sections" of cells, by focusing on designated planes through the cells. This methodology allows clear separation between the fluorescence staining of membranes, nuclear and cytoplasmic components without interference from overlying structures. Non-permeabilized bronchial epithelial cells and fibroblasts demonstrated no significant staining in response to the IL-13Rα2 antibody consistent with very low

level of surface expression (Figures 5.10, 5.12). In contrast, permeabilized primary bronchial epithelial cells and fibroblasts demonstrated bright uniform distribution of cytoplasmic fluorescence staining, confirming that IL-13Rα2 is an intracellular rather than a membrane bound protein (Figures 5.11, 5.13). To rule out the possibility that the absence of IL-13Rα2 surface expression was not the result of cell membrane damage or surface receptor cleavage during the experiments, we stained non-permeabilized primary bronchial fibroblasts with a monoclonal anti-ICAM-1 antibody. As shown in Figure 5.14, non-permeabilized fibroblasts stained with the anti-ICAM-1 antibody demonstrated intense membrane surface staining. In all experiments, appropriate isotype-matched control antibodies were used which demonstrated no staining.

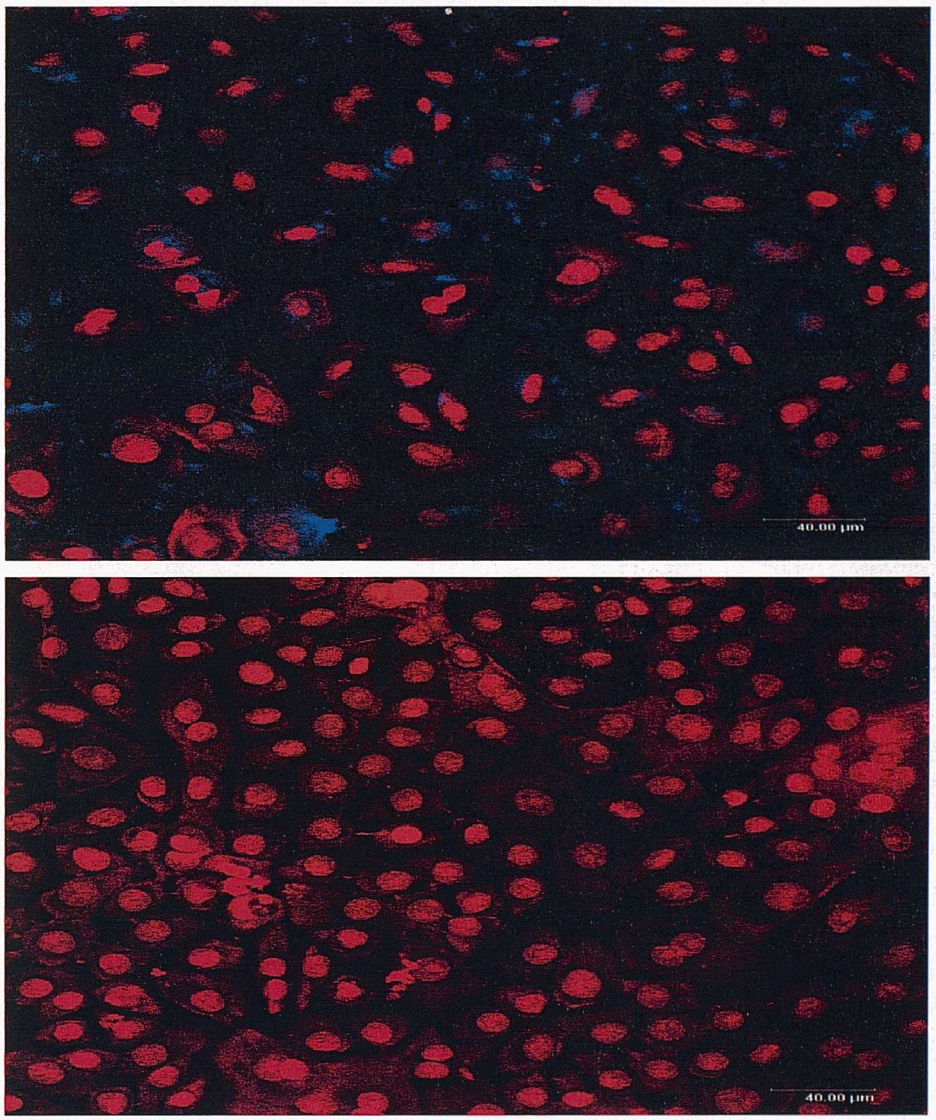


Figure 5.10. IL-13Rα2 surface expression on intact primary bronchial epithelial cells. Cells derived from a mild asthmatic subject were cultured on chamber slides, fixed with 1% paraformaldehyde, and stained with a specific monoclonal anti-IL-13Rα2 antibody (A), or an isotype-matched control antibody (B). Maximal projection of all sections taken at 1 µm by confocal laser scanning microscopy is shown. Specific IL-13Rα2 stain is shown in blue fluorescence, whereas the nuclei, counterstained with SYTOX Orange, show red fluorescence. There is only minimal IL-13Rα2 surface staining (A), whereas the specimen incubated with the control antibody shows no staining (B). Confocal images are representative of triplicate experiments. Scale bar = 40 µm.

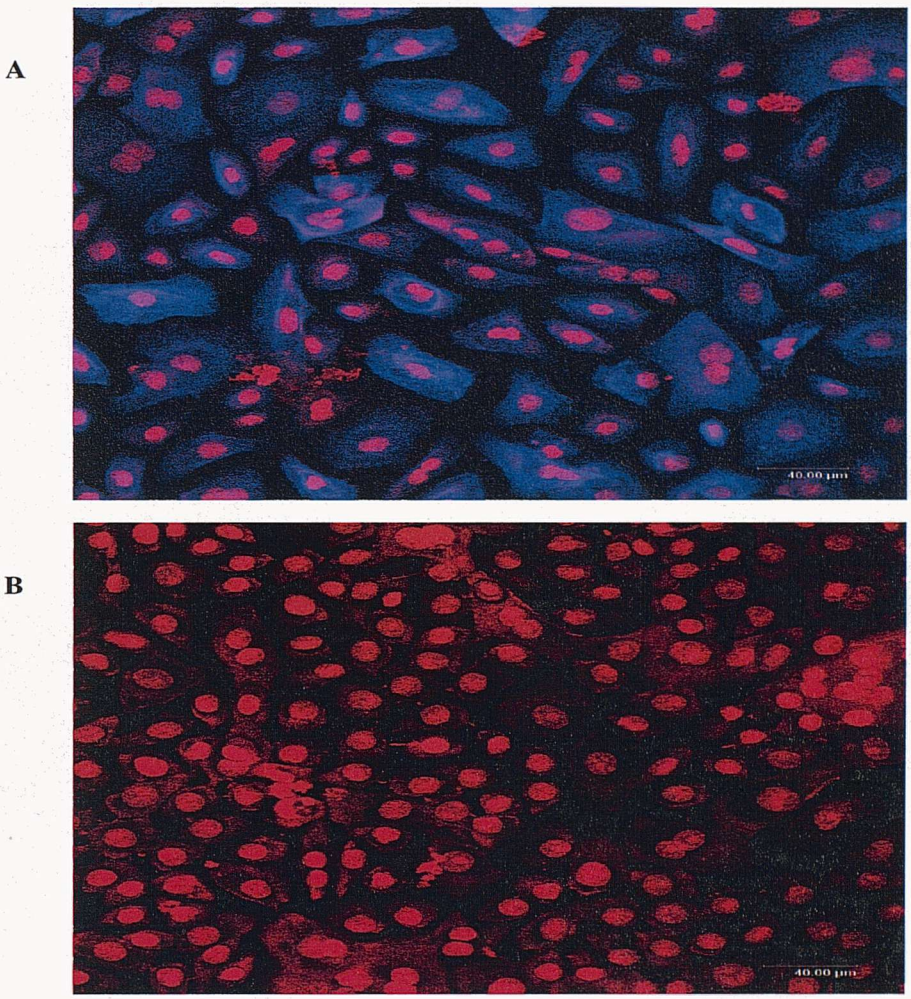


Figure 5.11. Localisation of IL-13Rα2 in permeabilized primary bronchial epithelial cells. Cells derived from a mild asthmatic subject were cultured on glass coverslips, fixed, permeabilized, and stained with a monoclonal anti-IL-13Rα2 antibody (A), or an isotype-matched control antibody (B). Maximal projection of all sections taken at 1 µm by confocal laser scanning microscopy is shown. Specific cytoplasmic IL-13Rα2 stain is shown in blue fluorescence, whereas nuclei, counterstained with SYTOX Orange, are shown in red fluorescence. IL-13Rα2 demonstrates a uniform distribution of granular cytoplasmic fluorescent staining (A), whereas the specimen incubated with the control antibody shows no cytoplasmic staining (B). Representative images from three separate experiments are depicted. Scale bar = 40 µm.

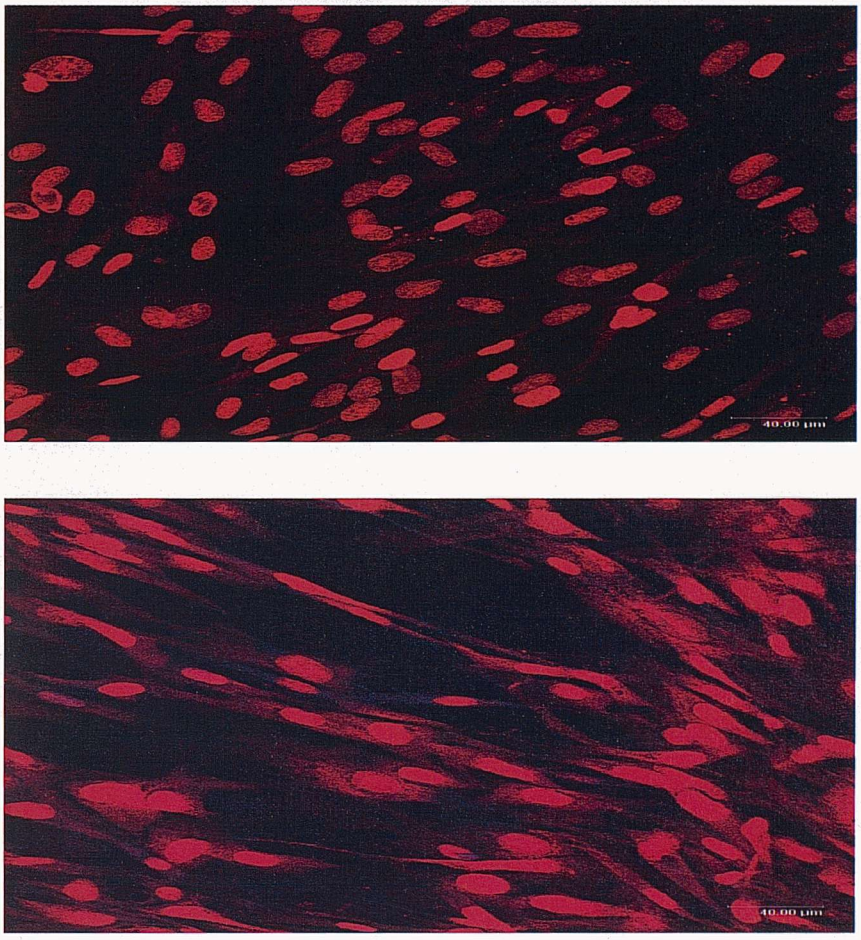
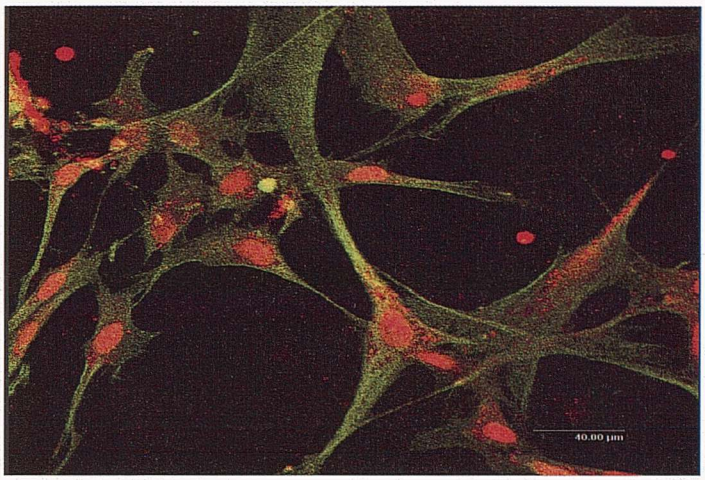


Figure 5.12. Lack of IL-13Rα2 surface expression on intact primary bronchial fibroblasts. Cells derived from a mild asthmatic subject were cultured on glass coverslips, fixed with 1% paraformaldehyde, and stained with an anti-IL-13Rα2 antibody (A), or an isotype-matched control antibody (B). Maximal projection of all sections taken at 1 µm by confocal laser scanning microscopy is shown. Nuclei counterstained with SYTOX Orange demonstrate red fluorescence. There is no IL-13Rα2 surface staining on primary lung fibroblasts (A), whereas the specimen stained with an isotype-matched control antibody shows no surface staining as well (B). Confocal images are representative of three separate experiments. Scale bar = 40 µm.

