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The role of CCR4 and CRTH2 in asthma

by

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Abstract

Chemokine receptors CCR4 and CRTH2, both expressed preferentially on Th2 cells, have been proposed to play an important role in the recruitment of T cells in asthma. The aim of this thesis was to see if further evidence of the role of CCR4 and its ligands CCL17 and CCL22 in allergic asthma could be obtained in a combination of descriptive and functional studies. Differences in the expression of CCR4 and CRTH2 on peripheral blood T cells from healthy non-atopic subjects and mild atopic asthmatic patients were looked for. Also the expression of a selection of activation/memory markers on CD4+ T cells were studied to determine whether there are any differences in the co-expression of these markers on CCR4+ T cells between the two subject groups. In order to try and confirm or deny findings in peripheral blood, immunohistochemistry was used to look for any differences in the expression of CCR4 in bronchial biopsy tissue sections of healthy control and asthmatic subjects. A bronchial explant model was used to look for differences in the production of a selection of cytokines and chemokines (the main focus being on CCL17 and CCL22) between healthy and house dust mite allergic asthmatics. Bronchial biopsies from healthy and asthmatic subjects were cultured for 24 hr with/without allergen and supernatants were assessed for cytokines and chemokines. The chemotactic responses of a CCR4+ T cell line and peripheral blood T cells, polarised *in vitro* torwards a Th2 phenotype were assessed; to recombinant chemokines and bronchial biopsy supernatants from asthmatic and healthy subjects stimulated *ex vivo* with allergen. A final aim, using peripheral blood mononuclear cells from allergic asthmatics was to elucidate whether CCR4+ cells contain allergen specific T cells by assessing IL-5 production following stimulation with allergen of whole PBMC and PBMC depleted of CCR4+ cells.

There were no significant differences between healthy subjects and asthmatic patients in levels of expression of CCR4 and CRTH2 on CD4+ T cells and expression of activation/memory markers on either CD4+ or CCR4+/CD4+ T cells. Sections of bronchial biopsies from healthy or asthmatic subjects were found not to contain any CCR4 positive cells. Analysis of bronchial explant supernatants showed significantly decreased IL-2 production in allergen-challenged when compared to unchallenged explants in healthy controls. Allergen induced a significant increase in IL-5 (p=<0.001) in asthmatic explants; this was significantly higher when compared to IL-5 measured in stimulated explants from healthy controls (p=<0.001). Allergen stimulation of explants from asthmatics but not control subjects also resulted in increased IL-4 release (p=0.015). Comparison of challenged explants from asthmatics and healthy control subjects also showed an increase in IL-13 production in asthmatic patients (p=0.014). CCL17 production increased significantly in the asthmatic explants only with significant differences between healthy subjects and asthmatics for both unchallenged and challenged samples (p=0.008 and 0.001 respectively). Further significant differences (i.e. increased production in asthmatic explants were seen for CCL22 (p=0.004), CCL2 (p=0.013), CCL19 (p=0.04), CCL20 (p=0.013) and CCL11 (p=0.002). Stimulation of PBMC from asthmatic donors with allergen resulted in increased IL-5 which was significantly reduced by selective depletion of CCR4+ cells (p=0.021), strongly suggesting that CCR4+ cells were the source of Th2 cytokines. In a final series of experiments where explant supernatants were used as the chemotactic stimulus, chemotactic activity that was dependent on CCR4 was not shown. The supernatants from healthy and asthmatic subjects failed to induce chemotactic activity of either the CCRF-CEM cells line with constitutive CCR4 expression or Th2 T cells polarised *ex vivo*.

In conclusion despite being able to demonstrate that the CCR4+ cell population contains T cells which produce Th2 cytokines and that bronchial tissue from asthmatic individuals stimulated *ex vivo* produces the CCR4 ligands, CCL17 and CCL22, chemotactic activity for T cells to explant supernatants was not observed.

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Abbreviations

AD	Atopic Dermatitis
AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
BAL	Bronchoalveolar Lavage
BALT	Bronchus Associated Lymphoid Tissue
BSA	Bovine Serum Albumin
CCL...	CC Chemokine Ligand
CCR...	CC Chemokine Receptor
CM	Central Memory
CLA	Cutaneous Lymphocyte-associated Antigen
CRTH2	Chemoattractant Receptor-homologous molecule expressed on Th2 cells
CTLA	Cytotoxic T Lymphocyte Antigen
CXCL..	CXC Chemokine Ligand
CXCR..	CXC Chemokine Receptor
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DP	D Prostanoid receptor
ECF	Eosinophil Chemotactic Factor
EDC	1-ethyl-3 (3-dimethylaminopropyl) carbodiimide
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbant Assay
EM	Effector Memory
FACS	Fluorescence Activated Cell Sorting
FcεR1	High Affinity IgE Receptor
FCS	Foetal Calf Serum
GMA	Glycol Methacrylate
GPCR	G-Protein Coupled Receptor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor

HDM	House Dust Mite
HA	Hyaluronan
HEV	High Endothelial Venule
HLA	Human Lymphocyte Antigen
ICAM	Intercellular Adhesion Molecule
IFNγ	Interferon Gamma
Ig	Immunoglobulin
IL-	Interleukin
LFA	Lymphocyte Function Antigen
LT	Leukotriene
MACS	Magnetic Automated Cell Sorting
MBP	Major Basic Protein
MDC	Macrophage Derived Chemokine
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NCF	Neutrophil Chemotactic Factor
NF-AT	Nuclear Factor of Activated T cells
NHS	N-hydroxy succinamine
NOD	Non-Obese Diabetic
OVA	Ovalbumin
PAF	Platelet Activating Factor
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGD_2	Prostaglandin D_2
PHA	Phytohemagglutinin
PSGL-1	P-Selectin Glycoprotein-1
ROW	Reverse Osmosis Water
TARC	Thymus and Activation Regulated Chemokine
TBS	Tris Buffered Saline

Tc	Cytotoxic T cell
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	Helper T cell
TIMP	Tissue Inhibitors of Metalloproteinases
TNF	Tumour Necrosis Factor
UHQ	Ultra High Quality
VCAM	Vascular Cell Adhesion Molecule
WT	Wild Type

The systemic and original nomenclature of chemokines

SYSTEMIC	ORIGINAL	SYSTEMIC	ORIGINAL
CCL1	I-309	CXCL1	GROα
CCL2	MCP-1	CXCL2	GROβ
CCL3	MIP-1α, LD78	CXCL3	GROγ
CCL3L1	LD78β	CXCL4	PF4
CCL4	MIP-1β	CXCL5	ENA-78
CCL5	RANTES	CXCL6	GCP-2
CCL7	MCP-3	CXCL7	NAP-2
CCL8	MCP-2	CXCL8	IL-8
CCL11	Eotaxin-1	CXCL9	Mig
CCL13	MCP-4	CXCL10	IP-10
CCL14	HCC-1	CXCL11	I-TAC
CCL15	HCC-2, Lkn-1, MIP-1δ	CXCL12	SDF-1α/β
CCL16	HCC-4, LEC	CXCL13	BCA-1
CCL17	TARC	CXCL14	BRAK
CCL18	PARC, DC-CK1, AMAC1		
CCL19	ELC, MIP-3β, exodus-3		
CCL20	LARC, MIP-3α, exodus-1		
CCL21	SLC, 6Ckine, exodus-2		
CCL22	MDC, STCP-1		
CCL23	MPIF-1, CKβ8		
CCL24	Eotaxin-2, MPIF-2		
CCL25	TECK		
CCL26	Eotaxin-3		
CCL27	CTACK, ILC		
CCL28	MEC		

Chapter 1

Introduction

Chapter 1

Introduction

1.1   Definition of asthma

Asthma is one of the most common chronic diseases of affluent societies and despite lacking a standard definition, the National Asthma Education and Prevention Program defines asthma as "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment" (GINA, 2004).

1.2   Epidemiology

There is increasing public concern over the rising trends in allergic diseases. They display large geographical differences in their prevalence. Britain, Australia and New Zealand have figures 10 to 15 times higher than those of central and Eastern Europe and Asia. In the United Kingdom alone, the prevalence of asthma is nearly double that of the European Union average and is six times higher in young adults than it was 25 years ago. It continues to increase in parallel with other allergic diseases such as eczema and hay fever.

Asthma is responsible for 1,500 deaths annually in the UK alone and treatment costs to the National Health Service are around £850 million. The increase in allergic disease has been noticed predominantly in young people and has been suggested as being linked to a western lifestyle, implicating homes with carpeted flooring and other soft furnishings, central heating, double glazing and atmospheric pollution. The rate of asthma increases as communities adopt a western lifestyle and become urbanised. With the projected increase in the proportion of the world population that is urban from 45% to 59% by 2025, there is likely to be a marked increase in the number of asthmatics worldwide over the next two decades, with an estimated 100 million persons with asthma by 2025 (GINA, 2004). Changes in diets, reduced exposure to

antibiotics in infancy and avoidance of indoor air pollutants have also been stated as being key steps to combat the rising trends (Peat and Li, 1999). Atopy, the heightened responsiveness to common aeroallergens, defined by the presence of positive skin prick tests to at least one of these allergens, has now reached epidemic proportions, with almost one in two people in the developed world being affected. As one of the worlds' most common chronic diseases, it affects approximately 300 million people and this figure is increasing by 50% each decade (GINA, 2004).

1.3   Background

Human asthma has long been viewed as being characterised by allergic airway inflammation with increased mucus production and lung mucosal remodelling, intermittent airway obstruction and airway hyperresponsiveness (Arm and Lee, 1992). The inflammation is responsible for the increased responsiveness to a number of stimuli such as air pollutants, allergens, exercise, viral infections of the respiratory tract and occupational chemicals. A number of different forms of asthma have been identified, each of which can be triggered by these different stimuli, all acting on a background of genetic predisposition.

The foundations of allergic diseases such as asthma, rhinitis, eczema and food allergies have evolved slowly, beginning with the description of Catarrus aestivus or hay fever in 1819 by John Bostock. Subsequent to this in 1873 was Charles Blackley's recognition of pollen grains as causative agents (Blackley, 1873). A transferable tissue sensitising factor was discovered in serum by Prausnitz and Küstner in 1921 (Prausnitz and Kustner, 1921) which was identified as immunoglobulin E (IgE) in 1967 by Johansen in Sweden and the Ishizakas in the United States (Ishizaka and Ishizaka, 1967).

It is thought that the contribution of environmental factors, on top of a genetic predisposition increases the likelihood that an individual will develop asthma and/or allergy. Research into the contribution of environmental influences and also studies

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identifying candidate genes are helping shed light on the development of allergic disorders.

1.3.1 Environmental Basis

For atopy and asthma to develop there is a requirement for interactions between genetic factors and environmental factors. The generation of IgE is a characteristic feature of the allergic tissue response and is thought to be part of the pathogenic processes in asthma. IgE is an antibody directed against peptide sequences which are found on common environmental allergens, the most important of which are those from house dust mites (*Dermatophagoides* (*D.*) *pteronyssinus* and *D. farinae*), domestic animals, (especially cats, rabbits, horses and rodents) and fungi (*Cladosporium alternaria* (*C*) and *C. Penicillium*). Outdoor allergens, such as birch pollen, prevail in the cooler northern climates and play a more important role in these responses. In areas where the atmosphere is much drier and does not permit the survival of house dust mites, fungal allergens and those from domestic animals are the more common irritants (Burrows *et al.*, 1989). While a strong link between allergen exposure and the development of asthma has been suggested, the relationship is not simple. One study, assessing the concentration of mite allergen in dust from homes of newborn children failed to show an association between mite levels and the development of asthma symptoms up to the early school years (Lau *et al.*, 2000). Another study found that the prevalence of asthma in areas where mites are not abundant is not lower than that observed in areas of high mite infestation (Sporik *et al.*, 1995). Outdoor allergens such as those derived from grasses and wind-pollinated trees appear to be more closely linked to hay fever and allergic conjunctivitis, whereas asthma and eczema are more closely associated with exposure to indoor allergens such as dust mites, animal dander and fungi (Moffatt and Cookson, 1996).

1.3.2 Genetic Basis

Allergic diseases, including asthma, have long been known to run in families, indicating a strong genetic component in addition to an environmental contribution.

Genome screens including classical linkage and fine mapping approaches suggest that the susceptibility of asthma is determined by many genes, each of which is having a moderate effect and in combination plays a major role. Important loci exist on chromosomes 5q 23-31 (Lonjou *et al.*, 1999), 11q (Doull *et al.*, 1996) and 12q (Wilkinson *et al.*, 1998) and new genes identified on chromosomes 14q 24 and 20p 13 (ADAM 33) are showing increasing importance (Van Eerdewegh *et al.*, 2002). These genetic influences can be divided into two components. Firstly, they determine the ability of a given individual to recognise an environmental allergen as foreign through the recognition and presentation of antigen to T lymphocytes by B lymphocytes via Human Lymphocyte Antigens (HLA or Major Histocompatibility Complex II [MHC II]) molecules HLA-DR, DP and DQ. A second set of genetic influences is important in cytokine responses and that is a region on chromosome 5, comprising a group of "allergic (Th2) cytokines", Interleukin-3 (IL-3), IL-4, 5, 9, 10, 13 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). The region encoding these cytokines is closely linked with inheritance of an increased IgE response and bronchial hyperresponsiveness due to presence of Th2 cytokines, IL-4 and IL-13 being essential for the production of IgE from B cells. A polymorphism in the IL-4 gene promoter is implicated in increased binding of the nuclear transcription factor, Nuclear Factor of Activated T cells (NF-AT), to its consensus sequence of the DNA promoter resulting in a greater IgE response (Rosenwasser *et al.*, 1995). It is thought possible that in allergic diseases such as asthma, there is either an increase in the expression of genes regulating Th2 cytokines (pivotal in the asthmatic/allergic response) or a decrease in the expression of genes regulating interferon gamma (IFNγ) and/or IL-12 (Th1 cytokines) production, or possibly a combination of both. The existence of a reciprocal relationship between Th1 and Th2 responses, whereby IL-10 and IL-4 (derived from Th2 cells) inhibit Th1 responses and IL-12 and IFNγ (generated by Th1 cells) inhibits Th2 responses, makes this hypothesis plausible and might suggest that there may be some disregulation in the immune system allowing predisposed persons to mount inappropriate responses and others not.

1.4 Orchestration of the allergic asthmatic response

The response of the immune system to allergens comprises several key stages (Fig 1.1) and involves two essential signals. Upon inhalation of allergen, the dendritic cell (DC), a professional antigen presenting cell (APC), located in the epithelium at the interface between the external and internal environments (Semper and Hartley, 1996), takes up allergen by pinocytosis or receptor mediated internalisation and digests it into peptides. A small peptide sequence resulting from digestion is then expressed on the DC surface by an MHC class II molecule. This occurs most likely in the lymphoid tissue, where allergen peptides are presented to T lymphocytes in combination with essential binding of a co-stimulatory molecule, B7, expressed on the DC surface and CD28, on the T cell surface (Palmer and van Seventer, 1997). This ligation results in the differentiation of a naïve T cell to one that produces a range of cytokines (Bellini *et al.*, 1993) which are able to upregulate cell function and production of antibodies involved in the allergic response. These cytokines are encoded in a small cluster on the long arm of chromosome 5 and are produced by T cells that differentiate down the Th2 route.

Figure 1.1

Mucus

Eosinophil

Tissue
damage

Epithelial cell

Chemokines

Protease

Chemokines

IL-4
IL-13

IL-5
IL-9

Naive T cell

Mast cell

Th2

IL-9

IL-4

FcεR1

APC   IL-13?