A



B

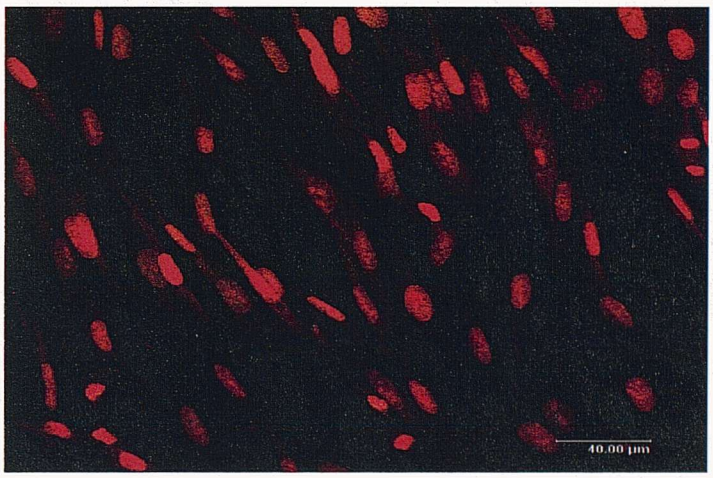
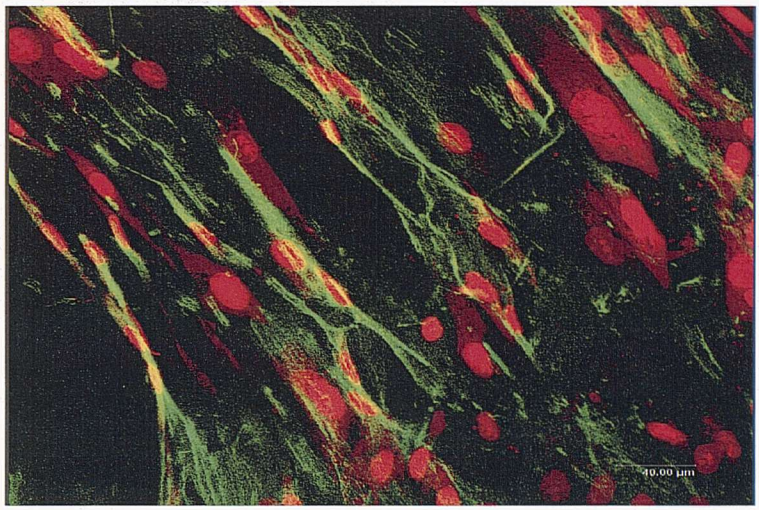


Figure 5.13. Localisation of IL-13Rα2 in permeabilized primary bronchial fibroblasts. Cells derived from a mild asthmatic subject were cultured on glass coverslips, fixed, permeabilized, and stained with a monoclonal anti-IL-13Rα2 antibody (A), or an isotype-matched control antibody (B). Maximal projection of all sections taken at 1 µm by confocal microscopy is shown. Specific IL-13Rα2 staining is shown in green fluorescence, whereas nuclei counterstained with SYTOX Orange demonstrate red fluorescence. IL-13Rα2 demonstrates a uniform distribution of granular cytoplasmic fluorescent staining (A), whereas the specimen incubated with a control antibody shows no staining (B). Representative images from three separate experiments are shown. Scale bar = 40 µm.

A



B

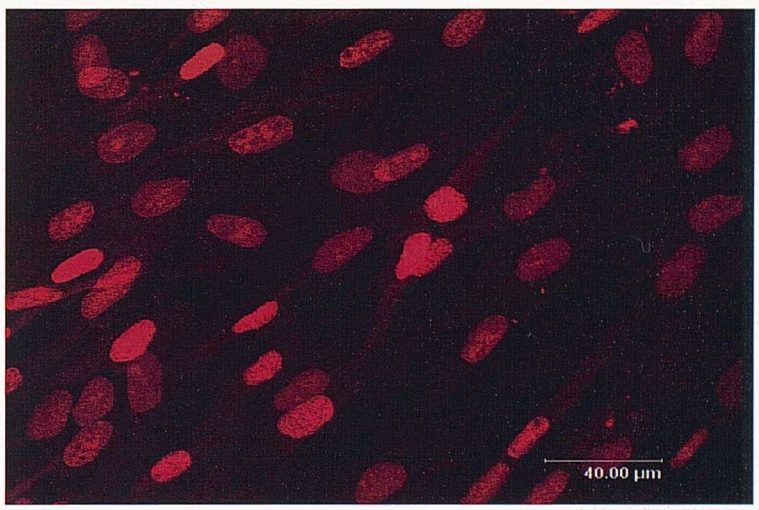


Figure 5.14. Expression of ICAM-1 on intact primary bronchial fibroblasts. Cells derived from a mild asthmatic subject were cultured on chamber slides, fixed with 1% paraformaldehyde, and stained with an anti-IL-13Rα2 antibody (A), or an isotype-matched control antibody (B). Maximal projection of all sections taken at 1 µm by confocal laser scanning microscopy is shown. Specific IL-13Rα2 staining is shown in green fluorescence, whereas nuclei counterstained with SYTOX Orange demonstrate red fluorescence. There is strong ICAM-1 surface staining (A), whereas the specimen stained with the control antibody shows no staining (B). Confocal images are representative of triplicate experiments. Scale bar = 40 µm.

5.3.6. IL-4Rα expression in airway cells

IL-4Rα chain is a component of the functional IL-13 receptor complex forming a heterodimer with the IL-13Rα1 chain. The same receptor complex also serves as an alternative IL-4 receptor in cells lacking IL-2Rγ expression. We evaluated the mRNA expression of IL-4Rα and IL-2Rγ using RT-PCR analysis and found that specific transcripts for both IL-4Rα and IL-2Rγ are present in primary bronchial fibroblasts, primary bronchial epithelial cells, white blood cells, as well as in various bronchial epithelial and haematopoietic cell lines (Figure 5.1). Next, we evaluated whether IL-4Rα protein is also expressed in unstimulated primary bronchial epithelial cells and primary bronchial fibroblasts by immunostaining. Bronchial epithelial cells demonstrated strong IL-4Rα immunoreactivity, whereas IL-4Rα expression was relatively weak in bronchial fibroblasts (Figures 5.2, 5.3). We subsequently investigated the expression of IL-4Rα protein *in vivo*, by staining bronchial biopsies derived from three mild asthmatic subjects. Immunohistochemistry revealed that the bronchial epithelium was the major site for IL-4Rα immunoreactivity with strong staining of the columnar epithelial layer and relatively weak staining of the basal layer (Figure 5.15).

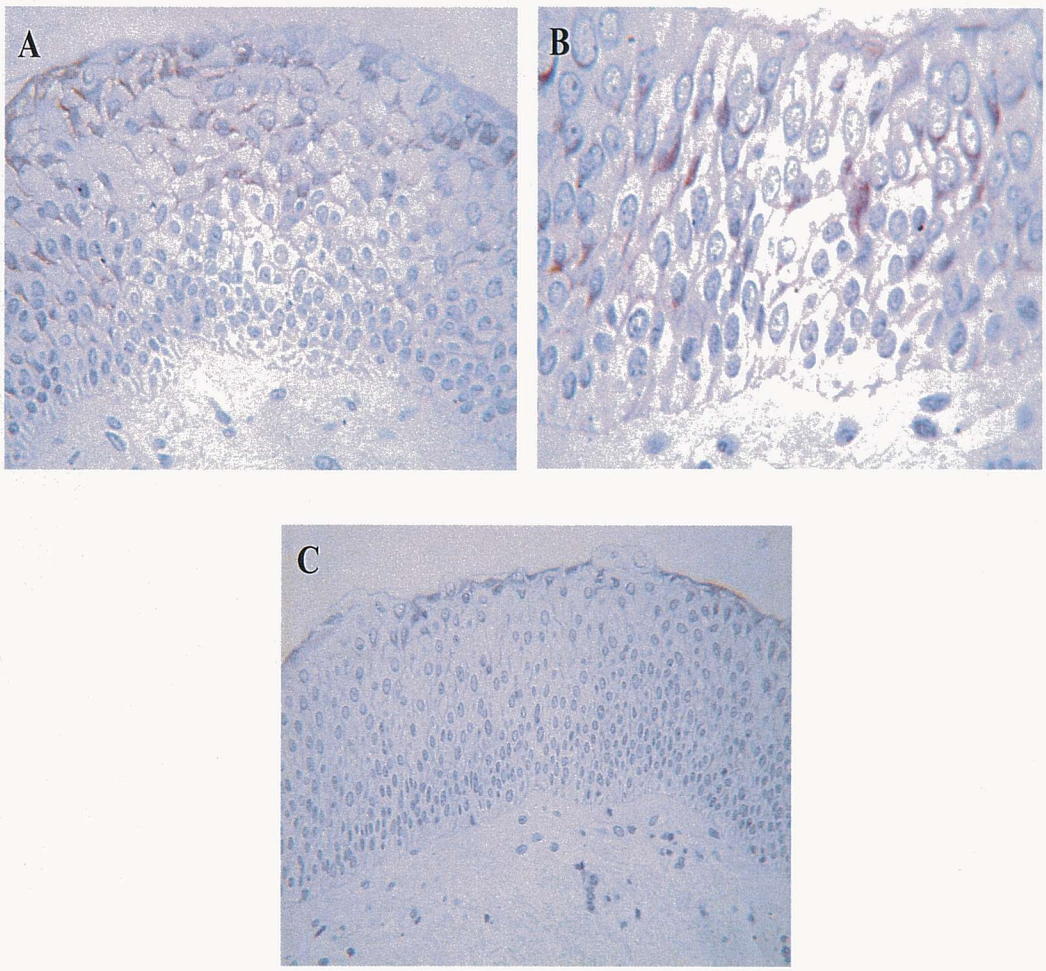


Figure 5.15. Immunoreactivity of IL-4Rα in the asthmatic bronchial mucosa. Bronchial biopsies obtained from a mild asthmatic subject by fiberoptic bronchoscopy were sectioned and stained for the presence of IL-4Rα using a monoclonal anti-IL-4Rα antibody. The panels show immunohistochemical staining of IL-4Rα in bronchial epithelium (A, B), and a control section stained with an isotype-matched control antibody (C). The columnar epithelial layer was the main target for IL-4Rα immunoreactivity, whereas basal cells demonstrated weaker staining (A, B). Representative sections from three separate experiments are depicted. Original magnification: x40 (A, C), or x63 (B).

5.4. DISCUSSION

Our experiments demonstrate that primary bronchial epithelial cells and primary bronchial fibroblasts constitutively express both mRNA and protein for IL-13Rα1, IL-4Rα, and IL-13Rα2. Immonohistochemical studies of bronchial biopsies revealed that the columnar epithelial layer is the major site of IL-13Rα1, IL-13Rα2, and IL-4Rα immunoreactivity in the asthmatic bronchial mucosa. Flow cytometry analysis demonstrated that IL-13Rα2 is an intracellular molecule in primary bronchial epithelial cells, primary lung fibroblasts, and bronchial epithelial cell lines. Studies using fluorescence confocal microscopy revealed that IL-13Rα2 is an intracellular molecule with a uniform cytoplasmic distribution in primary bronchial epithelial cells and primary bronchial fibroblasts.

5.4.1. IL-13Rα1 expression in the airways

The presence of functional IL-13 receptor in primary bronchial epithelial cells and primary bronchial fibroblasts was shown by a number of *in vitro* studies measuring different outcomes following IL-13 stimulation (Lordan et al., 2002, Doucet et al., 1998). In our study, we demonstrated that IL-13Rα1 mRNA is expressed in primary bronchial epithelial cells, primary bronchial fibroblasts, as well as in the bronchial epithelial cell lines NCI-H292, 16HBE, and A549. IL-13Rα1 mRNA expression was also shown in U937 cells and Jurkat cells. RT-PCR analysis was validated by including RT minus products, genomic DNA and Milli Q water as negative controls in all experiments. Following PCR amplification of negative controls, no product was seen on the agarose gel. In addition, DNA sequencing confirmed that the PCR product corresponded to the reference *IL13RA1* sequence. The quality of the RNA samples was tested by amplification of the *APRT* gene and was found to be optimal in all experiments.

Using immunocytochemistry, we demonstrated that IL-13Rα1 protein is also present in primary bronchial epithelial cells and primary bronchial fibroblasts. Cells were obtained from three mild asthmatic subjects by fiberoptic bronchoscopy. The level of IL-13Rα1 expression was high in primary bronchial epithelial cells and moderate in primary

bronchial fibroblasts. We also demonstrated that IL-13Rα1 protein is expressed *in vivo* in the asthmatic mucosa by performing immunohistochemistry in bronchial biopsies. The monoclonal antibody UU15 used in our studies, was previously characterised by Western blotting and immunostaining of positive and negative cell lines and found to be specific for IL-13Rα1 and suitable for immunohistochemistry (Akaiwa et al., 2001). Results by RT-PCR analysis in our study are in accordance with two previous studies, which examined IL-13Rα1 mRNA expression in various human tissues using Northern blot analysis and found that IL-13Rα1 mRNA is expressed in multiple tissues including the lung (Aman et al., 1996, Gauchat et al., 1997). Moreover, our immunohistochemical findings are also in accordance with a previous study showing expression of IL-13Rα1 in the bronchial mucosa (Heinzmann et al., 2000).

5.4.2. IL-13Rα2 expression in the airways

Specific IL-13Rα2 transcript was detected in primary bronchial epithelial cells, primary bronchial fibroblasts, U937 cells and bronchial epithelial cell lines, including NCI-H92, A549, and 16-HBE. In contrast, no IL-13Rα2 mRNA expression was found in Jurkat cells, in accordance with a previous study (Caput et al., 1996). RT-PCR analysis showed weak IL-13Rα2 mRNA expression in white blood cells. It is conceivable that part of IL-13Rα2 expression in white blood cells derived from peripheral monocytes, given that the promyelomonocytic cell line U937 expressed specific IL-13Rα2 transcript. This is further supported by a previous study showing that primary peripheral monocytes express specific IL-13Rα2 transcript (Daines et al., 2002). In our study though, we have not performed RT-PCR analysis in individual white blood cell populations.

IL-13Rα2 protein was expressed in both primary bronchial epithelial cells and primary bronchial fibroblasts using immunostaining. Staining of bronchial biopsies, derived from three mild asthmatic subjects, revealed that IL-13Rα2 is strongly expressed in the columnar cells of the mucosa and weakly expressed in the basal cells. Inflammatory cells infiltrating the mucosa showed no IL-13Rα2 innunoreactivity, in keeping with very low IL-13Rα2 mRNA expression in white blood cells previously shown by RT-PCR analysis.

The absence of IL-13Rα2 protein expression on the surface of primary bronchial epithelial cells and primary bronchial fibroblasts using flow cytometry, is in accordance with previous studies showing no IL-13Rα2 surface expression on primary monocytes and U937 cells (Daines et al., 2002), bronchial epithelial cells (Yasunaga et al., 2003), and various human fibroblast lines (Yoshikawa et al., 2003). However, when intact primary bronchial epithelial cells were visualized by confocal microscopy in our experiments, there was a rim pattern of staining consistent with weak surface expression. In contrast, no surface expression was appreciated on intact primary bronchial fibroblasts by confocal microscopy. Bernard and co-workers (Bernard et al., 2001) found high surface IL-13Rα2 expression in human glioma cell lines by means of flow cytometry, suggesting that IL-13Rα2 surface expression may vary among different human cell types. Flow cytometry showed that IL-13Rα2 is an intracellular molecule in primary bronchial epithelial cells, primary bronchial fibroblasts, various airway epithelial cell lines and U937 cells, in accordance with previous studies (Daines et al., 2002, Yasunaga et al., 2003, Yoshikawa et al., 2003). We have shown that the intracellular pool of IL-13Rα2 had a diffuse granular-like pattern of cytoplasmic distribution in primary bronchial epithelial cells and primary bronchial fibroblasts, when cells visualized by confocal laser scanning microscopy. We also demonstrated that intracellular IL-13Rα2 binding, using a monoclonal anti-IL-13Rα2 antibody, was specific, since no IL-13Rα2 protein expression was detected in the intracellular compartment of permeabilized Jurkat cells, previously shown to lack IL-13Rα2 mRNA expression by RT-PCR analysis.

5.4.3. IL-4Rα and IL-2Rγ expression in the airways

Using similar approaches as above, we found that the second component of the IL-13 receptor complex, the IL-4Rα chain, is expressed in bronchial epithelial cells and bronchial fibroblasts at both the mRNA and protein levels. Staining of bronchial specimens of three mild asthmatic subjects showed the presence of IL-4Rα in the columnar layer of bronchial mucosa. We have demonstarted that the other component of the classical IL-4 receptor system, the IL-2Rγ chain, is expressed on mRNA level in both primary bronchial epithelial cells and primary bronchial fibroblasts. Our findings are in accordance with previous studies, which have shown the presence of IL-4Rα (van der

Velden et al., 1998, Heinzmann et al., 2000, Kotsimbos et al., 1998) and IL-2Rγ in structural airway cells (van der Velden et al., 1998, Doucet et al., 1998). The classical IL-4 receptor in hematopoietic cells is a heterodimer composed of IL-4Rα (140-kDa) and IL-2Rγ (65-kDa). The IL-13 receptor complex, which consists of IL-13Rα1 and IL-4Rα, also serves as the alternative form of IL-4 receptor in non-hematopoietic cells that lack IL-2Rγ expression (Murata et al., 1997). Despite the notion that non-hematopoietic cells do not express the IL-2Rγ chain, our experiments showed that specific IL-2Rγ transcript is expressed in both primary bronchial epithelial cells and primary bronchial fibroblasts. This strongly suggests that IL-4 can signal through both classical and alternative IL-4 receptor complexes in the airways, and the balance between the level of expression of IL-13Rα1 and IL-2Rγ may determine which receptor complex is mostly utilised by IL-4.

Summary

- RT-PCR analysis established the presence of specific IL-13Rα1, IL-13Rα2, IL-4Rα, and IL-2Rγ transcripts in primary bronchial epithelial cells and primary bronchial fibroblasts.

- Immunohistochemical analysis of IL-13 receptor components in bronchial biopsies derived from asthmatic subjects showed that the epithelial layer of the airway mucosa expresses protein for IL-13Rα1, IL-13Rα2, and IL-4Rα *in vivo*.

- Using flow cytometry and confocal laser scanning microscopy we have shown that IL-13Rα2 is predominantly an intracellular molecule with diffuse granular cytoplasmic distribution in primary bronchial epithelial cells and primary bronchial fibroblasts.

- A rim pattern of surface IL-13Rα2 expression was seen on intact primary bronchial epithelial cells, whereas no surface IL-13Rα2 staining was present on intact primary bronchial fibroblasts, when cells were visualized by confocal laser scanning microscopy.

CHAPTER 6

Intracellular stores of IL-13Rα2 in airway cells

6.1. Overview

Previous studies provided evidence that IFN-γ, TNF-α, IL-13, and IL-4 play a crucial role in the regulation of IL-13Rα2 expression in various cell types. Studies were undertaken to determine whether various cytokines could upregulate surface IL-13Rα2 expression in NCI-H292 cells, primary bronchial fibroblasts, and primary bronchial epithelial cells.

Results: Stimulation of NCI-H292 cells and primary bronchial fibroblasts with either IFN-γ, PMA, TNF-α, IL-13, or IL-4 did not induce IL-13Rα2 surface expression on these cells. In addition, there was no upregulation of surface IL-13Rα2 on primary bronchial epithelial cells following treatment with IFN-γ or PMA.

Daines and Hershey reported that IFN-γ and PMA mobilize the intracellular pool of IL-13Rα2 on the surface of U937 and primary human monocytes (Daines and Hershey, 2002). In the above study, the IFN-γ-dependent increased IL-13Rα2 was not dependent on protein synthesis, and IFN-γ pre-treatment of U937 cells resulted in diminished IL-13-dependent STAT-6 activation. In human fibroblasts derived from nasal polyp, lung and skin, IL-13Rα2 surface expression was upregulated in response to TNF-α and IL-4 through induction of gene expression and mobilization of intracellular receptors (Yoshikawa et al., 2003). In the HaCaT keratinocyte cell line IL-4 and IL-13 were shown to upregulate IL-13Rα2 at both the mRNA and protein level (David et al., 2001). In another study, the same group of investigators demonstrated that TNF-α potentiates IL-4/IL-13 induced IL-13Rα2 expression in HaCaT cells at both the mRNA and protein level, resulting in induction of IL-13Rα2 surface expression (David et al., 2003). In contrast, Yasunaga and coworkers reported that although IL-4 and IL-13 upregulate intracellular expression of IL-13Rα2 protein in human bronchial epithelial cells, they do not induce expression of IL-13Rα2 on the cell surface (Yasunaga et al., 2003).

Having established that IL-13Rα2 is an intracellular molecule in primary bronchial epithelial cells, bronchial epithelial cell lines and primary bronchial fibroblasts, we sought to investigate whether the intracellular pool of IL-13Rα2 could be mobilised to the cell surface in response to IFN-γ, PMA, TNF-α, IL-13, or IL-4.

6.2. Experimental design

Primary bronchial epithelial cells and primary bronchial fibroblasts were obtained from three mild asthmatic subjects by fiberoptic bronchoscopy. Cultures were established as described in sections 2.16 and 2.17. Primary bronchial epithelial cells were used for experimentation at passage 3, whereas fibroblasts were used between passages 3 and 6. To ensure similar cell densities in different assays, cells were seeded in fixed numbers per cm^2 (20×10^3). When cultures reached ~90% confluency cells were rendered quiescent by replacing the medium with basal medium supplemented with antibiotics (penicillin and streptomycin) in the presence or absence of IFN-γ, PMA, TNF-α, IL-13, or IL-4 for various incubation periods. Following cytokine treatment, viability of cells was assessed by exclusion of trypan blue dye and found to be consistently >90%, suggesting that treatments did not cause cell toxicity. IL-13Rα2 and ICAM-1 cell surface expression was evaluated by flow cytometry. Primary bronchial epithelial cells and primary bronchial fibroblasts were dissociated from flasks using trypsin, while NCI-H292 cells were dissociated using a non-enzymatic solution (Sigma-Aldrich, Poole, UK). To evaluate surface expression of IL-13Rα2 or ICAM-1, cells were incubated with a specific monoclonal anti-IL-13Rα2 antibody, or a monoclonal anti-ICAM-1 antibody, or appropriate isotype-matched control antibodies. Each sample was washed and incubated with a secondary goat anti-mouse IgG-FITC antibody. Samples were subsequently washed, re-suspended in PBS and immediately analysed with a FACScan (Becton Dickinson Biosciences, Oxford UK). To analyse IL-13Rα2 intracellular pool, cells were fixed with paraformaldehyde and permeabilized with PBS containing 0.2% saponin. Saponin (0.2%) was also added to the antibody and wash solutions. Data were collected on 10,000 events. Median index of fluorescence was determined after non-viable cells and debris were excluded by PI exclusion gating. Data were analysed for significance using a one-way analysis of variance (ANOVA). When a difference

among multiple treatments was found, the Bonferroni test was used to identify which of the means were significantly different from the other. When variances were not homogenous, non-parametric tests were used. Data were considered significant at $P<0.01$.

6.3. Results

6.3.1. IL-13Rα2 surface expression on primary airway cells is not induced by a variety of cytokines

Having previously found a large intracellular pool of IL-13Rα2 in primary bronchial epithelial cells and primary bronchial fibroblasts, we investigated whether this intracellular pool of receptors could be mobilised to the cell surface in response to cytokine stimulation using flow cytometry. Analysis of data by flow cytometry was performed after propidium iodide exclusion of non-viable cells, since analysis of the whole cell population would inevitably include a number of dead cells, which would bind the IL-13Rα2 antibody intracellularly providing false data.