IL-4
IL-13

IgE

Histamine
PGD$_2$/LTC$_4$

B lymphocyte

Smooth muscle
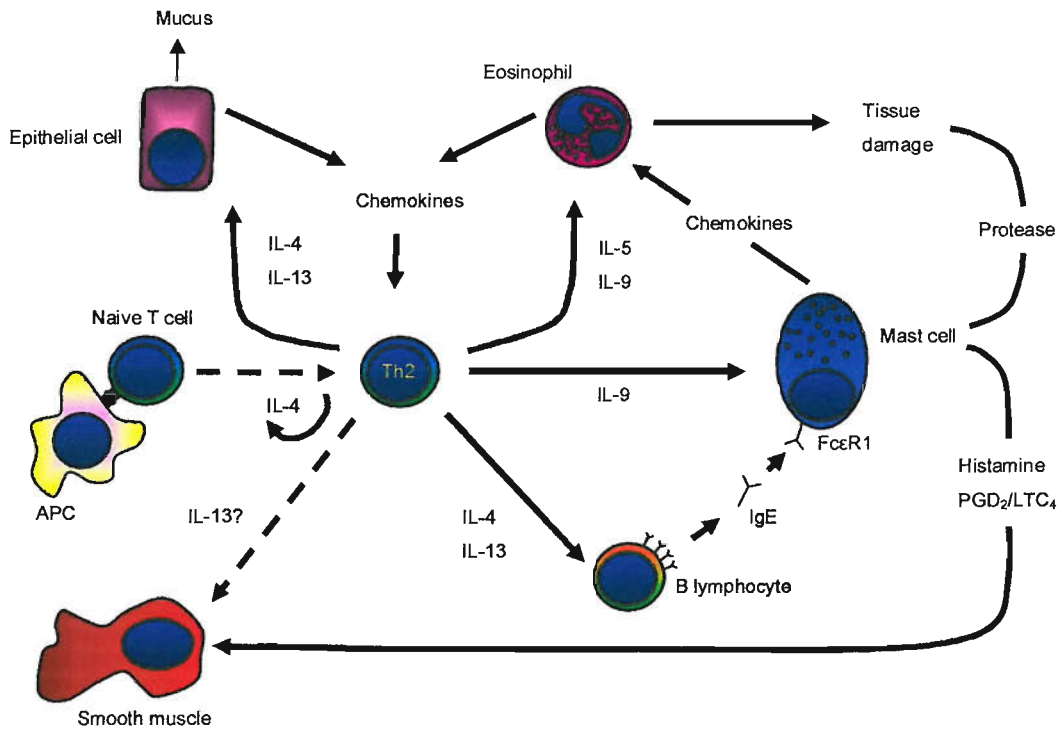
Figure 1.1   The pleiotropic activities of Th2 cytokines in allergic asthma.

Adapted from Renauld (Renauld, 2001).

Interleukin-4 plays a prominent role the allergic immune response (Aversa *et al.*, 1993), because for B cells to produce IgE they require an interaction with T cells and Th2 cytokines. Activated Th2 cells express CD40 ligand which binds to CD40 on the B cell (Aversa *et al.*, 1993) and this second signal, in the presence of IL-4 and/or IL-13, causes splicing of an ε heavy chain to the immunoglobulin hypervariable region (Fab region), resulting in an immunoglobulin class switch from IgM to IgE production, and B cell proliferation. This leads to the generation of plasma cells, which secrete allergen-specific IgE antibodies. These IgE antibodies are then able to bind to the high affinity IgE receptor (FcεR1) on the surface of mast cells and basophils. Cross-linking

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of IgE antibodies on the surface of mast cells gives rise to the early and late phases of the allergic response that are typically seen when sensitised individuals with hyper-responsive airways are challenged with specific allergen in the laboratory (Holgate *et al.*, 1991a). Whilst these models of controlled exacerbation of asthma in the laboratory may not be representative of all forms of naturally occurring exacerbations, especially those caused by viruses, they have, nevertheless provided valuable insight into inflammatory mechanisms of asthma. The early asthmatic response is a consequence of mast cell degranulation, causing a release of inflammatory mediators. Preformed and rapidly synthesised factors mediate acute inflammatory events (the early phase) which results in acute bronchoconstriction due to spasm of bronchial smooth muscles that is self-terminating, i.e. it resolves spontaneously within 1 to 2 hours. Four to 8 hours after allergen challenge, cytokines and lipid mediators induce the late phase asthmatic response (LAR) with an influx of Th2 cells, monocytes, eosinophils and neutrophils (Durham, 1998). This is associated with a second wave of bronchoconstriction that consists of vascular leakage, mucus hypersecretion, epithelial shedding and widespread airway narrowing (Chiappara *et al.*, 2001).

1.5   Airway remodelling

Airway remodelling is best described as a change in structure of tissue which is seen in chronic inflammatory diseases. It was first appreciated over 80 years ago by Huber and Koessler in their classic description of fatal asthma (Huber and Koessler, 1922). However, the term still remains difficult to define. Remodelling of the airways consists of restructuring of the airways and evidence points to a proinflammatory role for structural cells, including epithelial cells, fibroblasts, endothelial cells and smooth muscle cells (Laberge and El Bassam, 2004) (Fig 1.2). Epithelial cells, fibroblasts and smooth muscle cells work alongside inflammatory cells by releasing an abundance of mediators, cytokines and chemokines, thus upregulating the inflammatory component of inflammation.

Figure 1.2

Basement membrane thickening

Mucus gland hyperplasia

Smooth muscle
hypertrophy

Desquamation
of epithelium

Vasodilation

Mucus plug

Infiltration of edematous mucosa
with eosinophils and T cells
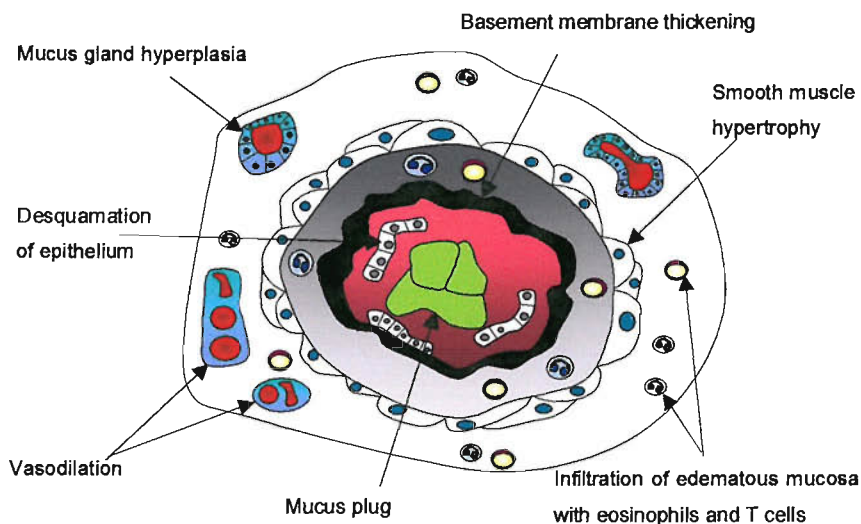
Figure 1.2   Cross-sectional diagram depicting the pathology of the airways
in asthma. Adapted from Roitt., 1997

1.5.1   Airway thickening

The thickness of the airways in fatal asthma is increased by 50-300% and is also
significantly thickened by 10-100% in cases of non-fatal asthma (James and Ryan,
1997). Increased airway wall thickness is a result of contributions from a number of
components including cellular influx (Djukanovic *et al.*, 1990), increased vascularity
(Li and Wilson, 1997), oedema (Dunnill, 1960), collagen and matrix deposition (Roche
et al., 1989) and smooth muscle hypertrophy/hyperplasia (Ebina *et al.*, 1993). The
majority of studies have concluded that wall thickening increases the airway narrowing
caused by smooth muscle stimulation, thereby promoting bronchospasm and
hyperresponsiveness to bronchospastic stimuli.

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1.5.2 Collagen deposition

Collagen deposition beneath the basement membrane is another prominent feature and a hallmark of asthma. It was first described by Dunnill (Dunnill, 1960) and has since been recognised as increased collagen types III and V along with matrix components laminin and fibronectin beneath the true basement membrane (composed of type IV collagen) (Roche et al., 1989). Myofibroblasts are the source of collagenous and non-collagenous matrix proteins and their numbers are increased in association with thickening of the sub-basement membrane collagen (Brewster et al., 1990). These cells appear quickly after antigen challenge, implying there may be a precursor cell that is able to acquire myofibroblastic features without dividing (Gizycki et al., 1997).

1.5.3 Smooth muscle cells

An increase in smooth muscle cells is a prominent feature in airway remodelling. In patients who died from an asthma exacerbation the increase in smooth muscle is far greater than in those who died from other causes. Animal models confirm that continued allergen exposure can increase smooth muscle thickness (Salmon et al., 1999). James and Ryan have identified that smooth muscle area in airway sections was increased by up to 43% in small cartilaginous airways in non-fatal asthma, but as much as 230% in fatal asthma (James and Ryan, 1997). Shortening of this airway smooth muscle by 40% may be sufficient to lead to airway closure (Wiggs et al., 1992).

1.5.4 Cytokines involved in remodelling

Many factors discovered in the airways in active asthma have been found to exhibit fibrogenic potential. One of the most powerful stimulators of collagen production is TGFβ. Its detection in the airways through immunohistochemical staining and the identification of mRNA encoding TGFβ in epithelium and eosinophils from inflamed airways helps substantiate the role of this cytokine in the airways (Ohno et al., 1992, Khalil et al., 1989). In addition, in most but not all studies, TGFβ expression correlates with basement membrane thickness and fibroblast number and/or disease severity (Minshall et al., 1997, Vignola et al., 1997, Ohno et al., 1996). Another

cytokine capable of stimulating collagen production and fibroblast replication is TNFα. This has also been found in asthmatic airways in substantial concentrations (Bradding et al., 1994). TNFα can also perpetuate the production of TGFβ, thus further stimulating production of collagen (Kohase et al., 1988). Another inducer of pulmonary inflammation with eosinophilia and fibrosis is GM-CSF. EGF and GM-CSF have both been detected in exaggerated quantities in airway biopsies from patients with asthma (Vignola et al., 1997). Many cytokines such as IL-1β, IL-2, 3, 4, 5 and 6 have been detected by different groups in the BAL of asthmatics in variable quantities (Walker et al., 1992, Broide and Firestein, 1991, Robinson et al., 1992), but their contribution to airway remodelling is speculative and debatable. To date, no single mediator has been able to recapitulate all of the features described in remodelled airways. Detection of GM-CSF (Brown et al., 1991), IFNγ (Corrigan and Kay, 1990) and IL-5 (Motojima et al., 1993) in the sera of asthmatics may be only indicative of a spill-over of cytokines present in the peripheral blood and any significance in determining the remodelling response in the airways is unknown.

1.5.5   Matrix metalloproteinases

Much research has gone into determining the role of matrix metalloproteinases and their inhibitors (tissue inhibitors of metalloproteinases [TIMP's]) in airway remodelling. The importance of these factors in the balance of fibrosis is well known. MMP-9 is found in exaggerated quantities in sputum and biopsies from asthmatics (Vignola et al., 1998, Hoshino et al., 1998). MMP-8 is important in normal wound healing but is found in raised quantities in non-healing wounds. TIMP-1 found in increased quantities in sputum and biopsies of asthmatics correlates with airway obstruction and the increased production of TIMP-1 may participate in the generation of the marked fibrosis characteristic of asthma (Hoshino et al., 1998, Mautino et al., 1999).

1.6   T lymphocytes

The cell mediated immune response involves CD8+ T cells, known as cytotoxic T cells (Tc). These cells take responsibility in protecting the immune system against intracellular pathogens. The CD4+T helper cells (Th) express the CD4 marker and mediate humoral responses, characterised by antibody production. The Th cell subset is further divided into two subsets, Th1 and Th2. This however, is a simplification, for there is great diversity amongst T cells, with many different subsets having been identified, such as Th1 and Th2, Tc1 and Tc2, Th0, Th3, NK T cells and regulatory T cells each of which have specific roles in immune responses.

After encounter with allergen presented by APC, naïve T cells clonally expand in response to IL-2 and begin to secrete cytokine patterns characteristic of Th1 and Th2 subsets. T helper type 2 cells represent a polarised form of the T helper cell, characterised by the production of IL-4, 5, 6, 9, 10 and 13. These cytokines induce the differentiation, activation and survival of eosinophils (through IL-5), promote production of high amounts of IgE (through IL-4 or IL-13) as well as growth of mast cells and basophils (through IL-4, 9 and 10). Interleukin-4, 10 and 13 are responsible for inhibition of several macrophage functions and the development of Th1 cells. IL-10 has recently attracted much attention for its involvement, both as a marker and contributor to immunoregulation involving regulatory T cells. In contrast, Th1 cells are characterised by the production of IL-2, IFNγ and tumor necrosis factor beta (TNFβ). Th1 responses are highly protective against infections mounted by the majority of microbes, especially intracellular pathogens because of the ability of Th1 type cytokines to activate phagocytes and promote the production of opsonising and complement fixing antibodies by B cells.

In mice, a CD4+ T cell producing IL-4 but not IFNγ is known as a Th2 cell and one making IFNγ but not IL-4 is termed a Th1 cell. This definition is clear in mice however, it is much less clear in humans. T cells producing exclusively Th1 or Th2 cytokines do not exist in humans and depending on the stimulus, human T cells can make both IL-4 and IFNγ (Holtzman *et al.*, 1996). The phenotype of the T cell is

therefore, defined based upon the ratio of IFNγ to IL-4. Another important Th2 cytokine is IL-5. Messenger RNA transcripts for IL-5 have been detected in bronchial biopsy T cells from atopic and non-atopic asthmatic subjects (Ying *et al.*, 1997a). As research into asthma has progressed the emphasis has shifted from mast cells and eosinophils playing a central role in driving inflammation associated with asthma, to T cells, particularly those of the Th2 subset (Castro *et al.*, 2000). These cells are now thought to be central in the orchestration of the airway inflammation that characterises asthma (Ray and Cohn, 1999, Corrigan, 2000) The importance of this T cell subset was first suggested by Mosmann in 1989 who proposed the Th2 hypothesis for asthma (Mosmann and Coffman, 1989). He based this on the fact that IgE and eosinophils play a major and crucial role in asthma pathogenesis and that factors that regulated IgE synthesis and eosinophil numbers and activity (namely Th2 cytokines) played a major role in asthma pathogenesis.

T cells detected through post mortem studies are present in increased numbers in the airway in fatal asthma (Dunnill *et al.*, 1969) and atypical intraepithelial lymphocytes have been described in mild asthma (Jeffery *et al.*, 1989). There are no differences in the ratio of CD4+:CD8+ T cells in the BAL (Wilson *et al.*, 1992) and bronchial mucosa (Azzawi *et al.*, 1990) in asthmatic patients. The possibility of an additional component is questioned for severe/fatal asthma where an increased number of CD8+ T cells is apparent (Faul *et al.*, 1997). A high majority of activated T cells has been identified in the peripheral blood of asthmatic patients challenged with allergen (Gerblich *et al.*, 1984), in both acute severe asthma (Corrigan *et al.*, 1988) and in mild atopic asthmatics (Walker *et al.*, 1992) and an observed association between both T cell activation and eosinophilia in bronchial lavage and asthma severity (Walker *et al.*, 1991). The proportions of activated, cytokine and chemokine producing T cells identified in various compartments implicates them as key players in the production of inflammatory mediators, all of which are attenuating the processes of airway remodelling.

1.6.1 T cell markers of activation, memory and homing

The mechanisms of memory T cell generation have been a subject of extensive research. T cells generated in the thymus are released into the blood stream and persist there in relatively small numbers as naïve T cells, expressing the marker CD45RA. When they encounter an antigen for which their T cell receptors have high affinity, co-stimulatory signals from an APC are essential. An array of co-stimulatory molecules has been classified, but it is not yet known whether all these molecules are required for each T cell response or whether some redundancy exists. Following co-stimulation, T cells proliferate, resulting in high numbers of effector cells. After clearance of foreign antigens, resulting from infection/allergic reactions, many of these effector T cells die by apoptosis. However, some remain in the circulation, surviving in greater numbers than naïve cells. These remaining cells are memory T cells and are positive for the marker CD45RO. Following encounter with the same antigen that initiated the primary response, these memory T cells serve to generate both a faster response and one which is increased in magnitude. This is termed the secondary response. There are a number of markers identified that are characteristic of their migratory behaviour and patterns, activation, memory and homing status. Some of these are detailed below, with additional reference to their roles in asthma and allergic disease. The migration of lymphocytes from the circulation into the secondary lymphoid organs and tissues is mediated by interactions between cell surface and endothelial adhesion molecules (Butcher, 1991). A multistep process involving tethering, rolling, activation and arrest, mediated mainly by selectins and integrins, precede the transmigration of cells by diapedesis into the tissues (Fig 1.3) and certain "homing markers" displayed on lymphocytes during this process are responsible for their recruitment into different components of the body, such as the skin, gut and lymph nodes.