Primary bronchial epithelial cells stimulated with either IFN-γ (20 ng/ml) or PMA (10 ng/ml) for 24 h did not show any induction of surface IL-13Rα2 by flow cytometry (Figure 6.1). To rule out the possibility that the lack of induction of IL-13Rα2 surface expression was due to poor cell response to cytokine stimulation, we assessed the induction of ICAM-1 on these cells in parallel experiments. In sharp contrast, incubation of primary bronchial epithelial cells with IFN-γ (20 ng/ml) or PMA (20 ng/ml) for 24 h enhanced ICAM-1 cell surface expression by 8-fold and 2-fold, respectively (Figure 6.2).

We next investigated whether IL-13Rα2 in the intracellular compartments of primary bronchial fibroblasts could be mobilised to the cell surface. Similar to bronchial epithelial cells, stimulation of primary bronchial fibroblasts with either IFN-γ (20 ng/ml) or PMA (10 ng/ml) for 48 h failed to induce IL-13Rα2 surface expression (data not shown). Moreover, stimulation of primary bronchial fibroblasts with TNF-α (20 ng/ml), IL-13 (20 ng/ml), or IL-4 (20 ng/ml) for 48 h also failed to enhance IL-13Rα2 surface expression (data not shown). In contrast, ICAM-1 surface expression

on primary bronchial fibroblasts was upregulated following 24 h exposure to TNF-α by 4-fold, to IFN-γ by 2.5-fold, and to PMA by 1.5-fold (Figure 6.2), demonstrating that cells were responsive to cytokine treatments in our experiments.

6.3.2. IL-13Rα2 surface expression on NCI-H292 cells is not induced by a variety of cytokines

We investigated whether the intracellular stores of IL-13Rα2 could be mobilised to the cell surface in the lung mucoepidermoid adenocarcinoma cell line NCI-H292. We carried out experiments on NCI-H292 cells since the number of cultured primary bronchial epithelial cells was not adequate for multiple assays. NCI-H292 cells were treated with either medium alone, IFN-γ (20 and 40 ng/ml), PMA (1 and 10 ng/ml), TNF-α (20 and 40 ng/ml), IL-13 (20 and 40 ng/ml), or IL-4 (20 and 40 ng/ml) for 1-, 6-, 12-, 24-, or 48 h. Intact cells were stained with an anti-IL-13Rα2 antibody followed by incubation with a FITC-conjugated secondary antibody and analysis by flow cytometry, after propidium iodide exclusion of non-viable cells.

No induction of IL-13Rα2 surface expression was seen following incubation of NCI-H292 cells with either IFN-γ, PMA, TNF-α, IL-13, or IL-4 at any time point (data not shown). In all experiments, the obtained fluorescence intensities in cells incubated with the specific anti-IL-13Rα2 antibody were similar to those of cells incubated with an isotype-matched control antibody. To investigate whether the lack of IL-13Rα2 surface induction in NCI-H292 cells was due to poor cell response to cytokine stimulation, we assessed the induction of surface ICAM-1 expression in parallel experiments. Previous studies have shown that IFN-γ, PMA, TNF-α, IL-13, and IL-4 enhance ICAM-1 expression in airway epithelial cells (Bloemen et al., 1993). We stimulated NCI-H292 cells with either medium alone (control), IFN-γ (20 ng/ml), PMA (1 ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), or IL-4 (20 ng/ml) for 12-, 24- and 48 h. Flow cytometry analysis was performed after propidium iodide exclusion of non-viable cells. ICAM-1 surface expression was enhanced in response to IFN-γ by 2-fold at 12 h ($P<0.0001$), by 3-fold at 24 h ($P<0.0001$), and by 5-fold at 48 h ($P<0.0001$). PMA increased ICAM-1 surface expression by approximately 1.5-fold at 24h ($P<0.001$) and 48h ($P<0.01$). TNF-α induced ICAM-1 expression by 1.5 at 24 h

(P<0.001). ICAM-1 expression was also upregulated by 1.5-fold following 24 h stimulation with IL-13 (P<0.005) or IL-4 (P<0.001) (Figure 6.3). The induction of ICAM-1 on NCI-H292 cells demonstrated that cells used in our experiments were responsive to cytokine stimulation.

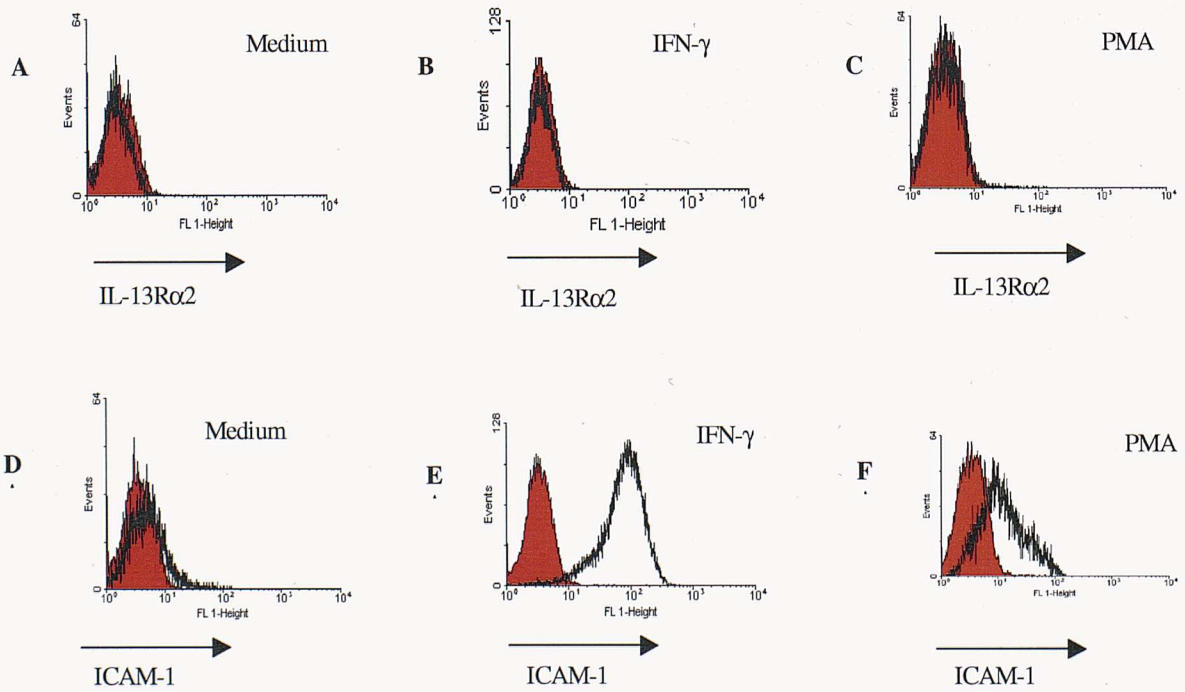


Figure 6.1. IL-13Rα2 surface expression is not upregulated by IFN-γ or PMA on primary bronchial epithelial cells. Cells derived from an asthmatic subject were incubated with medium alone (A, D), medium containing 20 ng/ml IFN-γ (B, E), or medium containing 10 ng/ml PMA (C, F) for 24 h. Cells were stained and analysed for surface expression of IL-13Rα2 (A, B, C) or surface expression of ICAM-1 (D, E, F). The panel shows that cells incubated with medium alone demonstrate no IL-13Rα2 surface expression (A), and minimal expression of ICAM-1 (D). Cells incubated with IFN-γ or PMA show no induction of IL-13Rα2 surface expression (B, C), however, they show significant enhancement of ICAM-1 expression (E, F), especially those treated with IFN-γ (E). Representative histograms of triplicate experiments are depicted.

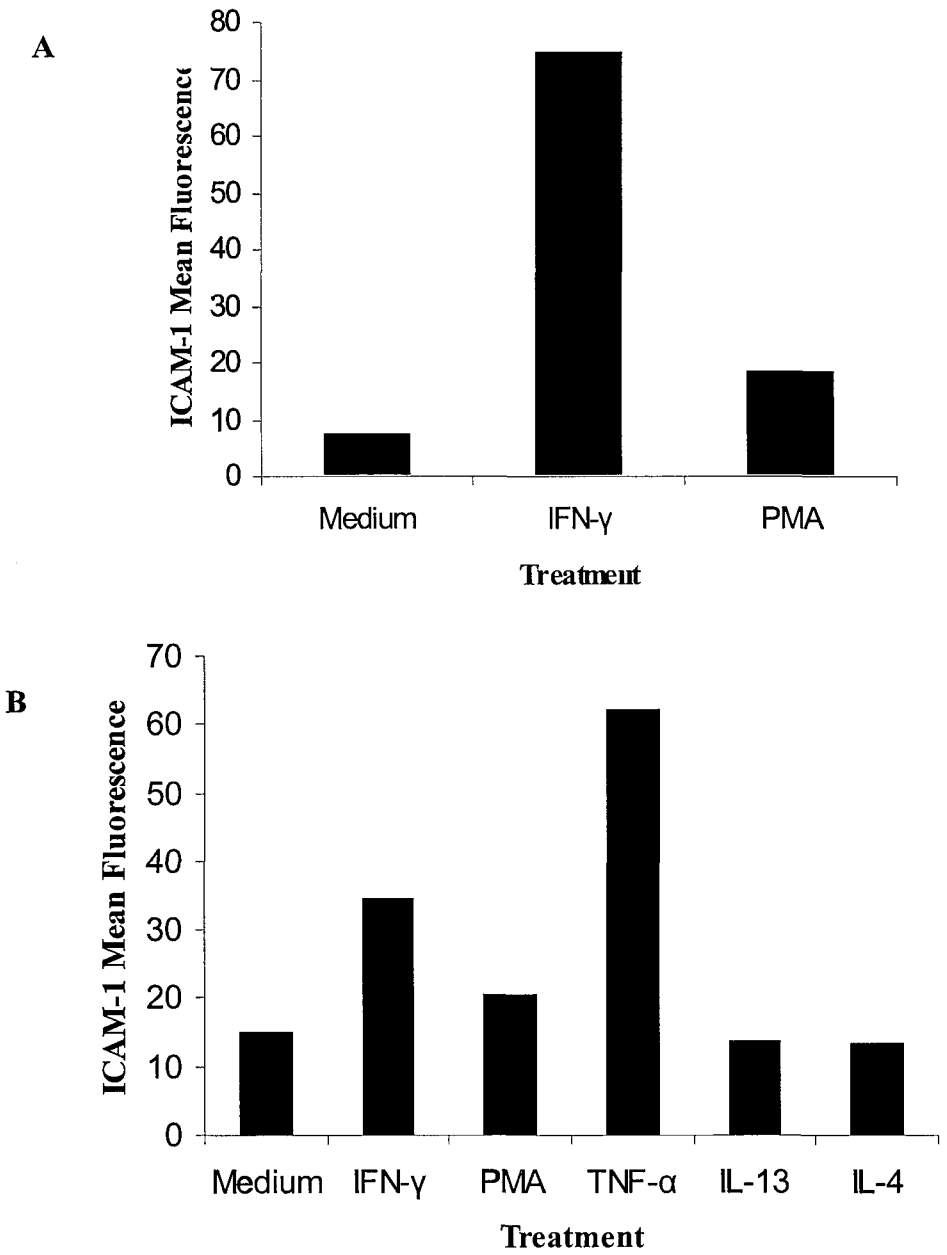


Figure 6.2. U pregulation of ICAM-1 s urface e xpression o n p rimary b ronchial epithelial cells and primary bronchial fibroblasts. Panel A: primary bronchial epithelial cells were incubated with IFN-γ or PMA for 24 h, and ICAM-1 surface expression determined by flow cytometry. Means from two independent experiments are shown. Panel B: primary bronchial fibroblasts were incubated with IFN-γ, PMA, TNF-α, IL-13, or IL-4 for 48 h, and ICAM-1 expression was assessed by flow cytometry. Means are from two separate experiments.

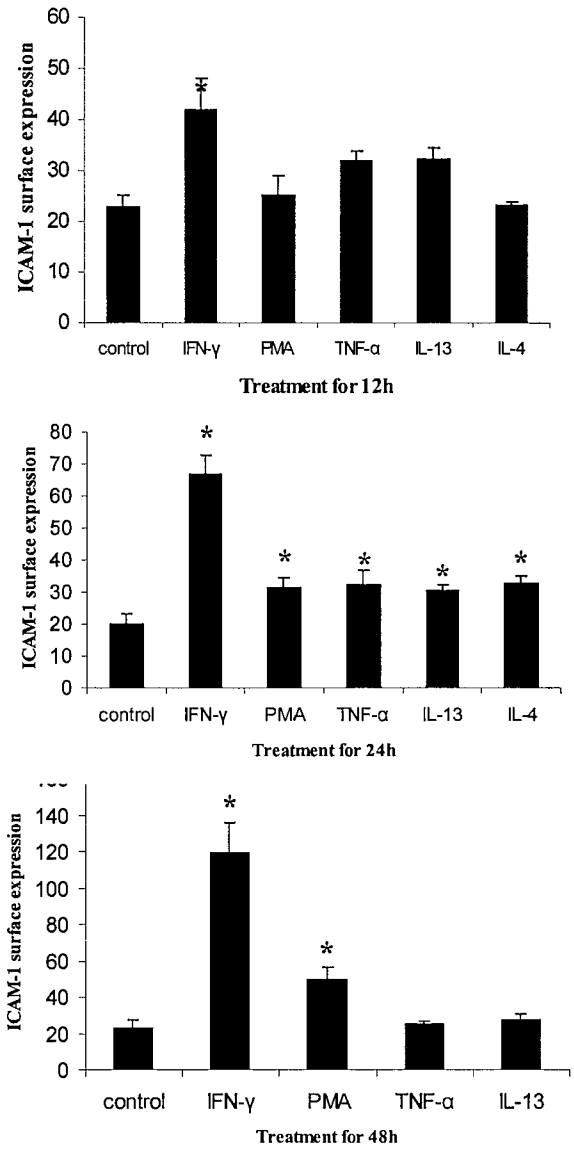
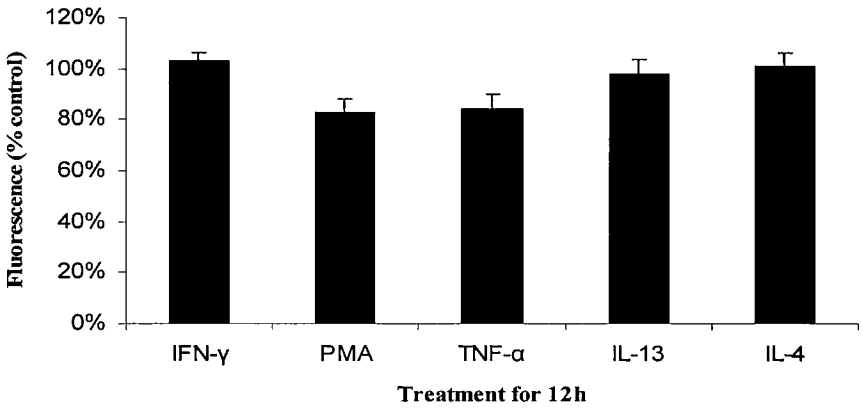


Figure 6.3. Upregulation of ICAM-1 surface expression on NCI-H292 cells following cytokine stimulation. NCI-H292 cells were incubated with either medium alone, IFN-γ (20 ng/ml), PMA (1 ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), or IL-4 (20 ng/ml) for 12 h, 24 h, or 48 h. Cells were stained with a monoclonal anti-ICAM-1 antibody and surface expression was evaluated by flow cytometry. Results represent mean plus standard deviation of 5 separate experiments. Significance was analysed by comparison between treated and untreated cells with ANOVA.

*$P<0.01$

6.3.3. Effect of cytokine stimulation on intracellular stores of IL-13Rα2

We investigated the effects of cytokine stimulation on the intracellular expression of IL-13Rα2 in NCI-H292 cells by flow cytometry. Cells were incubated with either medium alone, IFN-γ (20 ng/ml, PMA (1ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), or IL-4 (20 ng/ml) for 24 h. Five separate experiments were carried out for each cytokine treatment and significance was analysed by comparison between treated and untreated cells. There was no statistically significant change in IL-13Rα2 intracellular expression for any treatment (Figure 6.4). There was only a small reduction of IL-13Rα2 expression following treatment with IL-4, IL-13, or TNF-α for 24 h, which, however, was not statistically significant. We further investigated the effect of IL-13 and TNF-α on IL-13Rα2 intracellular expression by incubating NCI-H292 cells with either IL-13 (20 ng/ml) or TNF-α (20 ng/ml) for 48 h. There was only a small, not statistically significant reduction in intracellular IL-13Rα2 expression (Figure 6.5). Taken together, these results demonstrate that the expression of intracellular pool of IL-13Rα2 in NCI-H292 cells is not altered in response to either IFN-γ, PMA, TNF-α, IL-13, or IL-4.



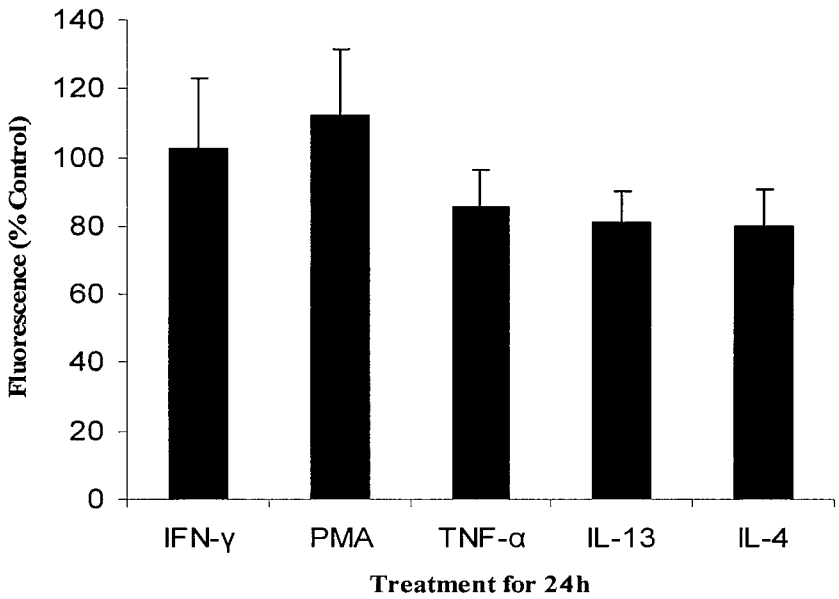


Figure 6.4. Effect of various cytokines on the intracellular pool of IL-13Rα2 in NCI-H292 cells. Cells were cultured in the presence of IFN-γ (20 ng/ml), PMA (1 ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), or IL-4 (20 ng/ml) for 12-, or 24 h. Cells were fixed, permeabilized, stained for IL-13Rα2 and analysed by flow cytometry. In panel A, cells treated for 12 h show no significant change in IL-13Rα2 expression. In panel B, cells treated for 24 h show decreased IL-13Rα2 expression following exposure to TNF-α, IL-13 and IL-4, which, however, did not reach statistical significance. Results show % percentage median fluorescence intensity and standard deviation compared to the control of four separate experiments. Significance was analysed by comparison between treated and untreated cells with ANOVA. Median fluorescence of unstimulated cells treated with medium alone=100%.

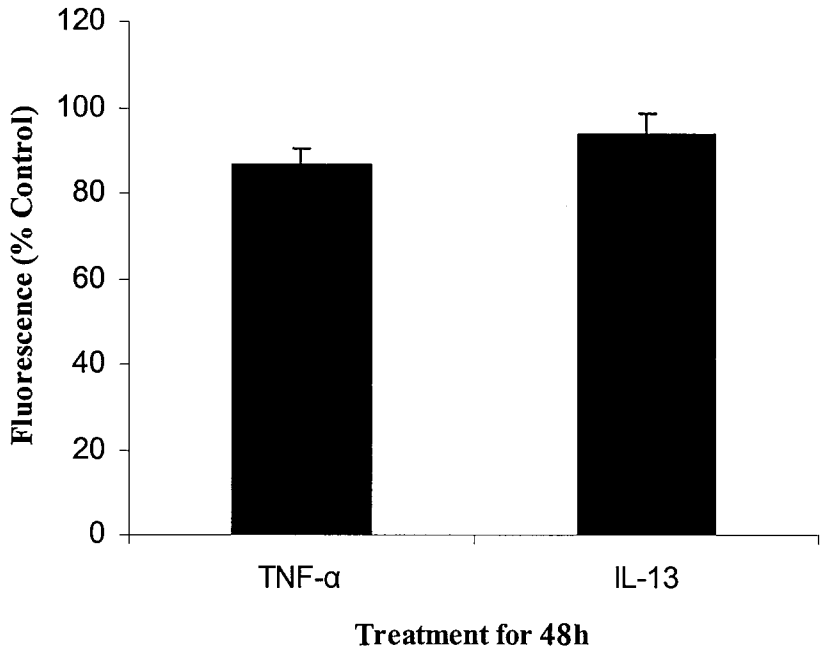


Figure 6.5. Effect of TNF-α and IL-13 on the intracellular pool of IL-13Rα2 in NCI-H292 cells. Cells were incubated in the presence of TNF-α or IL-13 for 48h, fixed, permeabilized, and stained for IL-13Rα2. Results show % percentage median fluorescence intensity and standard deviation compared to the control of three separate experiments. Significance was analysed by comparison between treated and untreated cells with ANOVA. Median fluorescence of unstimulated cells treated with medium alone=100%.

6.4. DISCUSSION

6.4.1. Lack of IL-13Rα2 surface induction on airway cells following cytokine stimulation

In our experiments, there was no induction of surface IL-13Rα2 on airway epithelial NCI-H292 cells and primary bronchial fibroblasts following stimulation with either IFN-γ, PMA, TNF-α, IL-13, or IL-4. Moreover, no induction of surface IL-13Rα2 expression was found on primary bronchial epithelial cells stimulated with IFN-γ or PMA. Our data are in agreement with the study of Yasunaga and co-workers who found that although IL-4 and IL-13 upregulated intracellular IL-13Rα2 protein, they did not induce expression of IL-13Rα2 on the cell surface of human bronchial epithelial cells (Yasunaga et al., 2003). In contrast, Daines and Hershey reported that treatment of U937 cells and primary human monocytes with IFN-γ or PMA increased IL-13Rα2 surface expression in a dose dependent manner (Daines and Hershey, 2002). In this study, IFN-γ treatment of U937 cells with physiological doses of 20 ng/ml resulted in a rapid increase of IL-13Rα2 surface expression as early as 4 h reaching maximum levels at 24 h. U937 cells subjected to IFN-γ stimulation showed approximately a 2-fold increase in surface IL-13Rα2, whereas primary human monocytes demonstrated a nearly 6-fold induction in surface expression. Similarly, in the study of Yoshikawa and co-workers, TNF-α and IL-4 induced surface IL-13Rα2 expression on human fibroblasts derived from nasal polyp, lung and skin, by upregulating gene expression and mobilizing intracellular receptors (Yoshikawa et al., 2003). IL-13Rα2 surface expression was induced in a time-dependent fashion and reached a maximum value after 96 h. However, in the studies of Daines and Hershey and the study of Yoshikawa and co-workers, flow cytometry analysis of cell surface IL-13Rα2 was performed without exclusion of non-viable cells, making very difficult to interpret the data. IFN-γ and PMA were shown to induce apoptosis in macrophages through a protein kinase C signaling pathway (Munn et al., 1995), as well as induction of tumor necrosis factor-α receptor and caspase-8 (Inagaki et al., 2002). Similarly, TNF-α induces apoptotic (Abe et al., 2000, Gupta et al., 2001) and nonapoptotic cell death (Lin et al., 2004) in many cell types. Therefore, experiments

assessing the surface expression of an intracellular molecule, such as IL-13Rα2, by flow cytometry, should incorporate exclusion of non-viable cells during analysis of the data. Non-viable cells can provide false data when assessing IL-13Rα2 surface expression, since damaged cell surface membrane can cause binding of the anti-IL-13Rα2 antibody to the intracellular compartment, which contains a large intracellular pool of IL-13Rα2. This intracellular binding may erroneously be interpreted as an increase of IL-13Rα2 surface expression. Further validity to our experiments was provided by assessment of cell responsiveness to cytokine stimulation by determining the induction of ICAM-1 surface expression. We demonstrated that ICAM-1 surface expression was enhanced following cytokine stimulation at various incubation periods, in accordance with previous studies.

6.4.2. Effects of cytokine stimulation on intracellular stores of IL-13Rα2 in NCI-H292 cells

Stimulation of NCI-H292 cells with IFN-γ, PMA, TNF-α, IL-13, or IL-4 for 24 h did not significantly change the expression of intracellular IL-13Rα2. There was a trend for a decrease in IL-13Rα2 expression following treatment with TNF-α or IL-13 at 24h (P=0.310, ANOVA) and 48 h (P=0.154, ANOVA), however, it did not reach statistical significance. Previous studies have shown that IL-13 and IL-4 enhance IL-13Rα2 mRNA expression in primary bronchial epithelial cells by four- to twelve-fold, respectively, compared to non-stimulated cells (Yuyama et al., 2002). Moreover, IL-13 and IL-4 induced IL-13Rα2 protein expression in human primary airway cells, as assessed by immunostaining (Yasunaga et al., 2003). These data suggest that the kinetics of intracellular IL-13Rα2 expression differ between primary bronchial epithelial cells and NCI-H292 cells.