Figure 1.3
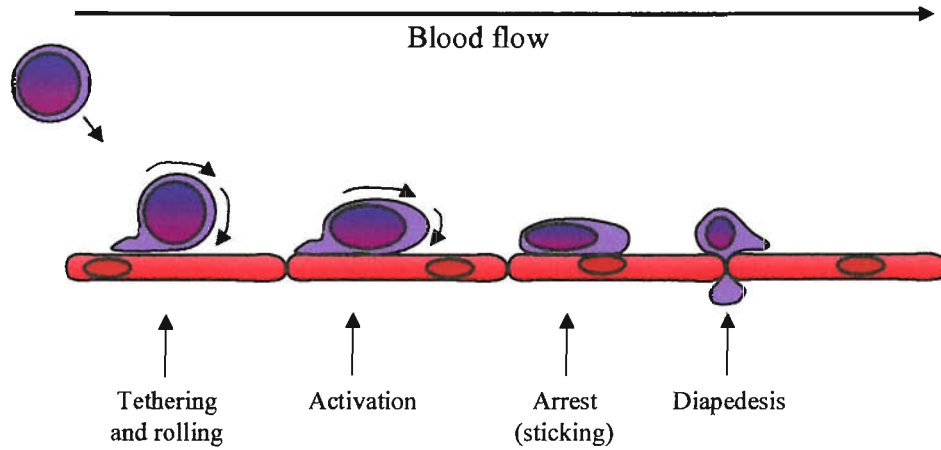
Figure 1.3   **Cell extravasation into the tissue.** L-selectin mediates tethering and rolling. HEV associated chemokines (e.g. secondary lymphoid chemokine (CCL21) [SLC]) which engage with the chemokine receptor CCR7 on lymphocytes leads to activation of the integrin LFA-1 on the lymphocyte. Firm arrest occurs when LFA-1 interacts with integrin ICAMs on the HEV and finally the lymphocyte migrates into the lymph node parenchyma.

1.6.1.1   CD62L (L-selectin)

The selectins are a family of membrane-bound lectins initiating the adhesion of leukocytes to endothelial cells, platelets or other leukocytes on the vessel walls of post capillary venules (Rosen and Bertozzi, 1994). Gallatin and colleagues discovered a cell surface antigen on lymphocytes, gp90MEL-14 (Gallatin *et al.*, 1983). This became the target of a function-blocking antibody and subsequent cloning lead to the identification of CD62L (Lasky, 1995). The rolling of naïve T cells in peripheral lymph node high endothelial venules is primarily mediated by CD62L which is shed upon activation. It binds with its amino-terminal C-type lectin domain to a group of glycoproteins known as addressins, expressed on the venules. The lymph node peripheral node addresssins (PNAd) include CD34 (Baumheter *et al.*, 1993), podocalyxin (Sassetti *et al.*, 1998), GlyCAM-1 (Imai *et al.*, 1993) and sgp200

(Hemmerich *et al.*, 1994). CD62L is widely distributed on most leukocytes in blood and its main role is that of lymphocyte homing to secondary lymphoid organs. However, it is now known to be implicated in many instances of inflammatory leukocyte trafficking (Winn *et al.*, 1998).

1.6.1.2   CD49d (VLA-4 [very late activating antigen-4])

CD49d is an integrin which has been implicated in allergic inflammation. The integrin name was derived from the role of these adhesion molecules in "integrating" the extracellular matrix with the cytoskeleton. Integrins are expressed on every mammalian cell type except red blood cells and consist of non-covalently associated α and β subunits. They play an important role in embryogenesis, wound healing, immune responses and the behaviour of malignant cells. Like most integrins, CD49d exists in both a high and a low affinity state. It has been proposed that its initial contact with its ligand, vascular cell adhesion molecule-1 (VCAM-1) is through low affinity, multi-valent binding interactions (Jakubowski *et al.*, 1995) and activation of the lymphocyte is thought to result in a conformational change to a high affinity state. It has been demonstrated that high affinity CD49d subsets are required for firm adhesion based on the finding that a mutant CD49d with reduced affinity supported rolling and tethering but failed in the firm adhesion of cells (Chen *et al.*, 1999).

1.6.1.3   Cutaneous Lymphocyte Antigen (CLA)

Lymphocytes that home to the skin express CLA. CLA is a carbohydrate modification of P-selectin glycoprotein-1 (PSGL-1) that recognizes E-selectin (Berg *et al.*, 1991). Cells that express high levels of CLA (CLA^{high}) migrate in response to chemokines TARC (thymus and activation regulated chemokine [CCL17]), the ligand for CCR4 and CCL27 (Cutaneous T cell-attracting chemokine [CTACK]), the ligand for CCR10, which are displayed on the surface of cutaneous post-capillary venules (Campbell *et al.*, 1999). This is in line with the finding that the skin-homing Th compartment is itself divided into distinct subpopulations (Soler *et al.*, 2003). Both receptors on Th cells are recognised in cutaneous homing; however, CCR4 is present on all skin homing cells and also on other non-skin homing cells. In contrast, CCR10 is

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associated with effector type Th lymphocytes (Soler *et al.*, 2003) and is absent on non skin-homing Th cells (Homey *et al.*, 2002) and only present on a small subset of skin-homing Th cells (Soler *et al.*, 2003). The importance of both CCR4 and CCL27 has been confirmed using a mouse model, whereby blocking of both inhibits lymphocyte recruitment to the skin (Reiss *et al.*, 2001). Through their investigation of leukocyte subsets Andrew and colleagues found that memory CLA+ CD4+ lymphocytes were all CCR4^{high} but they also noted the presence of CCR4^{high} CLA- and CCR4^{dull} CLA-CD4+ lymphocytes. In addition the subset of α4β7^{high} CD4+ lymphocytes which traffic to the gut, expressed little or no CCR4 (Andrew *et al.*, 2001). In conclusion, the expression of CCR4 on apparently non-cutaneous lymphocytes has led to the proposal that CLA and CCR4 must work together to convey skin-specific homing (Campbell *et al.*, 1999) but CLA alone appears to have no role in asthma.

1.6.1.4   Inducible Costimulator (ICOS)

ICOS has been identified as a costimulatory molecule related to CD28 and CTLA-4 which participate in other costimulatory pathways. ICOS delivers a co-signal through a novel member of the B7 (CD80/CD86) family called B7h (Swallow *et al.*, 1999), B7RP-1 (Yoshinaga *et al.*, 1999), GL50 (Ling *et al.*, 2000) or ICOS-ligand (Aicher *et al.*, 2000) which is expressed on antigen presenting cells. ICOS is expressed only on activated T cells and has been characterised as an important regulator of T cell activation (Hutloff *et al.*, 1999). Its expression on T cells must be induced by antigenic stimulation implying a delivery signal to activated rather than naïve T cells. Stimulation of ICOS promotes proliferation of murine and human T cells without increased IL-2 production, in contrast to stimulation involving CD28 (McAdam *et al.*, 2000).

The expression of ICOS is an integral part of the T cell differentiation program. ICOS- T cells, typical of naïve T cells (Yang *et al.*, 1998) predominantly produce TNFα. Löhning and co-workers were able to correlate the levels of expression of ICOS with the class of cytokine produced. In this work they claim that a low expression of ICOS was associated with the generation of IL-2, 3, 6 and GM-CSF,

whereas cells with an intermediate level of expression of ICOS were largely responsible for the production of IL-4, 5 and 13 (Lohning *et al.*, 2003). The strongest finding was that of cells displaying a high level of ICOS expression. In this population they noted that approximately 50% of T cells capable of generating IL-10 *in vivo* expressed ICOS. Moreover, IL-10 was the dominant cytokine produced by T cells with a high level of ICOS expression (Lohning *et al.*, 2003). Not all work in this area follows the original paradigm that ICOS is preferentially involved in Th2 responses. Increases in IFNγ and TNFα have been documented upon ICOS co-stimulation (Hutloff *et al.*, 1999), and even an increase in IL-2 production has been seen (Riley *et al.*, 2001). On investigating the relative contribution of ICOS-B7RP-1 binding in DC-mediated activation of T cells, Vieira and colleagues analysed changes in cytokine production upon addition of an ICOS-Ig fusion protein to superantigen-(*Staphylococcus aureus* enterotoxin B) primed DC-Th cell co-cultures (Vieira *et al.*, 2004). They found that an ICOS-Ig fusion protein preventing ICOS-B7RP-1 binding continues to support the Th2 theory by resulting in a decreased IL-4, 5, 10 and 13 production, but no changes in the levels of IL-2, GM-CSF and TNFα (Vieira *et al.*, 2004).

1.6.1.5   CCR7

It is widely understood that CCR7 helps T cells and DC's migrate into the T cell zone of lymphoid organs. Binding of CCR7 to ligands SLC (secondary lymphoid tissue chemokine [CCL21]) and ELC (EBI-1 ligand chemokine [CCL19]) is a means for antigen specific T cells and DC's to interact and initiate an adaptive immune response. The first of these two chemokines identified as a CCR7 ligand was CCL19 which is produced by cells throughout the T zone (Zlotnik *et al.*, 1999), whereas CCL21 is produced by stromal cells and also by HEV distributed throughout T zones (Zlotnik *et al.*, 1999). The pivotal roles and immunological importance of CCR7 have been demonstrated in many studies. For instance, CCR7-deficient mice fail to mount a delayed-type hypersensitivity response (Forster *et al.*, 1999). A further complexity to the CCR7 paradigm is the more recent proposition by Sallusto and colleagues that CCR7 can assist in the identification of two distinct memory cell populations (Sallusto

et al., 1999). Central memory T cells (T_{CM}) are CD45RO+ and express CCR7 in combination with CD62L whereas effector memory T cells (T_{EM}) are also RO+ but have lost the constitutive expression of CCR7 and are heterogeneous for CD62L and further define themselves by having a distinct set of chemokine receptors and adhesion molecules necessary for homing to inflamed tissues. CCR7 probably functions alongside these chemokine receptors and adhesion molecules found on effector/memory T cells to determine the distribution of functionally heterogeneous populations of cells within both lymphoid and non-lymphoid tissues (Kim *et al.,* 2001).

1.6.1.6   CD134 (OX40)

The engagement of CD28 on T cells by APC expressed CD80/CD86 provides an initial signal critical for cell cycle progression, IL-2 production and clonal expansion. More recently considerable evidence has shown that other co-stimulatory molecules play equally important roles in T cell responses, notably OX40 and ICOS with emphasis on CD4+ and Th2+ cells. Originally identified in 1987 by an antibody that reacted with activated rat CD4+ cells (Akiba *et al.,* 1998), OX40 is now the focus of much attention. It is a 50kDa type 1 trans-membrane protein of the TNF receptor family that is expressed primarily on activated T cells (Mallett *et al.,* 1990) and receives a signal through engagement by OX40-ligand on DC's, B cells and macrophages (Ohshima et al., 1997, Stuber et al., 1995, Murata et al., 2000). It can modulate T cell function by way of enhanced proliferation (Gramaglia *et al.,* 2000) and cytokine production (as seen *in vitro*) and preferential survival of CD4+ T cells (as seen *in vivo*) (Rogers *et al.,* 2001). Absent on resting T cells, it becomes expressed 12-48 hours after stimulation of naïve T cells, peaks at days 2-3 and is then down-regulated (Gramaglia *et al.,* 2000).

Salek-Ardakani and colleagues demonstrated that co-stimulation through OX40 is critical for several aspects of lung inflammation driven by memory Th2 cells (Salek-Ardakani *et al.,* 2003). Using OX40-/- TCR transgenic and wild-type mice they have shown that the exposure of pre-sensitised mice to aerosolised OVA results in large increases in the number of OX40 expressing memory/memory effector Th2 cells in the

lungs in association with airway hyperresponsiveness (Salek-Ardakani *et al.*, 2003). Not surprisingly, inhibiting this co-stimulatory interaction has been shown to prevent eosinophilia, airway hyperreactivity, mucus secretion, and Th2 cytokine production (Salek-Ardakani *et al.*, 2003).

1.6.1.7 CD44

CD44 is a class 1 transmembrane glycoprotein described originally on leukocytes but now known to be expressed by virtually all vertebrate cells (Ponta *et al.*, 2003). Upon activation, the CD44 expression profile of T cells changes from one characterised by the expression of low levels of CD44, high levels of CD62L and high molecular weight isoforms of CD45 (RA) to the expression of high levels of CD44, low levels of CD62L and low molecular weight isoforms of CD45 (RO) (Budd *et al.*, 1987, Jung *et al.*, 1988, Birkeland *et al.*, 1989). The amino-terminal globular domain of CD44 serves as a docking site for multiple components of the extracellular matrix, namely hyaluronan, collagen, laminin and fibronectin. The binding of these components to CD44 on CD4+ T cells, implicates CD44 in numerous processes involved in health and disease (Ponta *et al.*, 2003). Looking at the expression of CD44+ cells in bronchial biopsies by immunohistochemistry, Peroni and co-workers discovered an increase in the number of CD44 and LFA-1 positive leukocytes in asthmatics as compared with healthy controls (Peroni *et al.*, 1996). Using two different monoclonal antibodies, a CD44 neutralising reagent and one that induced CD44 shedding, Katoh and colleagues showed a dose-dependent inhibition of multiple aspects of experimental asthma (Katoh *et al.*, 2003b). Most markedly reduced were leukocyte recruitment and the production of IL-4 and IL-5 and the chemokines CCL17 and CCL11 (Katoh *et al.*, 2003b). Conversely, Teder and colleagues noted that CD44-/- mice developed exaggerated levels of lung leukocytes believed to be caused by absence of a macrophage-CD44 mediated clearance mechanism of apoptotic cells in the lung (Teder *et al.*, 2002). More recently it has been stated that CD44 and hyaluronan are key to both Th1 and Th2 lymphocyte interactions with the endothelium and that rolling and adhesion of lymphocytes on endothelium requires CD44 interactions with hyaluronan (Bonder *et al.*, 2006).

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1.6.1.8   CD45RA and CD45RO

CD45 is one of the most abundant surface glycoproteins, comprising up to 10% of the cell surface area of leukocytes (Thomas, 1989). It exists as multiple isoforms, due to alternative splicing of exons A, B and C. The highest molecular weight isoform consists of all three exons, and is termed CD45RA, a marker of naïve T cells. The lowest molecular weight isoform, CD45RO, is found on memory T cells, and lacks exons A, B and C. Upon cell activation a switch occurs and T cells change from expressing the high molecular weight isoform, RA, to the low molecular weight RO form. However, much research has gone into trying to strictly classify a T cell to that of naïve or memory and it is widely accepted that these markers cannot be used alone to discriminate between these two cell types and multiple combinations of CD45 with other cell surface molecules aids discrimination and much of this attention has focused on patterns of CCR7, CD44 and CD62L expression (section 1.6.1.5, 1.6.1.7 and 1.6.1.1).

Once a cell attains a memory phenotype it has less stringent requirements for activation than do naïve cells. Memory cells may be reactivated by a lower concentration of antigen (Rogers *et al.*, 2000) and evidence suggests that they are less dependent on co-stimulation than naïve cells. The fact that memory cells are able to respond to antigen presented to them by cells other than DC's (Croft *et al.*, 1994), as opposed to naïve cells which are solely reliant on DC's for antigen presentation supports this.