6.4.3. Induction of ICAM-1 on airway cells

ICAM-1 expression was enhanced in response to IFN-γ and PMA on primary bronchial epithelial cells, in accordance with a previous study (Bloemen et al., 1993). Similarly, incubation of primary bronchial fibroblasts with IFN-γ, PMA, or TNF-α induced ICAM-

1 expression in accordance with a previous study (Spoelstra et al., 1999). We have also shown that treatment of NCI-H292 cells with IFN-γ, PMA, TNF-α, IL-13, or IL-4 resulted in induction of surface ICAM-1 at different incubation periods. The most powerful inducer of ICAM-1 surface expression on NCI-H292 was IFN-γ with a maximum 5-fold increase at 48 h. PMA induced ICAM-1 expression at 24 h (1.5-fold increase) and 48 h (2-fold increase). TNF-α induced a small (1.5-fold), but statistically significant increase after 24 h incubation time. Similarly, IL-13 and IL-4 stimulation resulted in a small (1.5-fold), but statistically significant upregulation of ICAM-1 at 24 h. Interestingly, IL-13, but not IL-4, increased ICAM-1 expression by 1.4-fold as early as 12 h. These results are in accordance with previous studies showing induction of ICAM-1 surface expression in response to IFN-γ, PMA, or TNF-α on NCI-H292 cells (Bloemen et al., 1993, Togashi et al., 1998). IL-13 and IL-4 have previously been shown to induce ICAM-1 expression on primary bronchial epithelial cells (Striz et al., 1999). The above experiments served as positive control experiments providing clear evidence that all cells used for experimentation were responsive to cytokine stimulation.

6.4.4. Potential role of the intracellular pool of IL-13Rα2 in airway cells

IL-13Rα2 is a high affinity receptor for IL-13 (Kd = 250 pM), having a 100- to 300-fold higher affinity for IL-13 compared to IL-13Rα1 (Caput et al., 1996). However, its short cytoplasmic tail of 17 amino acids though does not conserve the box-1 region, critical for association with JAKs (Ihle et al., 1995), and cannot induce signalling via the STAT6 pathway after binding IL-13 (Kawakami et al., 2001). It is currently thought that IL-13Rα2 functions as decoy receptor for IL-13 providing tighter regulation of IL-13 by rapidly removing excess IL-13 from the system (Feng et al., 1998).

The lack of mobilisation of the intracellular stores of IL-13Rα2 to the cell surface in primary bronchial epithelial cells, primary bronchial fibroblasts, and NCI-H292 cells following stimulation with a variety of cytokines suggests that the intracellular pool of IL-13Rα2 in these cells may be released only after cellular damage or cellular death, thereby attenuating on-going extracellular IL-13 activity. Another hypothesis is that the intracellular pool of IL-13Rα2 in bronchial epithelial cells and bronchial fibroblasts

represents a truncated form of the receptor lacking the signal sequence, therefore, being unable to anchor to the cell surface. This receptor pool is either trapped within the cell and is only released after cell damage, or alternatively, it is slowly secreted serving as a soluble receptor. The latter hypothesis is supported by detection of soluble IL-13Rα2 in the serum and urine of mice (Zhang et al., 1997). However, to date, no such soluble form of IL-13Rα2 has been found in humans. Alternatively, IL-13Rα2 intracellular receptor pool in bronchial epithelial cells and airway fibroblasts might be mobilised to the cell surface under certain physiologic conditions and cytokine stimulations not used in our experiments.

Summary

- Treatment of NCI-H292 cells and primary bronchial fibroblasts with either IFN-γ, PMA, TNF-α, IL-4, or IL-13 did not upregulate surface IL-13Rα2.

- Stimulation of primary bronchial epithelial cells with IFN-γ or PMA did not induce IL-13Rα2 surface expression.

- In parallel experiments, treatment of primary bronchial epithelial cells, primary bronchial fibroblasts, and NCI-H292 cells with either IFN-γ, PMA, TNF-α, IL-13, or IL-4 resulted in significant enhancement of surface ICAM-1 at various incubation periods, providing strong evidence that cells used for experimentation were responsive to cytokine stimulation.

CHAPTER 7

GENERAL DISCUSSION

7.1. Mutation scanning of the *IL13RA1* and the *IL13RA2* genes

The analysis of DNA sequence variation is of fundamental importance in genetic studies (Lander and Schork, 1994). Despite the significant progress of SNP consortium, a large number of lower frequency (<10%) SNPs, especially those that are specific to a single population, have not yet been detected (Tabor et al., 2002). This is because both the private sequencing effort and the public SNP consortium collection are based on the sequence of a small number of individuals. This was shown in a systematic sequencing effort of 84 ethnically diverse individuals, examining 313 genes in 720 kb of genomic sequence (Stephens et al., 2001). Only 2% of the SNPs identified in that study were deposited to dbSNP at that time, suggesting that there are many more SNPs than the 1.71 million unique SNPs already deposited to dbSNP. According to the authors of the above study, the number of SNPs in the human genome, defined by a rare-allele frequency of 1% or greater, is likely to be at least 15 million (Tabor et al., 2002). Thus, the low sensitivity of SNP detection in SNP consortium is currently insufficient for performing association studies on candidate genes (Xiao and Oefner, 2001). Therefore, it remains important to perform mutational analysis of genes of interest using techniques with high sensitivity and specificity, such as SP-CCM and DHPLC used in our study.

At the time of writing, our own search has found that, as of to date, 126 non-coding SNPs and a single coding SNP in the *IL13RA1* gene, as well as 32 non-coding SNPs in the *IL13RA2* gene have been deposited to LocusLink. 119 non-coding SNPs in the *IL13RA1* gene are located in intervening introns, and 7 non-coding SNPs are located in the 3' UTR of *IL13RA1*. Only a single non-synonymous G to T substitution in the third nucleotide of codon 3 in the *IL13RA1* gene resulting in an amino acid alteration in which tryptophan is substituted for cysteine has been submitted to LocusLink, however, this and most of the deposited SNPs to LocusLink have not been validated by a second independent submission. The non-synonymous 9G>T SNP in exon 2 of the *IL13RA1* gene submitted to LocusLink was not detected in our mutation scanning using DHPLC. Moreover, two previous studies screening the *IL13RA1* gene for common variants did not identify this particular variant, suggesting that this might be a rare polymorphism or may represent a false SNP. The large number of non-coding

variants in the *IL13RA1* SNPs submitted to dbSNP compared to the coding variants found in our study, is explained by the fact that intronic regions in the *IL13RA1* gene span approximately 75.5 kb, whereas the coding region of *IL13RA1* spans only about 1.3 kb.

As of to date, 32 SNPs in the *IL13RA2* gene submitted to LocusLink. All 32 SNPs are located in intervening introns. To the best of our knowledge, at the time of submission of this thesis no SNPs in the 5' UTR or the coding region of *IL13RA2* have been submitted to dbSNP. We screened the promoter, coding, and proximal 3' UTR regions of *IL13RA1*, as well as the coding region of *IL13RA2* for common polymorphisms. We have not screened the intervening intronic regions and the distant 3' UTRs of the genes, as well as the promoter region of *IL13RA2* since its structure had not been described at the time this study was undertaken.

Our screening strategy was to identify variants that change protein function or regulate gene expression. Therefore, we placed a high priority on identifying SNPs that are located in the coding and promoter regions. Although asthma is a complex disease influenced by a repertoire of genes, data from Mendelian phenotypes show that these phenotypes are associated predominantly with alterations in the normal coding sequence of proteins and to lesser extent to the regulatory promoter region (Botstein and Risch, 2003). As of June 2002, the human gene mutation database, HGMD, listed over 27,000 mutations in 1,222 genes associated with human diseases and traits. These mutations were classified by the HGMD by type of change (Krawczak et al., 2000). Amino acid altering polymorphisms (non-synonymous) and changes to premature termination codons (nonsense) were the most frequent type of mutations (59% of total), whereas deletions accounted for 22% of all changes and insertions/duplications accounted for 7%. About 1% of mutations were found in promoter-regulatory regions and 10% were splice-site mutations found in intron-exon boundaries. It is conceivable that the effect of non-coding polymorphisms cannot be predicted with our current knowledge (Botstein and Risch, 2003). Exceptions are the splice site polymorphisms that can alter mRNA splicing (Cartegni et al., 2002) and the 3' UTR polymorphisms that can potentially alter mRNA stability (Conne et al., 2000). These data support the notion that it is reasonable to identify and subsequently

genotype variants that result in changes to the amino acid sequence or affect the promoter regulatory regions, since these variants are most likely to affect the function or the level of expression of a protein and be involved in disease aetiology (Tabor et al., 2002).

The lack of polymorphisms in the coding region of *IL13RA2* is not surprising. Firstly, it is in accordance with a previous study, which found no polymorphisms in the coding region of *IL13RA2* among human cell lines, including 19 glioblastoma multiforme, 1 normal astrocytic, and 2 fibroblast cell lines (Kawakami et al., 2000). Secondly, there is considerable variation in the number of coding SNPs in different genes. In a study exploring the distribution of SNPs among 106 genes relevant to cardiovascular, endocrinology, and neuropsychiatry diseases, the number of SNPs ranged from 29 for F5 (factor V) to none for 13 genes (Cargill et al., 1999).

According to the latest report by the International SNP mapping group (Sachidanandam et al., 2001) the overall density of SNPs in the human genome is about one SNP every 1,331 bp. The density of SNPs in exons is estimated to be one SNP per 1.08 kb according to the same study (Sachidanandam et al., 2001). These estimates are based on the identification of about 1.4 million SNPs by the public SNP consortium. However, both the private sequence effort and the public SNP consortium collections are based on a small number of subjects and SNPs with lower frequency (<10) have not been detected. Therefore, it is estimated that the total number of SNPs, including the rare ones, is likely to be at least 15 million (Botstein and Risch, 2003). More systematic surveys of SNPs assessing variations in large sample of individuals of different ethnicity but in a smaller number of genes were able to detect SNPs with low frequency (Cargill et al., 1999, Halushka et al., 1999). In these studies, the average number of coding SNPs (including those in the 3' UTR) was approximately four per gene. Cargill and co-workers (Cargill et al., 1999) have estimated in their study of 106 genes, that non-synonymous (amino acid altering) variants were detected at only 38% the rate of synonymous (non-amino acid altering) variants. Moreover, more than half (59%) of non-synonymous coding SNPs were found at a frequency below 5%.

7.2. Association studies of *IL13RA1* variants with asthma and asthma-related phenotypes

Having identified two common variants in the *IL13RA1* gene, the –281T>G polymorphism in the promoter region and the 1365A>G SNP in the proximal 3' UTR, we conducted a family-based and case-control studies to assess for potential association of these variants with asthma-associated traits. Genetic association studies aim to correlate differences in disease frequencies between groups with disease frequencies at a SNP. Thus, the frequencies of the two variant forms (alleles) of a SNP are the primary interest for identification of genes affecting disease. In this approach, a hypothesis is generated initially about the candidate genes that may have a role in the aetiology of the disease. Subsequently, variants are identified in or near those genes and genotyped in a population. Finally, by applying statistical methods potential correlations between these variants and the phenotype are assessed.

In complex diseases, the identification of functional variants in the coding and regulatory regions of candidate genes followed by case-control or family based association studies has many advantages compared to other approaches. For example, segregation analysis is very sensitive to biases; whereas linkage analysis regularly leads to dubious results due to heterogeneity, need to specify a precise mode of inheritance, and different penentrance of each genotype (Gambaro et al., 2000). Furthermore, linkage signals in complex diseases are usually weak due to a large number of disease-associated variants each one conferring a low relative risk. Although the affected sib-pair strategy used in the majority of linkage studies is a robust method, however, it has low statistical power and requires large collections of well-characterised sibling pairs (Corvol et al., 1999). Thus, association studies are the most used for identifying susceptibility loci in complex diseases (Cardon and Palmer, 2003), since they are easier to conduct and their methodology is well suited for detecting modest genetic effects within a complex and heterogeneous disease (Corvol et al., 1999).