1.7   Mast cells

Mast cells originate from CD34 positive hematopoietic progenitor cells in the bone marrow. They circulate as undifferentiated mononuclear cells in the peripheral circulation and subsequently mature under local influences following migration into tissues. They are widely distributed throughout the body in both connective tissue and at mucosal surfaces. They form a heterogeneous population of cells with apparent differences in development, mediator content, ultrastructure and functionally in their

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ability to interact within their local environment (Church *et al.*, 1994). Upon crosslinking of receptor bound allergen specific IgE antibodies on the surface of mast cells, the cells become activated and release a plethora of inflammatory mediators.

The immediate/early phase response initiates through the activity of preformed or rapidly synthesised toxic mediators. These mediators are released from granules within the mast cell upon activation. These are comprised of histamine, heparin proteoglycan, chemotactic factors (eosinophil chemotactic factor [ECF] and neutrophil chemotactic factor [NCF]), acid hydrolases (β-glucuronidase, hexosaminidase and β-galactosidase) and the proteolytic enzymes tryptase and chymase (Holgate, 1998). The late phase reaction, described above in the context of allergen challenge in the laboratory, is attributable to induced synthesis of mediators, including leukotrienes, and further production of chemokines and cytokines. These late phase mediators are lipid derivatives of arachidonic acid and are formed via two different pathways of metabolism. The enzyme cyclo-oxygenase acts on arachidonic acid, leading to the production of an array of prostaglandins. Alternatively the enzyme lipoxygenase leads to the production of a number of leukotrienes (LTs), with LTC_4 being the most relevant to allergic responses (Holgate *et al.*, 2003). Interaction of both preformed and newly synthesised mediators with receptors expressed on target tissues produces the acute allergic symptoms including contraction of airway smooth muscle, leakage of small blood vessels causing swelling, stimulation of glands to secrete excess mucus and irritation of nerve endings to create symptoms of itching, sneezing and coughing. Subacute and chronic components amplify the condition through recruitment of eosinophils, Th2 cells and neutrophils to the areas of inflammation.

1.8   Eosinophils and Basophils

Eosinophils are derived from the bone marrow, from pluripotent stem cells. They were thought to play a pivotal role in asthma (Seminario and Gleich, 1994) but this concept has been questioned recently when administration of an anti-IL-5 monoclonal antibody to asthmatics was able to markedly reduce eosinophil numbers in both the airways and induced sputum of asthmatics without any attenuating effect on either the early or late

phase responses following allergen in the laboratory (Leckie *et al.*, 2000). The association between eosinophilia and asthma was observed shortly after eosinophils were first discovered (Ellis, 1908). They are increased in the blood of asthmatics (Charles *et al.*, 1979), sputum (Vieira and Prolla, 1979) and BAL (Diaz *et al.*, 1989), relative to non-asthmatic/atopic controls. The cytokine IL-5 is a major player in eosinophil infiltration, in addition to IL-13 and GM-CSF. These cytokines prime eosinophils for locomotor attraction towards mast cell platelet activating factor (PAF), leukotriene B$_4$ (LTB$_4$), regulated upon activation normal T-cell expressed and secreted (RANTES [CCL5]), eotaxin-1 (CCL11) and IL-16. IL-5 also inhibits the natural apoptosis of eosinophils, thus prolonging their activity and survival. Chemoattractants recruit eosinophils into the airways and once in the airways a number of destructive toxic granules are released leading to tissue damage, smooth muscle contraction and increased vascular permeability which in turn leads to the recruitment of even more eosinophils and T cells to the site of inflammation. Major basic protein (MBP) and eosinophil cationic protein (ECP) are cytotoxic to respiratory epithelium and MBP can induce degranulation of mast cells. The eosinophil chemoattractants, CCL11, eotaxin 2 (CCL24) and CCL5 produced in the airways, recruit eosinophils and Th2 cells which produce cytokines that perpetuate inflammation and attract even more eosinophils to the site.

Basophils are the least numerous of the circulating leukocytes, representing less than 1% of the typical blood differential count. Under physiological conditions, basophils have a short life-span of several days. IL-3 is pivotal for basophils and can promote their production and survival (Valent *et al.*, 1989). Cytokines GM-CSF and IL-5 also play important roles. Basophils share many similarities with eosinophils and mast cells and they also release the same toxic mediators. The basophil, in addition to eosinophils, releases major basic protein (MBP) which induces degranulation of other basophils and eosinophils. As a consequence of allergen challenge basophil numbers in the airways can increase, often by more than 100-fold and can account for a much as 3% of the cells (Bochner, 2000). A marker of systemic effects of allergic disease is a

stable basophilia in allergic subjects and the number of circulating basophils increases even more during the allergy season (Bochner, 2000).

1.9 Treatment of asthma

The current treatment options available for allergic diseases are based on allergen avoidance, the use of corticosteroids to control inflammation and antihistamines and sympathomimetics to treat symptoms. The search is on for new, effective and novel treatments which target new mediators, such as cytokines and chemokines, in the hope that this would provide better and more targeted anti-inflammatory therapies. Mouse monoclonal antibodies have been generated that prevent the binding of IgE to its high affinity receptor, FcεR1. These have been humanised and are now available for the treatment of severe asthma (Bousquet et al., 2005). The binding of this antibody to circulating IgE prevents binding of IgE to surfaces of cells that contain the high and low-affinity receptors for IgE; this leads to depletion of surface IgE which prevents cross-linking by allergen. As a consequence, the degranulation of mast cells and basophils (Patalano, 1999, Holgate et al., 1991b) is reduced and this probably also reduces facilitated antigen presentation by presenting cells which have IgE bound on their surface. Upon intravenous administration of 9 weekly injections in patients with allergic asthma the early and late phase responses to allergen were almost abolished (Patalano, 1999). An alternative strategy is to target the mediators after their release from the cell by neutralising them. The targeting of pro-allergic cytokines has resulted in the generation of a soluble form of the IL-4 receptor and in mice prevents the development of both immediate hypersensitivity (Renz et al., 1996) and atopic asthma (Steinke and Borish, 2001). Results on the effect of an IL-5 blocking monoclonal antibody show that a single dose decreased blood eosinophils for up to 16 weeks and sputum eosinophils for four weeks. However there was no effect of this antibody on the late asthmatic response or on airway hyperresponsiveness to histamine (Leckie et al., 2000). Allergen specific immunotherapy is also an available treatment option. The process involves vaccinating with allergen or its derivatives in order to produce selective inhibition of allergen specific responses. One trial indicated that grass pollen immunotherapy improves quality of life in seasonal allergic rhinitis and reduces

seasonal asthma symptoms and bronchial hyperresponsiveness (Durham and Walker, 2000).

1.10 Chemokines

The term "chemokine", a short form of "chemotactic cytokine" was coined in 1992 after the International Immunology Meeting in Budapest (Bacon et al., 2002). Chemokines are small secretory or membrane bound proteins with molecular masses of 6-14kDa (Baggiolini and Dahinden, 1994). Their main function is the chemoattraction (the migration of cells along a concentration gradient of lipid or protein factors) of leukocytes and regulation of leukocyte trafficking. They also play roles in many other biological functions (Fig 1.4). They have been described as pro-inflammatory cytokines because the expression of many chemokines is inducible in inflammatory conditions and they attract leukocyte and thereby mediate inflammatory reactions.

Figure 1.4

Cell recruitment   Angiogenesis/angiostasis

Lymphoid organ   Metastisis
development

CHEMOKINES

Lymphoid trafficking
                                                  Wound healing
        Th1/Th2 development   Inflammation
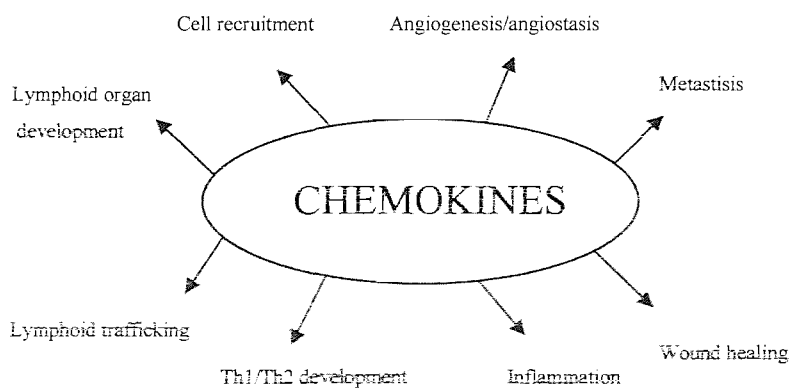
Figure 1.4   Biological roles of chemokines. Adapted from (Rossi and Zlotnik, 2000).

Over 50 human chemokines (Table 1.1) have been identified consisting roughly of 70-130 amino acids with four conserved cysteines (Baggiolini and Dahinden, 1994) from two main subfamilies. They are placed into either subfamily based on the position of

the first two cysteines. For the "CXC" chemokines, the first two cysteine residues are separated by an amino acid and for "CC" chemokines. the first two cysteines are adjacent. Two chemokines identified, namely lymphotactin (XCL1) and fractalkine (CX3CL1), do not fit into either of these two subfamilies. XCL1 has only two cysteines and is therefore known as a "C" chemokine and CX3CL1 has a three amino acid break between the first and second cysteine and is known as a CX3C chemokine.

These chemoattractant proteins are additionally classified as inflammatory or lymphoid. Inflammatory chemokines primarily attract neutrophils, monocytes and other innate immune cells. Their major sources are activated endothelial cells, epithelial cells and leukocytes, but virtually any cell type can generate chemokines when stimulated by lipopolysaccharides (endotoxin) or inflammatory cytokine.

Table 1.1

RECEPTORS	LIGANDS	RECEPTOR EXPRESSING CELLS
CCR1	CCL3, 5, 7, 8, 13, 14, 15, 23	Mø, DC (immature), T, Nø, Eø, MC and platelet
CCR2	CCL2, 7, 8, 13	Mø, DC, T, Bø, NK, fibroblast, endothelial cell
CCR3	CCL5, 7, 8, 11, 13, 14, 15, 24, 26,	Eø, Bø, MC, T (Th2), platelets, airway epithelial cell
CCR4	CCL17, 22	DC, Bø, T (Th2, Treg, Skin homing), platelets
CCR5	CCL3, 4, 5, 8, 11, 13, 14	T (Th1), DC, Mø, NK
CCR6	CCL20	DC (immature), T, B
CCR7	CCL19, 21	DC, T cell, B cell, NK
CCR8	CCL1, 16	T (Th2, Treg) Mø, NK, B, endothelial cell
CCR9	CCL25	T (gut homing)
CCR10	CCL27, 28	T (skin homing), melanocyte, langerhans cell, dermal endothelium, dermal fibroblast
CCR11	CCL19, 21, 25	Astrocyte
CXCR1	CXCL5, 6, 8	Nø, Mø, endothelial cell, astrocyte
CXCR2	CXCL1, 2, 3, 5, 7, 8	Nø, Mø, Eø, endothelial cell
CXCR3	CXCL9, 10, 11	T (Th1), B, mesangial cell, smooth muscle cell, microglia
CXCR4	CXCL12	T, DC, Mø, B, Nø, platelet, astrocyte
CXCR5	CXCL13	B, T, astrocyte
CXCR6	CXCL16	T (Th1)
XCR1	XCL1, 2	T
CX₃CR1	CX₃CL1	T, NK, astrocyte

Table 1.1   Chemokine receptors and their ligands, (D'Ambrosio *et al.*, 2003).

Mø (monocyte), DC (dendritic cell), T (T cell), B (B cell), Nø (neutrophil), Eø (eosinophil), Bø (basophil) and NK (natural killer cell).

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1.11   Chemokine receptors

Chemokine receptors (Table 1.1, Fig 1.5), are members of a superfamily of receptors, known as G-protein coupled receptors (GPCR). These receptors span the membrane 7 times and are known as 7-transmembrane serpentine receptors. They signal through coupled heterotrimeric G proteins. Several chemokine receptors have been identified so far. Chemokine receptors are classified into the same four groups as chemokines, namely CC, CXC, CX3C and XC because each class of chemokine receptor binds ligands from a single class of chemokines only. So CC chemokine receptors can bind a certain number of chemokines from the CC chemokines class with high affinity, for example CCR1 will bind MIP-1α (macrophage inflammatory protein 1α [CCL3]) (Sherry *et al.*, 1988), CCL5 (Neote *et al.*, 1993), MCP-2 (monocyte chemoattractant protein-2 [CCL8]), (Gong *et al.*, 1997) and MCP-3 (CCL7) (Combadiere *et al.*, 1995), leukotactin (CCL15) (Youn *et al.*, 1997) and HCC-1 (hemofiltrate CC chemokine [CCL14]) (Tsou *et al.*, 1998).

Figure 1.5
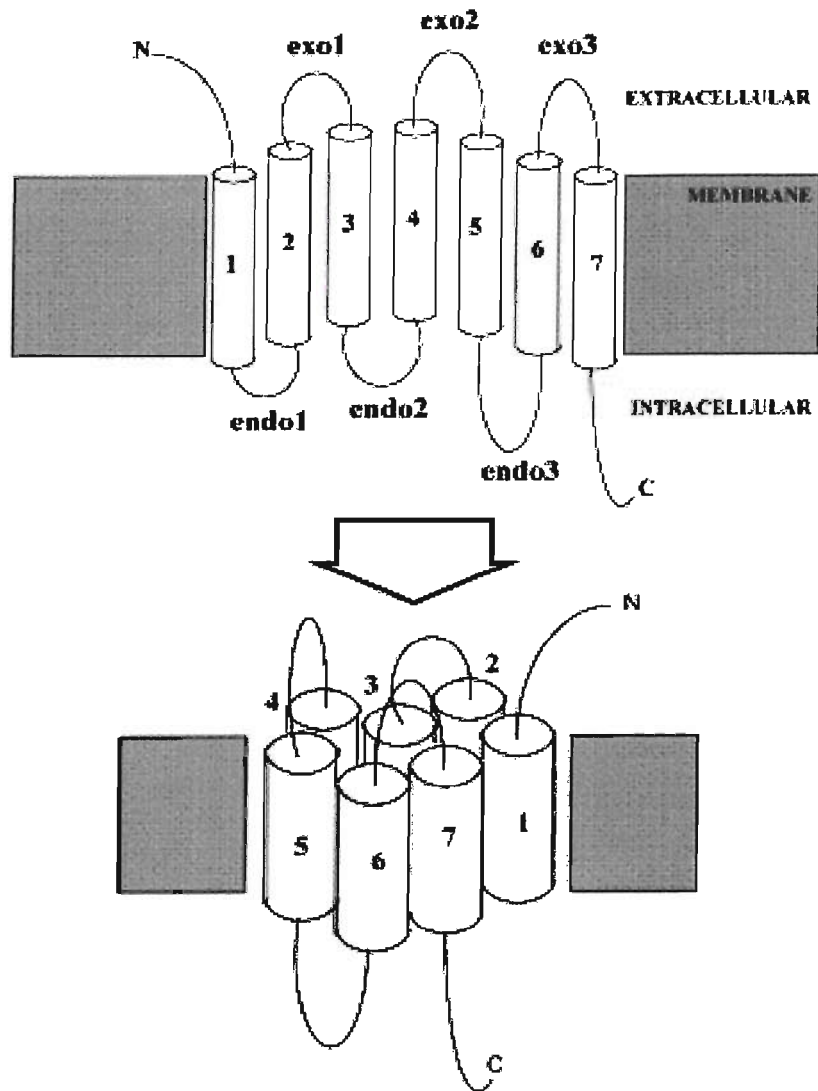
Figure 1.5   **Chemokine Receptors.** Shown in the upper panel is an unfolded diagram of a GPCR. Transmembrane helices are numbered 1–7. The lower diagram is showing how these helices pack together in the membrane. Chemokine Receptors (Flower, 1999).

Chemokines and their receptors are thought to contribute to allergic disorders (Kita and Gleich, 1996, Lukacs *et al.*, 1996).

Chemokines that arise under inflammatory conditions and act on CCR1, CCR2 and CCR5 attract immature dendritic cells (DC) to the affected sites. In inflamed tissue DC acquire the capacity to process and present antigen. This causes the DC to express CCR7 and CCR4. The DC migrate towards CCL21 and CCL19, which bind CCR7 and to CCL17, which binds CCR4, in the draining lymph nodes to initiate an immune response. The identification of CCR6 (on immature DC) (Power *et al.*, 1997) and CCR7 (on mature DC) confirms their role based on their specific binding of CCR6 and CCR7 ligands.

It has been suggested that DC's play a role in the recruitment of Th2 lymphocytes and allergic inflammation through the production of chemokines. DC's are a cell type responsible for the production of large amounts of CCL22 (macrophage derived chemokine) (Vissers *et al.*, 2001). Very high levels of CCL22 and CCL17 are selectively produced by myeloid DC while plasmacytoid DC were shown to produce very little (Penna *et al.*, 2002). CCR4, the chemokine receptor for CCL22 and CCL17, has been proposed as a receptor that is selective for allergic responses, including those seen in the late phase allergen responses that follow allergen challenge (Baggiolini and Dahinden, 1994). The discovery of a high production of CCR4 ligands by myeloid DC suggests their capacity to recruit Th2 cells and/or those with regulatory properties to sites of inflammation (Penna *et al.*, 2002). CCR4 has also been found to be up-regulated on mature DC (Vecchi *et al.*, 1999), thus implying a role for CCL22 and CCL17 in co-localisation and interaction of DC and T cells (Vulcano *et al.*, 2001).

The receptor CCR3 is important in the attraction and activation of eosinophils (Daugherty *et al.*, 1996), basophils (Uguccioni *et al.*, 1997), mast cells (Quackenbush *et al.*, 1998) and a subset of Th2 lymphocytes (Sallusto *et al.*, 1997) through

interactions with an array of chemokines, primarily CCL11, CCL24 and MCP-4 (CCL13). Because these cells are thought to play important roles in allergic diseases, CCR3 has been proposed as an important chemokine receptor in allergic responses. Plausibly, the CCR3 ligands that are released by airway epithelial cells could preferentially attract eosinophils through CCR3. An increase in the levels of CCL11 and CCL13 is found in the lungs of atopic asthma sufferers and is correlated with an increase in the number of infiltrating eosinophils (Ying et al., 1997b). This is supported by the fact that neutralising antibodies to both CCL11 (Gonzalo et al., 1998) and CCR3 (Uguccioni et al., 1997) induce a great reduction in eosinophil infiltration in the lung. It is also supported by the observation that the chemotactic activity that is detected in induced sputum and which correlates with asthma severity can be inhibited by a neutralising antibody against CCL11 (Dent et al., 2004).

Within the different T cell subsets, numerous chemokine receptors are differentially expressed (D'Ambrosio et al., 1998). Cells of the Th1 subset preferentially express CCR5 and CXCR3, binding the ligands MIP-1β (macrophage inflammatory protein-1β [CCL4]), IP-10 (γ-interferon-inducible protein-10 [CXCL10]) and I-TAC (interferon-inducible T cell chemoattractant [CXCL11]) respectively. Pivotal in allergic diseases, the Th2 subset preferentially express CCR3 (Sallusto et al., 1997), CCR4 and CCR8 (D'Ambrosio et al., 1998). All three of these receptors function in the migration of Th2 cells via ligands CCL11, CCL22 and CCL17, and I-309 (CCL1) respectively. The more recently identified receptor, CRTH2 (Chemoattractant Receptor-homologous molecule expressed on TH2 cells) is also selectively expressed on Th2 cells (Nagata et al., 1999) and binds the ligand prostaglandin D_2 (PGD$_2$) (Hirai et al., 2001).

There has been significant effort to discriminate between Th1 cells and Th2 cells by their selective or preferential expression of certain chemokine receptors, with a certain amount of success. Generally, Th1 cells preferentially express CXCR3 (to which CXCL10, monokine induced by IFN-gamma [Mig (CXCL9)] and CXCL11 bind) and CCR5 (to which CCL5, macrophage inflammatory protein-1 alpha [MIP-1α (CCL3)] and CCL4 bind), (Bonecchi et al., 1998). Th2 cells have been reported to express

CCR3 (Sallusto *et al.*, 1997), although there is still some disagreement over how selective the expression is for Th2 cells. CCR4 is preferentially expressed on Th2 cells (Sallusto *et al.*, 1998), and they are the only known subset of T cells displaying expression of CCR8 (Zingoni *et al.*, 1998). However, considerable controversy still exists. Th1 cells, and small numbers of Th2 cells are found in the CCR4-CXCR3+ population (Kim *et al.*, 2001). In contrast, in the CCR4+CXCR3- population, the majority of cells are of the Th2 phenotype and very few are Th1 cells (Kim *et al.*, 2001). There are many double-positive cells, expressing both CCR4 and CXCR3. Intermediate numbers from Th1 and Th2 subsets are double-positive, but the greatest proportion of double-positive cells are found within the Th0 subset (Kim *et al.*, 2001). The majority (77%) of memory CD4+ T cells in human blood are non-polarised T cells with a capacity to produce both Th1 and Th2 cytokines (Kim *et al.*, 2001). It is proposed that the combination of the receptor expression on subsets of T cells may confer some specialised homing behaviour (Kim *et al.*, 2001). It has recently been discovered by Kurashima and colleagues that the proportion of CXCR3+ CD45RO+ T cells in blood increases alongside asthma severity but the proportion of CCR4+ CD45RO+ cells remains the same in mild and severe asthma (Kurashima *et al.*, 2006)

The expression of mRNA for CXCR3 (D'Ambrosio *et al.*, 1998) and other receptors such as CXCR4 (receptor for stromal derived factor alpha [SDFα (CXCL12)]) on Th1 cells is down-regulated following anti-CD3/-anti-CD28 stimulation (Jourdan *et al.*, 1998), whereas in Th2 cells CCR4 mRNA is up-regulated following stimulation (D'Ambrosio *et al.*, 1998). In addition, anti-CD3/anti-CD28 has been shown to up-regulate CCR4 on Th1 cells (D'Ambrosio *et al.*, 1998). A similar pattern has been seen in calcium mobilisation assays, where CXCR3 is down-regulated and CCR4 up-regulated following stimulation (D'Ambrosio *et al.*, 1998).

1.12.1   CCR4 and ligands CCL17 and CCL22 in asthma

CCR4 was originally cloned from a human basophilic leukaemia cell line library (Power *et al.*, 1995), and is expressed on T cells basophils, platelets and monocytes in peripheral blood (Power *et al.*, 1995, Abi-Younes *et al.*, 2001, Katschke *et al.*, 2001).

CCL17 (TARC) and CCL22 (MDC) are both high affinity ligands and high potency agonists for CCR4 (Imai *et al.*, 1997, Imai *et al.*, 1998). Many studies show that CCR4 is selectively and highly expressed on Th2 cells and is a selective marker for Th2 lymphocytes which is markedly upregulated upon T cell receptor activation (Sallusto *et al.*, 1998, Panina-Bordignon *et al.*, 2001, D'Ambrosio *et al.*, 1998). CCR4 is also thought to be an important receptor in Th2 cell trafficking in allergic diseases (Chvatchko *et al.*, 2000, Panina-Bordignon *et al.*, 2001). Mathew and colleagues looked at Th2 cell trafficking into the lung and lymph nodes and found that trafficking of Th2 cells into the lung, airways and lymph nodes in allergic pulmonary inflammation requires involvement of chemoattractant receptors expressed on Th2 cells (Mathew *et al.*, 2002). Treatment of spleen and lymph node CD4+ T cells from DO11.10 mice with Pertussis toxin, a known inhibitor of G-protein-coupled receptor-induced chemotaxis (Spangrude *et al.*, 1985), ablated Th2 cell trafficking into the lungs of BALB/c mice (Mathew *et al.*, 2002). This reinforces the importance of chemokine receptors in allergic lung inflammation, but does not determine the extent to which CCR4 is responsible. Surprisingly, blocking of CCR4 using a blocking antibody has barely altered the recruitment of inflammatory leukocytes to the lungs (Conroy *et al.*, 2003). Chvatchko and colleagues demonstrated that in mice deficient in CCR4, cellular recruitment and induction of airway hyperreactivity was unaltered (Chvatchko *et al.*, 2000), raising questions about the role that CCR4 plays.

Bronchial epithelial cells are a cell type known to produce large amounts of CCL17 (Berin *et al.*, 2001). CCL17 is increased in asthmatic bronchial epithelium where, somewhat surprisingly, very little CCL22 is detected (Kawasaki *et al.*, 2001). Considerable amounts of CCL17 have been found in the BAL of asthmatics following exposure to relevant antigen (Berin *et al.*, 2001) and also CCL22 has been found in the BAL of healthy subjects and those patients with eosinophilic pneumonia (Katoh *et al.*, 2003a). CCL17 is also shown to be expressed by inflamed endothelium (Campbell *et al.*, 1999), and is known to assist in the rolling, arrest and adhesion of CCR4 expressing cells to the endothelial cell wall prior to diapedesis (Campbell *et al.*, 1999).

In addition to there being a predominance of Th2 cells in blood samples of patients suffering with atopic dermatitis (AD) (Nakatani *et al.*, 2001). CCR4, but not CXCR3, is expressed preferentially on peripheral blood CD4+ T cells of AD sufferers (Wakugawa *et al.*, 2001). Skin lesions in sufferers of atopic dermatitis have also been shown to harbour cells with selective expression of CCR4 (Wakugawa *et al.*, 2001). It is generally accepted that CCL22 and CCL17 play an important role in the recruitment of Th2 cells to sites of inflammation, but it is unclear to what extent they account for the total chemotactic activity that exists *in vivo* in atopic patients.

It has recently been shown that histamine and prostaglandin E_2 (PGE_2), both of which are released by allergen activated mast cells, dose-dependently up-regulate CCL17 and CCL22 by monocyte-derived immature DC (McIlroy *et al.*, 2006). In the serum and sputum of asthmatic individuals CCL17 is significantly increased, when compared with healthy subjects (Sekiya *et al.*, 2002). It is also postulated that CCL17 plays a role in the exacerbation and maintenance of Th2 cell responses in asthma, in part due to its up-regulation by IL-4 (Sekiya *et al.*, 2002). CCL22 is supposedly produced by B cells, macrophages, and thymic medullary epithelial cells (Chang *et al.*, 1997, Godiska *et al.*, 1997, Zlotnik and Yoshie, 2000) and DC (Vulcano *et al.*, 2001, Kikuchi and Crystal, 2001). Levels of CCL22 correlate with severity of atopic dermatitis (Kakinuma *et al.*, 2002). Kakinuma and colleagues noted increased levels of CCL22 in serum from AD patients in comparison to Psoriasis vulgaris patients and healthy controls. The levels of CCL22, detected by Enzyme Linked ImmunoSorbant Assay (ELISA) also increased in parallel with increasing disease severity as assessed in mild, moderate and severe AD groups (Kakinuma *et al.*, 2002). The levels declined in AD patients after treatment with topical corticosteroids and antihistamine drugs (Kakinuma *et al.*, 2002).

1.12.2   CRTH2 and its ligand PGD_2 in asthma

Prostaglandin D_2 (PGD_2) is known to bind and activate two GPCR, CRTH2 and the prostanoid receptor, DP. Hirai and colleagues discovered that PGD_2 was a ligand for CRTH2 through use of a CRTH2-transfected rat T cell line and discovered that only

PGD$_2$ was capable of inducing Ca^{2+} mobilisation in a transfected mast cell line (Hirai et al., 2001). These two receptors share little sequence homology. The highest amino acid sequence identity for CRTH2 is found with members of the leukocyte chemoattractant receptor subfamily which includes the fMLP (the bacterially produced peptide formyl. Met. Leu. Phe, which directionally attracts leukocytes) and complement receptors C3a and C5a (Nagata et al., 1999). Further work has confirmed that CRTH2 functions in a different way to DP, one such difference being the signalling molecules that these two receptors employ. It has been shown that Gαi is used for CRTH2 whereas Gαs is used for DP (Hirai et al., 2001). PGD$_2$ binding to DP causes activation of Gαs and elevation of cyclic AMP, resulting in vasodilatation, which will enhance extravasation of leukocytes; along with chemotaxis of Th2 cells, eosinophils and basophils mediated by the use of CRTH2 via Gαi (Hirai et al., 2001). PGD$_2$ is involved in diverse biological processes including maintaining body temperature, preventing platelet aggregation, promoting sleep, functioning of nerve cells, relaxation of smooth muscle and vasodilation (Urade and Hayaishi, 2000). It is released in large amounts by the mast cell following IgE receptor cross-linking during asthmatic attacks and allergic stimulation, through the metabolism of arachidonic acid. It has also been proposed as a marker of mast cell activation in asthma (Murray et al., 1986).

PGD$_2$ was generally believed to exert its action through binding to the DP receptor however it has been demonstrated that PGD$_2$ can increase eosinophil and Th2 cell motility through binding to CRTH2 (Hirai et al., 2001, Monneret et al., 2001, Gervais et al., 2001). Matusoka and colleagues found that the numbers of eosinophils and lymphocytes infiltrating the lung were high in wild-type mice, but significantly decreased in DP receptor-deficient mice (Matsuoka et al., 2000). Overall they concluded that DP-/- mice do not develop asthmatic responses in an ovalbumin-induced asthma model (Matsuoka et al., 2000). However, recent research has suggested that other receptors may be involved. CRTH2 was discovered through a search for markers on CD4+ T cells to enable discrimination between Th1 and Th2 cells (Nagata et al., 1999). CD8+ T cells, most likely T cytotoxic type 2 cells (Tc2),

also express this receptor (Nagata *et al.*, 1999). Isolation of the cells expressing CRTH2 confirmed their Th2 phenotype, by the production of Th2 type cytokines (primarily IL-4, IL-5 and IL-6), and very low levels of Th1 (primarily IL-2, IL-12 and IFNγ) cytokines upon stimulation with PMA and ionomycin (Nagata *et al.*, 1999). It has also been shown that virtually all CD4+, CRTH2+ cells from peripheral blood of healthy control subjects and those with atopic dermatitis have a large proportion of circulating Th2 cells with a pure Th2 phenotype. These cells also migrate in response to PGD_2 in chemotaxis assays (Iwasaki *et al.*, 2002). CD4+ and CD8+ T cells expressing CRTH2 are also increased in patients with atopic dermatitis (Cosmi *et al.*, 2000). CRTH2 has also been implicated in cutaneous inflammatory responses. Satoh and co-workers found that when Ramatroban, a CRTH2 antagonist, was used to treat mice, there was a reduction in cutaneous inflammatory responses (Satoh *et al.*, 2006). These responses were associated with decreased infiltration of lymphocytes, eosinophils, and basophils and decreased production of CCL22 and RANTES at inflammatory sites (Satoh *et al.*, 2006).

CRTH2 is thought to be one of the most reliable markers of Th2 and Tc2 cells and is not detected on Th0, Tc0, Th1 or Tc1 cells (Cosmi *et al.*, 2000). CRTH2 has also been shown to be a more reliable marker of Th2 cells than CCR4 since CCR4 is expressed on Th0 and Tc0 and even Th1 and Tc1 cells (Cosmi *et al.*, 2000). Following polyclonal stimulation CRTH2+ cells produce IL-4 alone or IL-4, IL-5 and IL-13, but never IFNγ. (Cosmi *et al.*, 2000).

Taken together these findings describe a prominent role of Th2 cells in asthma and other allergic diseases and suggest preferential usage of a restricted number of chemokines and their complementary ligands in this disease. CCR4 and CRTH2 are both characteristic of Th2 and/or Tc2 cells, and investigation into their selectivity, redundancy and overlapping specificity will help the understanding of the roles that these receptors and their respective ligands play in asthma.