A carefully selected case group is essential for ensuring the validity of a molecular epidemiology study, minimising any source of selection bias (Campbell and Rudan,

2002). Subjects recruited for studies of common diseases, such as asthma, should represent an unbiased section of the population with respect to environmental risk factors, gender, socio-economic status, and lifestyle. Thus, clinical-based population samples are usually less desirable than community-based or population-based samples for studies of common diseases, and a surgery roster is a viable alternative to a traditional clinic sample (Cupples, 1998). Our cohort comprised 341 Caucasian families recruited from the Southampton area with at least two biological siblings (age 5-21 years) with current physician diagnosis of asthma who were taking asthma medication on a regular basis. Recruitment of families took place through local general practitioners (G.P's). We have not recruited patients through outpatient clinics who usually suffer from more severe asthma and may not represent the whole spectrum of the disease. We have also conducted a detailed clinical phenotyping based on current MRC, ATS, and ISAAC questioners.

Phenotyping heterogeneity in complex diseases, such as asthma, may underestimate an association of a genetic variant with disease. Consequently, it is possible that the influence of particular genetic variants will only be found in selected disease phenotypes or in selected clinical subgroups. Therefore, we systematically analysed the genetic variants of *IL13RA1* for association not only with asthma diagnosis but also with asthma-related phenotypes, including BHR, pulmonary function measured by FEV1, serum total and specific IgE levels. The use of intermediate, quantitative phenotypes, such as serum total IgE levels and measures of BHR has become widespread in asthma genetic research. These traits are genetically distinct and should therefore examined separately in genetic studies of asthma. It is also likely, that asthma-associated quantitative phenotypes will provide more power than will a dichotomous trait reflecting clinical asthma (Cookson and Palmer, 1998). We have also analysed the genetic variants for associations with asthma-related traits in four separate asthmatic study groups, including first affected female siblings, first affected male siblings, asthmatic mothers, and asthmatic fathers.

The selection of the control group is also essential to enable the optimal design of case-control studies. Ideally, a control sample should reflect the ethnic and genetic composition of the case sample. Although hospital-based controls are phenotyped in

the same way as the study group, they may have other comorbidities that make them different from the general population. The use of blood donors with no personal or family history of asthma in our study had the advantage of being a representative population-based sample. It is possible that a few of the blood donors may have had mild asthma with the potential towards misclassification bias towards finding no association for susceptibility. We believe that misclassification was minimal in our study since specific questionnaires used to screen blood donors including questions about asthma related symptoms, as well as personal and family history of asthma. Genotyping errors due to either poor typing assay design or poor DNA quality may lead to spurious result due to information bias. This is usually explored by checking that genotype frequencies among controls are in Hardy-Weinberg equilibrium. In our study, we have found that the control population was in Hardy-Weinberg equilibrium for both the –281T>G and the 1365A>G polymorphisms. Moreover, we sequenced ten random samples and found that the genotype data were identical to the sequencing data. Therefore, it is highly unlikely that any degree of information bias was introduced in our study.

Three common situations may account for an observed association between an allele and a phenotype: (i) due to a functional allele; (ii) due to linkage disequilibrium; and (iii) due to chance, bias, or confounding (Silverman and Palmer, 2000). First, the allele itself is functional and directly affects the expression of a phenotype. Our study has been designed to identify and subsequently test functional variants for association. Firstly, we have tested the –281T>G polymorphism in the *IL13RA1* promoter, which can potentially modulate IL-13Rα1 expression, as well as the 1365A>G SNP in the 3' UTR, which can potentially affect mRNA stability for association with asthma and asthma-associated phenotypes (Heinzmann et al., 2000). Secondly, an allele found to be associated with a phenotype might be in linkage disequilibrium with an allele at another locus. Indeed, a suggestive association found between the *IL13RA1* T-A haplotype and raised serum total IgE levels among asthmatic mothers in our study, might be due to linkage disequilibrium and a greater effect of an as yet unidentified allele or alleles either within or outside the *IL13RA1* locus. Third, a positive association could be due to chance, bias, or confounding. A positive result may be due to chance, since a statistically significant finding does not imply that chance cannot

account for the result, only that this is unlikely (Campbell and Rudan, 2002). Chance may also account for a positive association when multiple association are performed with publication of only those that show positive results (publication bias). False positive association may also be due to multiple testing and subgroup analysis not related to a priori hypotheses with selectively reporting only analyses that reach statistical significance. In our study, we have initially formulated the hypothesis and decided a priori about the statistical methods that were applied. Confounding factors are those that are associated with both the disease and the factor under study. Thus, an apparent association between a genetic marker and a disease phenotype may be due to confounding. Population stratification or population substructure represents the most well known confounding factor in genetic association studies (Colhoun et al., 2003). Population stratification is defined as differences in allele frequencies between subsets of a given sample, and can result from recent population admixture or differences in ethnicity between cases and control. It and can be a particular problem in genetically diverse populations such as those in the U.S. (Ewens and Spielman, 1995). Approaches to limit and control population substructure include the use of family-based association studies and the 'genomic control' approaches. The most popular and robust family-based association method is the transmission disequilibrium test (TDT), which has been used in our study (Spielman et al., 1993). TDT uses heterozygous parental genotype and provides a joint test of linkage and association that eliminates the effects of stratification. A side effect of the TDT is that it effectively throws away some genotype information owing to its reliance on heterozygous parents. This creates a loss of statistical power to detect genuine allelic association (Cardon and Palmer, 2003). The amount of loss of genotype information depends on the frequency of the minor allele and the chromosome location of the gene of interest. Given that the frequency of heterozygotes is 2pq (where p=minor allele frequency, q=minor allele frequency), it is obvious that variants with low minor allele frequency would require large family collection to achieve adequate statistical power. The same is true when the variant of interest is located on the X chromosome; in this case only maternal genotype information is used. Another limitation of the TDT is the difficulty in recruiting parents for late-onset disorders. Consequently, although more robust in the presence of population stratification, the TDT requires significantly more patients and family collections than case-control studies (Cardon and Palmer, 2003). Our own

TDT study was well powered to detect a genotypic risk ratio of 1.5 for the –281T>G polymorphism and a genotypic risk ratio of 2 for the 1365A>G polymorphism. Another way of limiting population stratification is the "genomic control" approaches, not used in our study, which utilize genotyping data from unlinked genetic markers in both case and control groups to detect, and even correct, for population stratification when it is present (Pritchard and Rosenberg, 1999, Pritchard et al., 2000).

We were unable to replicate the findings of Heinzmann and co-workers who reported a statistically significant association between the *IL13RA1* 1365A>G polymorphism and raised serum total IgE levels in British young males (Heinzmann et al., 2000). The lack of a positive association between the 1365A>G polymorphism and raised serum total IgE levels in our study might be due to differences in asthma severity and/or study design between the two cohorts. In our study, we evaluated potential association of serum total IgE levels and genotypes of 1365A>G in four asthmatic study groups, including first affected male siblings, first affected female siblings, asthmatic fathers and asthmatic mothers. In contrast, in the study of Heinzmann and co-workers the 1365A>G polymorphism was assessed for evidence of association with serum total IgE among a single group including both asthmatic and non-asthmatic subjects. This approach, however, might have led to spurious results since asthmatic subjects usually have higher serum total IgE levels compared to normal subjects. Moreover, many candidate-gene studies in complex disease have not been replicated when followed up in subsequent association studies (Tabor et al., 2002). In a meta-analysis of 379 studies addressing 36 genetic associations with diseases or traits, the authors found that association studies of the same disease are often inconsistent in their findings of association (Ioannidis et al., 2001). In another meta-analysis of 301 genetic association studies covering 25 frequently studied associations between common variants and common diseases, 17 proposed associations were not replicated (Lohmueller et al., 2003) Furthermore, in the vast majority of association studies the odds ratio of the first positive report were inflated when compared with the results of other published research on the same question (Ioannidis et al., 2001). Lohmueller and co-workers (Lohmueller et al., 2003) suggested that this consistent overestimation was a consequence of the 'winner's curse' phenomenon: the first 'positive' study provides inflated estimates compared to the reality. It is likely that

perhaps only a quarter of previously published associations represent real associations with common disease (Ioannidis, 2003).

No association of either the *IL13RA1* –281T>G or the *IL13RA1* 1365A>G polymorphisms with asthma or asthma-related phenotypes was found in our study, apart from a borderline association of the *IL13RA1* -281T to 1365A haplotype and raised serum total IgE levels in asthmatic mothers. However, power calculations revealed that our case-control study was underpowered to detect an odds ratio of 1.5, for both the –281T>G and the 1365A>G polymorphisms. The statistical power to detect a significant association depends on the size of the association and the frequency of the allele of interest. Thus, SNPs with low allele frequencies would need to have large relative risks associated with them to be detected in a candidate-gene study (Tabor et al., 2002). In a meta-analysis of 301 genetic association studies by Lohmueller and co-workers, most estimated odds ratio in follow-up studies were between 1.1 and 2 (Lohmueller et al., 2003). It is likely that most genuine genetic associations in complex disease represent modest effects with odds ratio of 1.1-1.5 (Ioannidis, 2003). This explains only 1-8% of the relative risk in the population; however, the additive effect of several variants could make up the 20-70% of the overall disease risk that is attributable to genetic factors. In this context, our case-control study might have been underpowered to detect modest effects with odds ratios of 1.1-1.5 for both the –281T>G and the 1365A>G polymorphisms. This highlights the challenge of recruiting larger cohorts of participants in order to detect modestly higher odds ratios.

Our finding that the *IL13RA1* –281T to 1365A haplotype is marginally associated with raised serum total IgE levels only in adult female asthmatics is not surprising. It is known that sex and age have a clear effect on allergic manifestation and serum total IgE levels (Marsh et al., 1981). Males have higher geometric mean serum total IgE levels than females throughout the entire age range of six to 75 or more years (Barbee et al., 1981, Barbee et al., 1987). Age has also a significant effect on serum total IgE levels, since serum total IgE levels reach a maximum at 10 to 13 years and then decline markedly with increasing age in both males and females (Marsh et al., 1981).

7.3. Expression of IL-13 receptor components in airway cells

T cell deficient mice are capable of inducing asthmatic phenotypes following administration of IL-13 (Grunig et al., 1998), suggesting that IL-13 acts directly on airway cells. Additional *in vitro* studies have shown that IL-13 has important functions on bronchial epithelial cells, by altering the ciliated cell differentiation process, increasing the proportion of secretory cells (Laoukili et al., 2001b), and inducing release of eotaxin (Li et al., 1999) and TGF-β2 (Richter et al., 2001). In human fibroblasts, IL-13 enhances IL-6 and chemoatractant 1 release and upregulates the expression of β1 integrins and VCAM1 (Doucet et al., 1998). Our findings, that primary bronchial epithelial cells and primary bronchial fibroblasts express IL-4Rα, IL-13Rα1, and IL-13Rα2 both at the mRNA and protein levels provides additional evidence that IL-13 induces many of its effects on the asthmatic mucosa by acting directly on the airway cells. Moreover, using immunohistochemistry we found that the columnar epithelium of asthmatic subjects expresses protein for IL-4Rα, IL-13Rα1, and IL-13Rα2 *in vivo*.

We provide strong evidence that IL-13Rα2 is an intracellular molecule in primary bronchial epithelial cells and primary bronchial fibroblasts. We demonstrated that a large intracellular pool of IL-13Rα2 is present in the intracellular compartment in these cells using flow cytometry and confocal laser scanning microscopy. Our findings are in accordance with previous studies that have investigated the cellular localisation of IL-13Rα2 and found large intracellular pools of IL-13Rα2 in A549 cells, primary nasal epithelial cells, U937 cells and primary monocytes (Daines and Hershey, 2002), as well as in human fibroblasts (Yoshikawa et al., 2003) and human bronchial epithelial cells (Yasunaga et al., 2003). These findings indicate that the presence of intracellular stores of IL-13Rα2 is not restricted to a given cell type, but appears to be widespread. Using confocal laser scanning microscopy we have shown that IL-13Rα2 has a uniform granular-like pattern of cytoplasmic distribution in primary bronchial epithelial cells and primary bronchial fibroblasts. Intact primary bronchial epithelial cells demonstrated only rim surface staining consistent with a low level of surface expression, which is in agreement with the study of Daines and Hershey (Daines and Hershey, 2002). The intracellular localisation of IL-13Rα2

shown in our experiments is highly unlikely to be the result of non-specific binding of the specific monoclonal anti-IL-13Rα2 antibody used. Firstly, flow cytometry analysis of Jurkat cells, previously shown to lack IL-13Rα2 mRNA expression, demonstrated no surface or intracellular expression of IL-13Rα2. Secondly, the uniform distribution of cytoplasmic staining of IL-13Rα2 is similar to the distribution of other receptors localised in the cytoplasm. For example, CTLA-4 (Linsley et al., 1996), which is expressed in T cells, and the iron transport proteins hephaestin and molbifferin (Simovich et al., 2002), which are expressed in various cell types, demonstrate a similar diffuse pattern of distribution within the cytoplasm. CTLA-4 is a decoy receptor that exists in large intracellular pools in T cells and is a key costimulatory receptor, which binds costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells with high avidity (Schwartz, 1992). In contrast to costimulatory receptor CD28, which strongly upregulates T cell cytokine production and cellular activation, CTLA-4 triggering down-regulates CD-28 mediate effects. Thus, CTLA-4 is an important negative regulator of T cell activation and acts as a decoy receptor for the B7 molecules (Krummel and Allison, 1995). Although CTLA-4 has sequence properties of a plasma membrane receptor, it is primarily an intracellular protein localised in intracellular vesicles cycling between the cell surface and intracellular stores (Linsley et al., 1996).