1.13   Aims

The overall aim of the thesis was to improve knowledge of the mechanisms of T cell chemotaxis and T cell responses in atopic asthma, focusing, more specifically on the role of the chemokine receptor, CCR4 and its ligands CCL17 and CCL22.

The hypotheses were as follows:

1. CCR4 is a major chemokine receptor responsible for the accumulation (chemotaxis) of Th2 cells in the airways of atopic asthmatics.

2. CCL17 and CCL22 are the major chemokines which together with their receptor, CCR4, account for the majority of chemotaxis that is generated by the airway tissue in response to allergen stimulation.

3. Inhibition of CCR4 using a specific blocking molecule attenuates the chemotactic activity for T cells generated in asthmatic airways following stimulation of bronchial tissue with allergen to which patients are sensitised. Implying that CCR4 is a valid target for development of novel drugs for asthma.

4. CCR4+ T cells are those cells which are allergen specific and responsible for the production of Th2 cytokines in response to allergen stimulation. The CCR4+ cells are also those which proliferate in response to stimulation with allergen to which the individual is sensitised to. Therefore, removal of CCR4+ cells from PBMC will ablate Th2 cytokine production and proliferation in response to allergen.

To address these hypotheses the following aims were applied:

- To investigate the expression of CCR4 in atopic asthmatics who have active disease but are not treated with inhaled or oral corticosteroids.

- To compare the T cell expression of CCR4 and CRTH2.

- To characterise the CCR4 expressing peripheral blood T lymphocytes from healthy controls and mild asthmatics in respect of their expression of a range of activation, memory and homing cell surface markers.

- To investigate the importance of CCR4+ cells in generating mediators of relevance to the allergic response by studying the effects of depletion of CCR4 expressing cells on cytokine responses of PBMC taken from allergic asthmatics and stimulated with house dust mite allergen to which the donors of these cells are allergic.

- To further elucidate the relative contribution of CCL22 and CCL17 by extensive examination of the profile of chemokine and cytokine responses in the bronchial mucosa of asthmatics in supernatants of bronchial biopsy cultures (bronchial explants) from mild asthmatics stimulated with house dust mite allergen.

- To elucidate whether and to what extent CCR4 is responsible for the migration of T cells in response to chemoatttractants produced in the bronchial explants.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1　　　　Subjects

All samples were taken from consented healthy non-atopic control subjects and atopic asthmatic subjects. The study was approved by the Southampton University Hospitals Ethics Committee.

The donors were carefully screened and characterised for the presence or absence of atopy and asthma severity prior to inclusion in the study based on clinical history and physical examination, lung function testing and skin prick tests to determine atopy. Atopy was defined as the presence of a positive skin prick test to at least one common aero-allergen. Because of the intention to stimulate blood T cells and bronchial biopsies with house dust mite allergen, all the atopic asthmatics had to be positive to that allergen.

Skin prick tests were conducted using a panel of common aeroallergens supplied by ALK (ALK, Norway), namely *Aspergillus fumigatus*, *Alternaria alternate*, mixed grass pollens, mixed tree pollens, *D. pteronyssinus* and *D. farinae*, mixed feathers, cat hair, and dog hair. Standard clinical research methods were applied. Briefly, single drops of allergen extract solution were placed on the forearm and a prick lancet used to penetrate the skin without causing bleeding. After 15 min the site was inspected and a wheal reaction with a diameter 3mm or more greater than negative control (saline solution) was interpreted as positive. Histamine was used as a positive control to ensure that subject skin responsiveness was not reduced, e.g., in case they were treated with an antihistamine.

The asthmatic subjects all had mild disease as indicated by asthma symptoms, (wheeze and / or breathlessness being present less often than daily and requiring less than 1 puff of reliever bronchodilator, the β-agonist salbutamol, per day). None of the asthmatics were using either inhaled or oral corticosteroids at least 6 weeks prior to the study and

none had a respiratory tract infection during the same period of time. The healthy control subjects were all non-atopic and none had any respiratory symptoms or any other relevant chronic disease.

2.2   Experiments with peripheral blood T cells

2.2.1   Isolation of PBMC from whole blood

Peripheral blood mononuclear cells were used for flow cytometric analyses of CCR4, CRTH2, activation markers and markers of homing and memory expressed on circulating T cells and for PBMC cell culture experiments in which the aim was to quantify Th2 responses to allergen. The PBMC were also used to generate polarised Th1 and Th2 cells using stimulation with IFNγ and IL-4 respectively (Section 2.2.4). Blood was collected in heparinised tubes and immediately centrifuged at 1400rpm for 10 min at room temperature and the plasma was removed in order to further purify the blood. The remaining blood was diluted 1:1 with phosphate buffered saline (PBS). The blood sample was then subjected to Ficoll-Hypaque (Lymphoprep Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation at 2150rpm for 25 min at room temperature. The PBMC layer at the interface was removed and cells washed with RPMI-1640 w/o L-glutamine (Life technologies, Paisley, UK) supplemented with 1% L-glutamine, 50IU/ml penicillin, 50μg/ml streptomycin, 10% heat-inactivated FCS and 10mM HEPES (all from Life Technologies, Paisley, UK) and finally resuspended in media.

2.2.2   Characterisation of CCR4+ T cells

Analysis of CCR4 expression and the co-expression of activation markers was conducted on peripheral blood T cells of atopic asthmatics and healthy control subjects by flow cytometry using the FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). Set up tubes were always included allowing detection and compensation to be set appropriately. Thresholds were set to exclude any debris and the majority of dead cells. A dot plot of the forward scatter (FSC) and the side scatter (SSC) of the cells was used to identify the population of lymphocytes to set the gates.

2.2.3   Membrane staining for the study of expression of CCR4 and CRTH2
             and other surface markers

Non-specific antibody binding was blocked by treating the cells at a concentration of
1×10^7/ml with a 50μg/ml solution of Fcγ fragments (in-house) for 30 min on ice. Cells
were centrifuged at 1500rpm for 10 min and adjusted to a concentration of 1×10^7/ml.
A volume of 50μl was added to each FACS tube, along with 1ml of FACS wash buffer
(PBS containing 0.01% Sodium Azide and 1% bovine serum albumin [BSA]) prior to
centrifugation at 1500rpm for 5 min. The supernatant was aspirated and the cells
re-suspended by tapping. The antibodies for membrane staining (full list provided in
chapter 3) were then added. The cells were left to stain for 30 min at 4°C in the dark,
washed again with wash buffer by centrifugation at 1500rpm for 5 min and re-
suspended by tapping. Finally, 500μl of wash buffer were added to each tube to give
the desired concentration for use in the FACSCalibur flow cytometer (Becton
Dickinson, UK). The tubes were kept at 4°C prior to analysis within 1 hr.

2.2.4   Polarisation of PBMC

Cells were adjusted to a concentration of 1×10^6/ml and 1ml added to the wells of a 24
well plate and stimulated with 1μg/ml of Phytohaemagglutinin (PHA) (Sigma, Dorset,
UK). Two solutions of supplemented RPMI were made (Table 2.1). A Th1 polarising
solution containing IL-12 (R&D Systems, Abingdon, Oxon, UK) and anti-IL-4 (BD
Biosciences, Oxford, UK) and a Th2 polarising solution containing IL-4 (R&D
Systems, Abingdon, Oxon, UK) and anti-IFNγ (BD Biosciences, Oxford, UK). In each
well which contained 1ml of cell suspension, 1ml of polarising media was added. Into
half of the wells Th1 polarising media was added and into the remaining wells the Th2
polarising media was added.

Table 2.1

Cytokines and antibodies	Th1 media	Cytokines and antibodies	Th2 media
IL-12 (R & D systems, UK)	5ng/ml	IL-4 (R & D systems, UK)	50ng/ml
Anti-IL-4 (BD Biosciences, Oxford, UK)	2μg/ml	Anti-IFNγ(BD Biosciences, Oxford, UK)	5μg/ml

Table 2.1   Manufacturers and concentrations of antibodies and cytokines used.

The protocol to polarise the cells was a 15 day procedure. During the 1st polarisation, cells were cultured in 5% CO_2 at 37°C and stimulated with PHA and the cytokines and antibodies (Table 2.1). On day 4, the plated cells were split by re-suspending the cells and removing 1ml, leaving approximately 1ml in each well. One ml of fresh RPMI and IL-2 (R&D Systems, Abingdon, Oxon, UK) was added to give a final concentration of 10ng/ml. This treatment was referred to as 1st expansion. Cells were cultured in this condition until day 8 when a repeat of the polarisation was performed, and referred to as the 2nd polarisation. At this stage the cells were again re-suspended and 1ml from each well was removed. One millilitre of the polarising cocktail was added to each well along with a repeat stimulation with 1μg/ml of PHA resulting in again, approximately 2ml/well. A final 2nd expansion was performed on day 12, when cells were resuspended, split and given a repeat treatment with 1ml of media containing IL-2, at a final concentration of 10ng/ml. Cells were ready to use at day 15.

2.3   Bronchial biopsies

In order to establish the bronchial explant, bronchial biopsies were obtained from healthy non-atopics and atopic asthmatics by fibreoptic bronchoscopy by Dr Pandurangan Viyayanand, a medical specialist registrar who was a collaborator on this project, using standard internationally accepted guidelines (Djukanovic et al., 2004). All subjects were premedicated in an identical manner with nebulised B_2-agonist, salbutamol (500mg) and the anti-cholinergic drug ipratropium bromide (2.5mg) in addition to intravenous sedation with midazolam (between 2 and 10mg aimed at

achieving mild sedation) and atropine (0.6mg). Up to eight biopsies around 1-2 mm in diameter were taken from bifurcations between segmental and subsegmantal airways using standard biopsy forceps. These were immediately processed as outlined below.

2.3.1   Bronchial biopsy culture (bronchial explant)

Media were prepared fresh prior to the bronchoscopy, consisting of AIM-V serum free media supplemented with 1% L-glutamine, 50IU/ml penicillin, 50µg/ml streptomycin, 2mM 2-mercaptoethanol, 50µl fungizone and 250µl sodium pyruvate at 100mM (all from Life technologies, Paisley, UK). The biopsies were loosened from the forceps by gentle shaking into a universal of PBS, on ice. Two biopsies were placed into each of 4 Eppendorfs and weighed. To 4 wells of a 24 well tissue culture plate, 550µl of the prepared media was added and two biopsies were placed into each of the 4 wells: 2 of the wells contained medium alone, i.e. the tissues were not challenged and 2 contained allergen (freshly prepared media supplemented with 5000SqU/ml of HDM allergen [ALK, Norway]) (Fig 2.1). The empty Eppendorfs were then weighed to determine the weight of the biopsies.

Figure 2.1
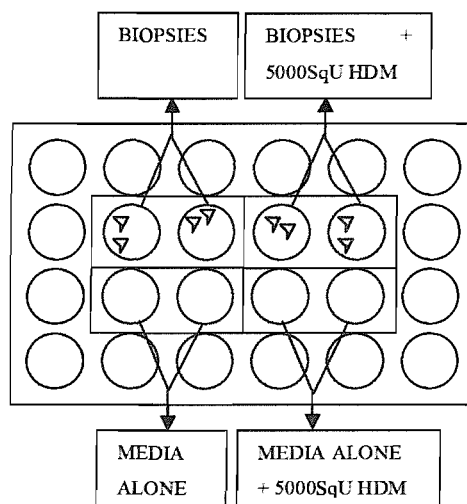
Figure 2.1   Bronchial biopsy culture.

Biopsies treated without allergen (top left) and with allergen (top right) and matched media alone (bottom left) and media plus allergen (bottom right) controls. Plate is left at 37°C for 24 hr.

Four wells containing media alone or media alone plus HDM allergen without any tissue were also prepared as controls on the plate. The plates were left in the incubator at 37°C, 5% CO_2 for 24 hr. After culture, the biopsies were removed and contents of each well were centrifuged at 1500rpm for 10 min. The supernatants were aliquoted into appropriate volume, to minimise freeze-thawing, and stored at -80°C until analysis of cytokine / chemokine concentrations and chemotaxis assays.

2.3.2 SearchLight assay for biopsy supernatant **chemokines**

The concentrations of a range of chemokines in biopsy culture supernatants were determined using a SearchLight custom human chemokine array (Pierce, Northumberland, UK) for which kits had been ordered from the supplier. Two assays were performed, with samples diluted 1:5 and 1:25 in order for all chemokine concentrations determined to sit within the range of the standard curve. The procedure was performed following the manufacturers instructions. Briefly, the 96 well plate provided in the kit arrived pre-spotted with chemokine specific antibodies. The lyophilised chemokines were reconstituted and diluted according to the information provided, to generate a 6 point standard curve including a diluent alone as the blank. 50µl of each standard concentration or 50µl of sample were added to the designated wells and the plate was covered with an adhesive plate sealer and incubated for one hr at room temperature with gentle shaking. The plate was then washed 3 times using the wash buffer supplied and 50µl of the prepared biotinylated reagent was placed into each well. The plate was covered with an adhesive sealer and incubated at room temperature for 30 min with gentle shaking followed by 3 washes. The SUPERSIGNAL® substrate was prepared and 50µl was added to each well and the plate was read using a 12-bit cooled CCD camera.

2.3.3 Luminex assay for biopsy supernatant **cytokines**

The concentrations of a range of other cytokines and two chemokines in biopsy culture supernatants were determined using the luminex assay. Luminex beads (Bio-Rad, Hemel Hempstead, UK) with different bead set numbers for each cytokine/chemokine

detected (table 2.2) were first coupled to the range of anti-cytokine (capture) antibodies chosen. This was achieved as follows. One vial of beads (1.25×10^7) of each bead set number was vortexed and then transferred to a 1.5ml Eppendorf and 500µl of activation buffer (0.1M sodium phosphate, pH 6.1) was added followed by centrifugation for 1 min at 10,000rpm. Supernatant was removed and the wash repeated. Then 320µl of activation buffer were added to the bead pellet and the Eppendorf was vortexed and sonicated. A 40µl volume of NHS (N-hydroxy succinamine), (Biacore, Stevenage, UK) at 11mg/ml in UHQ water and 40µl of EDC (1-ethyl-3 (3-dimethylaminopropyl) carbodiimide), (Biacore, Stevenage, UK) at 75mg/ml in UHQ water were added and the Eppendorf was wrapped in foil and incubated for 1 hr at room temperature on a rotator. 500µl of triton coupling buffer (5mM sodium acetate, pH 5, 0.025% triton) were added prior to centrifugation for 1 min at 10,000rpm. Supernatant was removed followed by two more washes with 500µl of coupling buffer, as above. A volume of 1ml of the separate capture antibodies for each bead set diluted in coupling buffer was added to the respective bead pellet to give a final antibody concentration of 50µg/ml (table 2.1) and the Eppendorf was vortexed, wrapped in foil and incubated overnight at room temperature on a rotator. Each bead/antibody set was washed with 500µl of wash buffer (50mM Na2HPO4, 150mM NaCl, 0.025% triton) for 1 min at 10,000rpm. The supernatant was removed and the wash repeated. Beads were then prepared for storage by adding 1ml of blocking/storage buffer (50mM Na2HPO4, 150mM NaCl, 0.025% triton, 10mg/ml BSA). Bead/antibody conjugates for each cytokine were counted using a haemocytometer and the stocks were diluted so that they will each require the same dilution for an assay.

A cocktail of recombinant cytokines and two chemokines for a 12 point standard curve was prepared with desired top standard concentrations (table 2.1) and diluted 3 fold in the plate. A 96-well filter plate (Millipore, Watford, UK) was moistened with 50µl of assay buffer (PBS, 1% BSA, 0.025% tritonX100) and liquid was removed by low vacuum filtration. The biopsy supernatants were diluted 1:10 (determined through previous assays) and 50µl of samples and standards were added to the appropriate

wells. The desired volume of a cocktail of the bead/antibody conjugates was prepared to give a final concentration of 1×10^5 beads/ml for each bead set and 50µl of the beads were placed into each well. The plate was covered with foil and placed on a shaker for 5 min at room temperature, 1000rpm and was stored at 4°C overnight. Liquid was removed and the plate was washed twice with 100µl of wash buffer using low vacuum filtration. A cocktail of detection antibodies, each diluted to give the desired final working concentrations (table 2.1), was prepared and 50µl added to each well. The plate was covered with foil and placed on a shaker for 60 min at room temperature, 1000rpm. Two further washes were performed and 50µl/well streptavidin PE (BD Biosciences, Oxford, UK) added at a final concentration of 1µg/ml. The plate was covered in foil and shaken for 30 min at room temperature, at 1000rpm. The liquid was removed, the plate washed twice and 120µl of sheath fluid added to each well. The plate was shaken vigorously for 5 min prior to reading on a Bioplex machine (Bio-Rad, Hemel Hempstead, UK).

Table 2.2

Antibody	Top standard concentration	Standard manufacturer	Bead set no.	Capture manufacturer	Final detection concentration	Detection manufacturer
IL-2	20ng/ml	R&D systems	30	Pharmingen	500µg/ml	Pharmingen
IL-4	10ng/ml	R&D systems	11	Pharmingen	500µg/ml	Endogen
IL-5	5ng/ml	R&D systems	70	Glaxo SmithKline (in house)	500µg/ml	Pharmingen
IL-10	10ng/ml	R&D systems	10	Endogen	500µg/ml	Endogen
IL-13	10ng/ml	R&D systems	2	Pharmingen	500µg/ml	Pharmingen
TNFα	10ng/ml	R&D systems	4	R&D systems	500µg/ml	R&D systems
IFNγ	10ng/ml	R&D systems	1	Endogen	500µg/ml	Endogen
CCL22	10ng/ml	R&D systems	31	R&D systems	250ng/ml	R&D systems
CCL17	10ng/ml	R&D systems	32	R&D systems	250ng/ml	R&D systems

Table 2.2   Summary table displaying reagent information for Luminex bead assay

2.4 Chemotaxis assays

The CCRF-CEM T cell line (European Collection of Cell Cultures, Salisbury, UK) was used for the initial chemotaxis assays and was chosen based on its high percentage of CCR4 expression. CEM cell suspensions were centrifuged at 1500rpm for 10 min at room temperature and resuspended in chemotaxis media consisting of RPMI-1640 w/o L-glutamine w/o phenol red, (Life technologies, Paisley, UK), supplemented with 1% bovine serum albumin (BSA, Sigma, Dorset, UK). Cells were re-suspended at a concentration of 1×10^7 cells/ml and stained at room temperature in the dark for 45 min with the fluorescent dye, calcein (Molecular Probes, Netherlands) at $5\mu g/1 \times 10^7$ cells. They were then washed by centrifugation in chemotaxis media at 1500rpm for 10 min at room temperature to remove any unbound calcein. Three-fold dilutions of the chemoattractant CCL22 in chemotaxis media (R&D Systems, Abingdon, Oxon, UK) from 300nM to a blank containing no chemokine, for a standard curve, were placed in triplicate in the bottom wells of the chemotaxis plate (Neuro Probe, Gaithersburg, USA), with 30μl of each concentration being added to each well and 30μl of the biopsy supernatant samples being placed in the free wells. A membrane filter was securely fitted on top of the raised meniscus of the chemoattractant. A volume of 50μl of cells at a concentration of 1×10^7/ml, labelled with calcein was loaded onto the top of the filter. The chemotaxis plate was then incubated at 37°C for 90 min. The fluorescence emitted by cells that had migrated from above the filter, into the lower wells containing the chemoattractant below the filter, was measured using a Cytofluor II fluorescence plate reader (CytoFluor II, Perseptive Biosystems, Framingham, MA, USA). The fluorescence emitted by the migrated cells was detected with an excitation wavelength of 485nm and an emission wavelength of 530nm, with a gain of 70.

2.5 Depletion experiments

2.5.1 Immunomagnetic separation of CCR4+ cells

These experiments were conducted to assess the contribution of CCR4+ T cells to the Th2 cytokine responses of PBMC from atopic asthmatics (for more details see chapter 6). To that effect, PBMC's were isolated from whole blood of HDM allergic volunteers using the method outlined above (section 2.2). Cells were counted and then pelleted by centrifugation at 1500rpm for 10 minutes at 4°C. The cells were resuspended in 80μl of ice cold Magnetic Automated Cell Sorting (MACS) buffer (PBS, pH 7.2, supplemented with 0.5% BSA and 2mM EDTA) and 50μl per 1×10^7 cells of anti-CCR4 PE-conjugated antibody (R&D Systems, Abingdon, Oxon, UK) was added. Cells were kept in the dark, on ice for 20 min. They were pelleted by centrifugation at 1500rpm for 10 min at 4°C and resuspended in 80μl of MACS buffer per 1×10^7 cells. Following this 20μl of anti-PE microbeads (Miltenyi Biotec, Surrey, UK) were added per 1×10^7 cells and they were left on ice for 20 min. A final centrifugation at 1500rpm for 10 min followed and the cells were kept in 500μl of MACS buffer. Cells were placed on ice in the dark and separated by AutoMACS (Miltenyi Biotec, Surrey, UK) using the "possel_d" positive selection program, finally resulting in the separation of PBMC into a CCR4+ population and a PBMC CCR4- population. A whole PBMC population was retained from fresh PMBC prior to separation.

2.5.2 Cell culture

Populations of PBMC or PBMC depleted of CCR4+ cells by MACS were cultured to see whether removal of CCR4+ cells resulted in a change in cell responses to allergen. Cells were adjusted to a concentration of 2×10^6/ml in RPMI 1640 supplemented with 2mM 2-mercaptoethanol, 1% L-glutamine, 50IU/ml penicillin, 50μg/ml, 250μl of 100mM sodium pyruvate and 5% human AB serum (all from Life technologies, Paisley, UK). One ml of cells from either population was added into desired wells of a 24 well plate on day 0, and treated as: media alone, 1μg/ml of Phytohaemagglutinin (PHA) (Sigma, Dorset, UK), three different concentrations of HDM allergen (ALK,

Norway): 2,000, 5,000 and 10,000 SqU/ml, three different concentrations of recombinant Der p (Indoor Biotec, UK): 0.5, 1.0 and 3.0µg/ml and three dilutions of tetanus toxoid (Diftavax, Aventis Pasteur, Berkshire, UK): 1:7,000, 1:9,000 and 1:11,000. The plate was incubated at 37°C, 5% CO_2 for 7 days which had been previously shown to be optimal for the assessment of cytokine production by PBMC stimulated with allergen. Cells were harvested and centrifuged at 1500rpm for 10 min at room temperature, and supernatants were aliquoted into appropriate volumes, to minimise freeze-thawing and frozen at -20°C. An ELISA was used to determine the concentration of IL-5 (described in section 2.5.4) in these supernatants.

2.5.3   Proliferation assay

A volume of 200µl of PBMC or CCR4- cells, suspended in the 5% AB serum supplemented media (as above) were placed into Corning-Costar U bottomed 96 well plates (Fisher, Loughborough, UK) at a concentration of 2×10^6 cells/ml. Both cell populations were treated on day 0 as: media alone, 1µg/ml of Phytohaemagglutinin, three different concentrations of HDM allergen (ALK, Norway), 2,000, 5,000 and 10,000 SqU/ml, three different concentrations of recombinant Der p (Indoor Biotec, UK) 0.5, 1.0 and 3.0µg/ml and three dilutions of tetanus toxoid (Diftavax, Aventis Pasteur, Berkshire, UK), 1:7,000, 1:9,000 and 1:11,000. HDM was used as the specific stimulus and PHA was used as a control for responses of all T cells, tetanus toxoid was used as a further control because it stimulates both Th1 and Th2 cells. The plate was incubated at 37°C, 5% CO_2 for 7 days. On day 3 [3H] thymidine (0.5µCi/well; Amersham Pharmacia Biotech, Buckinghamshire, UK) was added for the last 18 h, cells were harvested onto filter paper (Camo Ltd.) on day 4 with a semiautomatic cell harvester (Skatron Instruments). [3H] thymidine incorporation was then measured by scintillation counting (2500 TR liquid scintillation counter; Packard-Canberra), and results were expressed as average counts per minute for triplicate wells ± standard error (SE).

2.5.4   Multiplex ELISA

The unfractionated PBMC and the PBMC depleted of CCR4+ve cells were tested for their ability to produce a range of Th1 and Th2 cytokines using the multiplex ELISA method as described above.

2.6   Immunohistochemistry using Glycol Methacrylate (GMA) embedded tissue

The tissue sections were treated to inhibit endogenous peroxide with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide. This solution was left on the sections for 30 mins at room temperature. Each slide was then subjected to three, 5 min washes with tris buffered saline (TBS) followed by the addition of blocking medium which was left on the sections for 30 mins at room temperature. The primary antibody at appropriate dilutions was then added. Monoclonal antibodies were left to bind overnight at room temperature while polyclonal antibodies were left to bind overnight at 4°C. Each slide was then subjected to three, 5 min washes with TBS before the addition of the biotinylated secondary antibodies at the recommended concentration which were left to bind for 2 hours at room temperature. A streptavidin-biotin complex, consisting of a 1:200 dilution of both streptavidin horseradishperoxidase and biotin (Dako, Ely, UK), with tris HCl was then prepared and left to form a complex within this 2 hour incubation. After incubation with the secondary antibodies the slides were given three 5 min washes with TBS after which the streptavidin-biotin complex was added. This was left to bind to the biotinylated secondary antibody for 2 hours at room temperature. A final three 5 min washes were performed and then a chromagen was added, in this case aminoethylcarbozole (AEC) liquid system, in kit form (Menarini Diagnostics, Berkshire, UK) AEC consists of reverse osmosis water (ROW), hydrogen peroxide and the chromagen solution, which was added to the sections and incubated for 20 mins at room temperature. Slides were given a rinse with TBS and then transferred into a rack and washed in running tap water for 5 mins. The rack was immersed in Mayer's haematoxylin for 90 seconds to counterstain the cell nuclei and left to "blue" under running tap water for 5 mins. Crystal Mount was pipetted onto the sections on the slides and then they were left at 80°C until dry. The

Crystal Mount forms an impervious barrier to solvent-based mounting media and tissue sections exhibit enhanced contrast and reproducibility, and maintain their original clarity and colour intensity when properly mounted in this substance. Using DPX (Sigma, Dorset, UK), a mixture of distyrene (a polystyrene), a plasticiser (tricresyl phosphate), and xylene, cover slips were fixed onto the stained tissue sections.

Chapter 3

Results

Expression of CCR4 and CRTH2 and co-expression
of memory cell/activation markers on circulating
T cells and expression of CCR4 in bronchial biopsies
in atopic asthma

3.1   Introduction

The two subsets of helper (CD4) T cells, Th1 and Th2, have been strongly implicated in the regulation of allergic responses. In asthma, the subset initiating the allergen-specific response and generating cytokines involved in the production of IgE and the recruitment, activation and survival of infiltrating cells is the Th2 subset (Robinson *et al.*, 1992). Different expression patterns of homing, memory and activation markers exist between Th1 and Th2 subsets (Andrew *et al.*, 2001, Bonecchi *et al.*, 1998) and chemokines and their receptors are required for the trafficking of many cell types. For example the migration of DC which express CCR7 to the lymph nodes is mediated by CCL19 and CCL21. Basophils and eosinophils expressing CCR3 are directed to inflammatory sites by CCL11, CCL24 and CCL13. The expression of these markers on T cells can define those cells with tropism for particular tissues and microenvironments and can help identify T cell subsets with distinct functional properties (Butcher *et al.*, 1999). For example CLA+ CD4+ T cells are thought to define memory cells that traffic to the skin (Berg *et al.*, 1991), while α4β7+ lymphocytes traffic to intestinal sites (Butcher, 1991).

Evidence for T cell activation was obtained in the early 1990s shortly after bronchoscopy was first used in asthma research, with the demonstration of increased frequency of T cells expressing activation markers such as the interleukin 2 receptor (IL-2R, CD25), and the MHC class II antigen, HLA-DR, both in BAL (Wilson *et al.*, 1992) and bronchial biopsies (Robinson *et al.*, 1992). Increased numbers of T cells in the airways expressing activation markers are seen following *in vivo* allergen challenge (Bentley *et al.*, 1993, Robinson *et al.*, 1993).

In the last few years, there have been attempts to define T cell subsets based on the expression of certain chemokine receptors, two of which are CRTH2 and CCR4. Both have been proposed as playing important roles in the airway inflammatory response in asthma. The recently identified CRTH2, a receptor for PGD_2, is now known to be selectively expressed on Th2 cells (Nagata *et al.*, 1999). As the chemokine receptor for CCL17 and CCL22, CCR4 has been implicated in pulmonary allergic

inflammation, with the majority of T cells migrating into the lungs of allergen challenged asthmatics expressing CCR4 (Panina-Bordignon *et al.*, 2001) as shown by analysis of bronchial biopsies taken during the late phase response.

Other subsets of CD4 lymphocytes can be defined based on their expression of adhesion molecules such as CD62L, CLA and CD49d (Butcher and Picker, 1996) and other markers of memory cells with diverse functions (Lara-Marquez *et al.*, 2001).

The aim of the experiments shown in this chapter was to determine if there was an increase in the frequency of peripheral blood CD4 cells expressing CCR4 and/or CRTH2 in patients with mild asthma; with the underlying notion that a primary or secondary consequence of the asthmatic state might be a general increase in the frequency of cells which could migrate into the airways in response to their respective ligands, CCL17 and CCL22 (for CCR4) and PGD_2 (for CRTH2).

A second aim was to determine if the CCR4+ or CRTH2+ cells in blood of asthmatics bear markers of antigen-experienced memory cells (Table 3.1) which have encountered antigen, possibly in the airways, thus linking the process of activation to the presence of CCR4 on T cells that respond to allergen in the asthmatic airways. The cell surface-bound markers of memory, homing and activation were chosen based on a literature search, with a view to selecting a panel of such markers to study that could be involved in the allergic asthmatic response. The table below provides a brief description of the roles each marker plays and each marker is assessed in detail, based on its potential involvement in the allergic asthmatic response, in the discussion section.

A third aim was to enumerate CCR4+ T cells in bronchial mucosal tissue. Immunohistochemistry was applied to bronchial biopsies from asthmatic and healthy non-atopic control subjects in order to see whether there are any differences in the expression of CCR4 within the inflamed airways.

A fourth and final aim was to see whether CD4+ T cells from asthmatic subjects displayed and increased propensity to express CCR4 in comparison to the healthy non-atopic subjects, in response to stimulation with the polyclonal T cell mitogen, PHA.

Table 3.1 Summary of the functions of the memory/activation markers studied.

T cell marker	Primary ligand/s	Function
ICOS	ICOS-L	Co-stimulatory molecule. Proposed to be involved in Th2 responses. Promotes T cell proliferation and production of Th2 cytokines. Displays regulatory properties.
CLA	E-selectin	Homing marker involved in directing T cells to inflammed skin
CD62L	PSGL-1, PNAd	Homing of naïve cells to peripheral lymph nodes. Mediates the tethering and rolling of cells to HEV. Shed upon cell activation
CD44	Hyaluronin, multiple components of extracellular matrix	Activation enhanced memory marker. Involved in adhesion of lymphocytes to endothelium, and binds multiple components of extracellular matrix
CD45RA	CD45 is a tyrosine phosphatase that regulates signalling through antigen and cytokine receptors	Marker of naïve cells. Respond to Ag presented on DC only
CD45RO		Marker of activated/memory cells. Enables cells to have a faster and more effective recall response to Ag presented by APC
CD134	CD134-L	Promotes proliferation, cytokine production and survival of Ag specific CD4 T cells
CCR7	Chemokines CCL19, CCL21	Guides T cells into lymphoid tissue for Ag presentation by DC
CD49d	V-CAM	Mediates tethering and rolling of cells to HEV.