Previous studies have highlighted the role of IL-13Rα2 as a decoy receptor for IL-13 (Kawakami et al., 2001, Bernard et al., 2001). Nonsignaling cytokine receptors are expressed in a soluble- or membrane-bound and may function as decoy receptors to attenuate signal transduction by a particular cytokine (Rahaman et al., 2002). Decoy receptors are classified in three groups: the Toll/IL-1 receptor family, the TNF receptor family, and the chemokine silent receptor family (Mantovani et al., 2001). The Toll/IL-1 receptor family includes type II IL-1R (IL-1RII) and 18 binding protein (IL-18 18BP) (Mantovani et al., 2001). IL-1RII is found in membrane-bound and soluble form and acts as a decoy, capturing IL-1 and preventing it from interacting with IL-1RI (Martin and Falk, 1997). IL-18 BP is an Ig superfamily receptor, expressed in multiple isoforms, which captures IL-18 and blocks its activity. The TNF receptor family includes osteoprotegerin and decoy receptor 1 and 2. (DcR1, DcR2) (Ashkenazi and Dixit, 1999). Osteoprotegerin is a potent antiresorptive molecule

conferring protection to bone through binding to osteoprotegerin ligand. DcR1 and DcR2 act as decoys that prevent Apo2 ligand (Apo2L) from inducing apoptosis through death receptor 4 (DR4) and DR5. The chemokine silent receptor family includes Duffy and D6, which have a typical seven transmebrane receptor architecture and bind chemokines with high affinity but with no subsequent signaling activity (Mantovani et al., 2001).

Not only receptor molecules are found in the intracellular compartment, but also biologically active forms of cytokines, thought to be exclusively secreted, are bound to the cell surface. This small family of cytokines exhibiting a membrane form includes IL-1, TNF-α, IL-10 and IL-15 (Musso et al., 1999). Furthermore, an isoform of IL-15 with a short signal peptide is not secreted or found as a surface-bound form, but instead, it is localised primarily diffusely in the cytoplasm, as well as in the nucleus (Onu et al., 1997).

The presence of a large intracellular pool of IL-13Rα2 in airway epithelial cells and airway fibroblasts might be due to the existence of an alternative isoform of the receptor with disrupted signal peptide. This putative alternate isoform of IL-13Rα2 may result from exon skipping or usage of a hidden alternative exon. As a consequence, the IL-13Rα2 isoform associated with the disrupted signal peptide is stored intracellularly. Preferential expression of the alternate isoform might explain the large intracellular pools of IL-13Rα2 and the poor cell surface expression. Interestingly, although only a single 1.4 kb IL-13Rα2 mRNA was detected in various human tissues and cells using Northern blot analysis in three different studies (Caput et al., 1996, Guo et al., 1997, Gauchat et al., 1997), a recent study found several alternative spliced transcripts in the human keratinocyte cell line HaCaT, resulting from alternative usage of the first four 5' noncoding exons (David et al., 2003).

Our experiments demonstrated that IL-13Rα2 surface expression was not induced following stimulation of airway epithelial NCI-H292 cells and primary bronchial fibroblasts with either IFN-γ, PMA, TNF-α, IL-13, or IL-4. Similarly, IL-13Rα2 surface expression was not enhanced on primary bronchial epithelial cells in response to IFN-γ or PMA. Our data are in agreement with the study of Yasunaga and co-

workers who found that although IL-4 and IL-13 up-regulate intracellular IL-13Rα2 protein, they do not induce expression of IL-13Rα2 on the cell surface of human bronchial epithelial cells (Yasunaga et al., 2003). The lack of mobilization of the intracellular stores of IL-13Rα2 to the cell surface in airway epithelial cells and primary bronchial fibroblasts suggests that the intracellular pool of IL-13Rα2 in these cells may be released only after cellular damage or cellular death, thereby attenuating on-going extracellular IL-13 activity. Alternatively, the intracellular pool of IL-13Rα2 in airway epithelial cells and airway fibroblasts might be mobilized to the cell surface under certain physiologic conditions and cytokine stimulations not used in our experiments.

7.4. Conclusions

In conclusion, this study has identified a novel polymorphism in the *IL13RA1* promoter and confirmed the presence of two previously described non-amino acid altering polymorphisms in *IL13RA1*, 1050C>T and 1365A>G. In accordance with a previous study, no common variants in the coding region of *IL13RA2* were found. We investigated the role of the novel *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms, which can potentially modulate gene transcription and mRNA stability, respectively, in conferring susceptibility to the development of atopy and asthma. Neither the *IL13RA1* –281T>G polymorphism nor the *IL13RA1* 1365A>G variant were found to be associated with asthma or asthma-related traits in a large asthmatic cohort. Only a borderline association between the *IL13RA1* –281T to 1365A haplotype and raised serum total IgE levels was found in adult female asthmatics. We demonstrated that primary bronchial epithelial cells and primary bronchial fibroblasts express all IL-13 receptor components, including IL-4Rα, IL-13Rα1, and IL-13Rα2 both at the mRNA and protein levels, providing strong evidence that IL-13 can act directly on the airways. We have also shown that primary bronchial epithelial cells and primary bronchial fibroblasts contain large intracellular IL-13Rα2 stores with a diffuse granular-like pattern of expression, suggesting that a large reservoir of IL-13Rα2 can play a role in IL-13 regulation following release from the intracellular compartment.

7.5. Future directions

Our findings suggest that genetic variation in the coding and promoter regions of *IL13RA1* is not an important determinant of asthma susceptibility in our cohort. Future studies should examine the role of *IL13RA1* polymorphisms in the development of asthma and atopy in different ethnicities, given that genetic variants may be more or less important in different populations (Campbell and Rudan, 2002). Moreover, small effects resulting from the *IL13RA1* –281T>G and the *IL13RA1* 1365A>G polymorphisms may need larger sample size to detect.

Although the *IL13RA1* –281T>G polymorphism was not found to be associated with asthma–related phenotypes in our cohort, and the promoter analysis software predicted that neither the wild type nor the minor allele contains any known transcriptional binding site, its functional role is uncertain. The functional significance of the –281T>G polymorphism can be assessed by promoter reporter studies. These studies could involve cloning of promoter fragments, containing each of the two *IL13RA1* promoter alleles, to the 5' of a luciferase reporter gene within a basic reporter vector, followed by transient transfection of appropriate cell lines. Differences in basal transcriptional activity between the two alleles would highlight a functional role for the *IL13RA1* –281T>G polymorphism. Moreover, electrophoretic mobility assays (EMSAs), including antibody supershift, could determine whether any the G allele of the –281T>G variant disrupts or creates a transcriptional binding site.

As of to date, 7 additional *IL13RA1* polymorphisms located in the 3' UTR have been submitted to dbSNP. In several instances, the 3' UTRs may regulate gene expression through the modulation of mRNA stability. RNA stability is regulated through binding of specific proteins, which occurs mostly, but not exclusively, in 3' UTR AU-rich regions. These additional 3' UTRs can be evaluated for potential association to asthma phenotypes. However, given the significant number of false positive SNPs in dbSNP (Botstein and Risch, 2003) and the fact that some of these SNPs are rare, the presence and frequency of these *IL13RA1* 3' UTRs submitted to dbSNP should be first established, for example by direct sequencing of DNA samples.

We have not found any common polymorphisms in the coding region of the *IL13RA2* gene, in accordance with a previous study, which found no common variants in *IL13RA2* among human glioma cell lines (Kawakami et al., 2000). We have not carried out mutation scanning of the *IL13RA2* promoter, since at the time this study was undertaken the *IL13RA2* promoter had not been characterised. Recently, two different groups have identified two different regions, 20 kb apart from each other, as the putative *IL13RA2* promoter. The most distant putative *IL13RA2* promoter was found in the human keratinocyte cell line HaCaT and 5' RACE experiments identified a few mRNAs initiating from exon 2, suggesting the existence of an alternative promoter in HaCaT cells (David et al., 2003). The most proximal putative *IL13RA2* promoter was found in the glioma cell line U118 (Wu and Low, 2003). Taken together, these data suggest that either of the two recently identified putative *IL13RA2* promoters, or even others as yet unidentified alternative promoters, may be used preferentially in different cell types. It would be of great interest to investigate the transcriptional regulation of IL-13Rα2 in airway epithelial cells and airway fibroblasts. This can be accomplished by carrying out 5' RACE experiments and promoter reporter studies. Moreover, mutation screening of the *IL13RA2* promoter region may identify common genetic variants that could subsequently be tested for potential association with asthma and asthma-related phenotypes.

We have found a borderline association between the *IL13RA1* –281T to 1365A haplotype and raised serum total IgE levels in adult female asthmatics. However, a limitation of our study has been that families in our cohort were recruited on the basis of asthma. Therefore, there were too few atopic individuals without asthma to study the effect of *IL13RA1* genetic variation on atopy alone. Future studies should examine the role of *IL13RA1* polymorphisms in the development and severity of atopy in individuals without asthma in order to investigate their importance in the predisposition to allergy.

Asthma is a complex genetic disorder with a heterogeneous phenotype attributed to the interactions among many genes and the environment. Moreover, the true effect of

particular genes on asthma susceptibility could be masked by other genes or environmental factors. In this context, it will be of great interest to investigate gene-gene interaction between *ILRA1* and *IL4R* by evaluating the association of various combinations of genotypes at the *IL13RA1* and the *IL4R* loci.

We have shown that IL-13Rα1, IL-13Rα2, and IL-4Rα are expressed *in vivo*, in GMA embedded bronchial biopsies using immunohistochemistry. Due to insufficient numbers of biopsy specimens available, we were unable to investigate differences in expression of these receptors between different subject groups. Further studies are needed to determine whether IL-13Rα1 and IL-13Rα2 expression are altered in bronchial biopsies of asthmatics compared to normal subjects, using immunohistochemistry and/or in-situ hybridisation.

We have shown that primary bronchial epithelial cells and primary bronchial fibroblasts contain large intracellular stores of IL-13Rα2 with diffuse granular distribution, by confocal laser scanning microscopy using a specific monoclonal anti-IL13Rα2 antibody. Additional information on the cellular localisation of IL-13Rα2 can be provided by experiments, in which the human IL-13Rα2 cDNA would be either fluorescently labelled or coupled to a sequence tag. For example, the human IL-13Rα2 cDNA can be fused to the Aequorea victoria GFP and transfected to appropriate cell lines (Read, 1999). The IL-13Rα2-FP fusion protein can be subsequently traced by confocal laser scanning microscopy. Alternatively, a recombinant DNA construct can be generated by coupling the coding region of IL-13Rα2 and a tag sequence, such as the synthetic FLAG (Read, 1999), followed by transfection of the construct to appropriate cell lines. Confocal laser scanning microscopy using a fluorescently labelled antibody against the epitope tag can then trace the construct.

Contrary to previous studies showing mobilisation of the intracellular receptor pool of IL-13Rα2 to the cell surface in a variety of cell types in response to IFN-γ, PMA, TNF-α, IL-13, or IL-4, none of these cytokines induced surface IL-13Rα2 on airway epithelial

NCBI-H292 cells and primary bronchial fibroblasts. In addition, no mobilisation of the intracellular IL13Rα2 stores was seen in primary bronchial epithelial cells stimulated with IFN-γ or PMA. There are several cytokines known to participate in bronchial inflammation in asthma and it would be of great importance to examine the effects of additional cytokines on the mobilisation of the intracellular stores of IL-13Rα2 in airway cells.

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