Table 3.1   Memory and activation markers studied and their functions in the immune response

Methods

Flow cytometric analysis of PBMC

Surface staining of PBMC was performed as described in detail in chapter 2. Non-specific antibody binding was blocked using a solution of Fcγ fragments. Antibodies used for membrane staining were added, cells were washed by centrifugation prior to adding an appropriate volume of FACS buffer to give the desired cell concentration for use in the FACSCalibur flow cytometer. Following this, the extent of CD4+ T cell expression of CCR4 and CRTH2 with the markers listed in table 3.1 was investigated by flow cytometry. The extent of staining was expressed as a percentage of cells expressing the surface marker. PBMC from a separate group of 9 healthy control and 9 mild asthmatic subjects were cultured for 48h either with or without PHA.

Detection of CCR4 in nasal polyp tissue from subjects with allergic rhinitis, and bronchial biopsy tissue sections embedded in Glycol Methacrylate (GMA) from 6 healthy control and 6 atopic mild asthmatic subjects was performed using immunohistochemistry (described in detail in chapter 2). Briefly, 2μm thick sections were cut and placed onto microscopy slides. Primary antibodies (CD3, EGFR, and isotype controls) were left to bind overnight at room temperature while the polyclonal antibody (CCR4) was left to bind overnight at 4°C. Following this overnight incubation, the biotinylated secondary antibodies were then left to bind for 2 hours. A streptavidin-biotin complex was then prepared and left to form a complex and then placed on the sections. The sections were incubated with chromagen and then briefly immersed in haemotoxylin to counterstain the nuclei.

PBMC from 9 healthy non-atopic and 9 asthmatic subjects were isolated and adjusted to $1x10^6$/ml and then one population of cells from each subject were stimulated with 1μg/ml of the T cell mitogen PHA, and another population remained unstimulated. The PBMC in tissue culture plates were left at 37°C, 5% CO_2 for 48 hr. Subsequently, the cells were stained with fluorescently labelled antibodies to CD4 and

CCR4 / CRTH2 and the percentage of CD4+ cells which had induced CCR4 / CRTH2 expression following stimulation with PHA was calculated by subtracting the percentage of non-stimulated cells expressing CCR4 / CRTH2 from the percentage of stimulated cells expressing CCR4 / CRTH2.

3.2   Results

3.2.1   The frequency of CCR4+/CD4+ and CRTH2+/CD4+ cells in the blood
            of healthy controls and mild asthmatics

No differences between the asthmatic and healthy control groups were seen for both
CCR4 and CRTH2 expression on CD4+ T cells. However, there was a wider range in
the number of positive cells in the asthmatics for both receptors (Fig 3.1, 3.2). The
percentages of CRTH2 expressing cells ranged from between 0.3 and 1.1% in the
healthy controls and 0.4 to 2.2% for the asthmatics. The frequency of CCR4+ cells
ranged from 11.7 to 25.2% in the healthy controls and from 9.1 to 29.2% for the
asthmatics. There was also no difference in the median fluorescence intensity of either
CCR4 or CRTH2 on CD4 cells from either group.

3.2.2   The expression of activation and memory markers on CCR4+ cells in
            the blood of healthy controls and mild asthmatics

The differences in the expression of the studied markers between healthy and asthmatic
patients were negligible, as illustrated in figure 3.21, and not statistically significant.
A wider spread of expression was seen for the asthmatics in a number of the markers
investigated. However, the median and mean values remained close to that seen in the
healthy controls. Due to the expression being limited to only a very small population
of cells, the memory/activation markers discussed were not studied for CRTH2.

3.2.2.1   ICOS
The extent of expression of ICOS in circulating T cells in both the asthmatics and
control subjects was very low. Fewer than 0.8% of lymphocytes were ICOS+ CD4+ T
cells (Fig 3.3) and fewer than 1.4% of CCR4+ CD4 cells (Fig 3.4) expressed ICOS in
both the healthy and asthmatic subjects, with no significant differences between the
two groups of subjects.

3.2.2.2 CLA

On CD4+ T cells CLA was expressed at variable levels on the cell surface of CD4+ T cells (Fig 3.5), with the percentage of CD4+ CLA+ T cells ranging from 5.3 to 11.7% in controls and from 1.8 to 11.6% in the asthmatics. When inspecting the expression of CLA on CCR4+ CD4 T cells (Fig 3.6), only the CCR4^{high} expressing cells were positive for CLA in both subject groups.

3.2.2.3 CD62L

The percentage of CD4+ CD62L- cells ranged from 3.7 to 13.1% in the healthy controls and 1.8 to 9.1% in the asthmatics (Fig 3.7), which was not significantly different. The greatest proportion of cells was located within the CD4+ CD62L+ quadrant for the large majority of all subjects. For the CCR4+ CD4+ T cells (Fig 3.8) that were CD62L- the percentages ranged from 5.6 to 14.1% for the healthy controls and 1.4 to 13.3% for the asthmatics, but most of the CCR4+ cells were also CD62L+. The largest percentage of cells was found within the CD62L+ CCR4- population. Individual data points were equally distributed for both the healthy and asthmatic subjects.

3.2.2.4 CD44

CD44 was expressed on all CD4+ cells (Fig 3.9), with no differences between the asthmatic and control subjects. Between 33.9 and 62.6% of lymphocytes were CD4+ CD44+, with negligible differences in the distribution of individual subject percentages for both healthy and asthmatic subjects alike. All CCR4+ CD4 T cells were CD44^{high} with fewer than 1% of CD4+ being CCR4 and CD44-. The distribution of percentages in the asthmatics was wider than that in the healthy controls, ranging from 11.9 to 66.0% and 25.6 to 50.3% respectively (Fig 3.10).

3.2.2.5 CD45RA

The expression of CD45RA on CD4+ cells shown as a percentage of total lymphocytes was generally below 25% (Fig 3.11) and not significantly different when comparing asthmatics and healthy controls. A greater percentage of CD45RA+ cells could be

seen within the CD4- population. On inspection of the expression of CD45RA in relation to CCR4, fewer than 8% of cells are CCR4+ CD45RA+ of the total CD4 cells in all subjects and the vast majority of CCR4-expressing CD4 cells were negative for CD45RA (Fig 3.12).

3.2.2.6 CD45RO

In contrast to CD45RA, many more CD4+ cells expressed CD45RO (Fig 3.13) and the asthmatics displayed a wider range of percentage expression; however, there were no differences between the asthmatics and healthy subjects. Looking at the percentage of CCR4+ CD4+ cells (Fig 3.14), the majority of cells are CD45RO+. Moreover, the small percentage of cells that were CD45RO- CCR4+ only expressed low levels of CCR4 and an even smaller proportion of the CCR4^{high} cells were CD45RO-. Looking at the individual data points on the scatter plot the percentages for all healthy controls were within a 17.4% range (20.5 to 37.9%) and the asthmatic patients displayed a 51.7% (7.2% to 58.9%).

3.2.2.7 CD134

The expression of CD134 was minimal or absent. Fewer than 1% of total lymphocytes were CD4+ CD134+ cells in healthy patients and under 2% in the asthmatics (Fig 3.15). Fewer than 1.4% of CCR4+ CD4 cells (Fig 3.16) expressed CD134 in both healthy and asthmatic subjects, with the spread being more or less identical in both subject groups.

3.2.2.8 CCR7

Very little difference was seen between healthy controls and asthmatics with regards to the percentage of CCR7 expression on CD4 T cells from the total lymphocyte gate (Fig 3.17). As a percentage of CD4+ T cells, the large majority expressed CCR7 and a substantial percentage were also CCR7+ CCR4- but most of the CCR4+ cells were CCR7+ (Fig 3.18). In patients with a greater proportion of CCR7+ CCR4+ cells, a population of CCR4^{high} cells appeared to be CCR7-, although this was more striking in some patients than others, and was not associated with asthma. The asthmatics showed

a wider distribution in percentage expression than the healthy controls. The expression of CCR4 and CCR7 on CD4 cells ranged from 17.4 to 34.2% in the healthy controls and 8.6 to 49.0% in the asthmatics.

3.2.2.9 CD49d

The expression of CD49d was variable on CD4+ T cells to an extent that makes it hard to distinguish positive cells from negative cells (Fig 3.19). The majority of CD4+ cells of the total lymphocyte population expressing CD49d, were within a tight range of between 28 and 45%, with some subjects from each patient group displaying percentages outside this range. On inspection of the cells expressing CCR4 and CD49d as a percentage of total CD4 cells, the majority of CCR4+ cells expressed a consistent level of CD49d (Fig 3.20). There was a noticeable decrease in the level of expression of CD49d on CCR4^{high} cells in every subject.

3.2.3 Changes in the frequency of expression of CCR4 and CRTH2 on CD4+ circulating T cells with or without stimulation with PHA of healthy controls and mild asthmatics.

The expression of CCR4 on CD4+ T cells in the asthmatics increased by 11.38% after stimulation with PHA and the expression on CD4+ T cells in the healthy control subjects increased by 9%. For the expression of CRTH2, the healthy control patients had a higher percentage increase of 1.75% for CRTH2 expression upon stimulation with PHA where as the mild asthmatics showed that PHA stimulation had resulted in a 0.5% increase in CRTH2 expression (Fig 3.23).

3.2.4   Expression of CCR4 in nasal polyp of one representative allergic rhinitic subject and one representative mild asthmatic bronchial biopsy tissue sections

The negative control slides (TBS, mouse anti-IgG and goat anti-IgG) were found to be free from any non-specific staining both in the nasal polyp sections and bronchial biopsy sections (Fig 3.24a and Figs 3.24d-e). The epidermal growth factor receptor was found to be highly expressed as the positive control (Fig 3.24b) and a number of CCR4+ve cells stained were evident in the nasal polyp section (Fig 3.24c). Figure 3.24f demonstrates the presence of CD3+ T cells. CCR4+ve cells were absent in the bronchial biopsy section (Fig 3.24g).

3.3 Discussion

The main focus of this chapter was on the expression of CCR4 and CRTH2 on circulating T cells in asthma. Based on previous publications that these two surface molecules are preferentially expressed on Th2-type cells, it was hypothesised that higher expression of CCR4 and CRTH2 on CD4+ peripheral blood T cells would be seen in the asthmatics in comparison to the healthy control subjects. This hypothesis was supported by the view that there is expansion of Th2 T cells in atopic asthma, as shown in the airways (Robinson *et al.*, 1993), although a similar expansion of Th2 cells has not been documented in blood. However, the current study of mild asthmatics showed no differences in either the expression of CCR4 or CRTH2 and it did not show any differences in co-expression of a number of markers of T cell activation.

Previous research has shown that CCR4 is preferentially expressed on CD4+ cells, particularly of the Th2 phenotype, and CRTH2 is selectively expressed on Th2 cells, both observations being based on the production of Th2 cytokines by the expressing cells. Many studies investigating the expression of CRTH2 have focused on its role in allergic responses, investigating any differences between atopic and non-atopic patients, and information on the role of CRTH2 in asthma is limited. Cosmi and co-workers demonstrated an increased percentage of CRTH2+CD4+ T cells in the peripheral blood of allergic patients (Cosmi *et al.*, 2000) and Nagata *et al* further found that the proliferative response of PBMC to allergen was greatly reduced upon removal of CRTH2+ cells (Nagata *et al.*, 1999). It has been shown that the majority of CRTH2 expressing cells are enriched in the expression of CCR4, but not the reverse (Cosmi *et al.*, 2000). The existing research implies that that lymphocytes may be migrating into the airways by additional mechanisms or possibly even mechanisms that are independent of CCR4, based on the expression of CCR4 on all T cell subsets to a certain extent (Kim *et al.*, 2001), and that the activities of CCR4 and its ligands are not selective for pulmonary inflammation. For instance, it is strongly implicated in homing to the skin (Soler *et al.*, 2003), participates in the formation of T cell-DC clusters in lymph nodes as determined by the detection of both CCL22 (Katou *et al.*,

2001) and CCR4+ T cells, and targeted deletion of CCR4 has failed to ablate Th2 cell trafficking into the lung *in vivo* (Chvatchko *et al.*, 2000). This was confirmed by Conroy and colleagues who found only negligible changes in the numbers of CCR4+ CD4 cells in BAL of allergen challenged guinea pigs treated with a blocking antibody to CCR4 (Conroy *et al.*, 2003).

The lack of differences between the healthy and allergic asthmatic patients investigated in the present study, with regard to expression of both CCR4 and CRTH2, suggests that these receptors possibly do not play an important role in mild asthma. However, the fact that the two patient groups displayed no differences could suggest that these cells are important in pulmonary inflammation and are raised in asthma in their activated effector stage but persist mainly in the airways. Indeed a recent study by Panina-Bordignon (Panina-Bordignon *et al.*, 2001) showed a marked influx of CCR4+ T cells into the airways following allergen challenge of asthmatic volunteers. This was associated with a marked increase in CCL17 and CCL22 within the epithelium. IgE cross-linking by allergen on the surface of mast cells in the submucosa could also be a source of PGD_2, be it continually or following exposure to allergen. Based on this, the explanation for the presence of the CRTH2+ cells in the blood could be that these cells are only a basal population of circulating Th2 cells in both healthy and asthmatic patients. CCR4+ cells found in the airways of asthmatics may be in excess of those in the circulation which could be playing more of a role in other inflammatory and immune responses. Another point to consider is whether the expressing cells in the asthmatics somehow have a lower threshold for activation than those of the healthy patients.

In order to compare these flow cytometry results on the expression on PBMC, GMA embedded sections of nasal polyp from allergic rhinitis sufferers and sections of bronchial biopsies from mild asthmatic and healthy control subjects were used to investigate the expression of CCR4 in the airways. Several publications have described the presence of CCR4+ve cells in bronchial biopsy specimens. For instance Conroy and colleagues located CCR4+ve cells in the BAL of allergen challenged

guinea pigs (Conroy *et al.*, 2003), Pannina-Bordignon took endobronchial biopsies from 6 asthmatic patients, 24h following endobronchial allergen challenge and found that virtually all T cells expressed CCR4 and there was also some epithelial staining (Panina-Bordignon *et al.*, 2001). The stained cells were present, albeit in lower numbers in the unchallenged cryostat sections (Panina-Bordignon *et al.*, 2001). Additionally, Nouri-Aria and co-workers identified CCR4 and protein mRNA at baseline levels in the lungs of some atopics, the presence of which increased upon allergen challenge (Nouri-Aria *et al.*, 2002). CCR4 expression has also been found before and after allergen challenge by Kallinich and colleagues who discovered that challenge with allergen increased the numbers of CCR4+ve cells in the bronchial mucosa of mild asthmatic individuals (Kallinich *et al.*, 2005). In the current study, the first nasal polyp tested, stained positive for CCR4. Following this, polyps from a further 9 patients with allergic rhinitis were investigated. None of these 9 patients stained positive for CCR4. In order to clarify that there were no problems with antibodies and reagents, sections from the same patient for which positive staining for CCR4 was observed, were stained again and positive staining was again obtained. Having discovered that the staining procedure and CCR4 antibody were effective, bronchial biopsies from 6 healthy control and 6 mild asthmatic subjects were stained for CD3 and CCR4. The sections were cut consecutively in order to be able to localise the CCR4 to CD3+ve cells. The only staining found in all 6 patients was for CD3. The single patient for whom CCR4 was present in nasal polyp was tested in parallel with the biopsies serving as a positive control for CCR4 staining. Possible explanations for the lack of CCR4+ve cells in the biopsies could be, firstly, the use of Glycol Methacrylate (GMA) embedded tissue. Although this processing method has the advantage of providing thin sections that can be stained sequentially, thus allowing co-localisation of surface markers, it is plausible that tissues embedded in GMA may not have been the best to use, for if CCR4 is expressed sparsely on T cells in bronchial biopsies then the chances of detecting it on very thin tissue sections are reduced. It is also possible that antibodies for CCR4 do not work well in certain tissues processed into GMA. However, in preliminary experiments, anti-CCR4 antibodies from three different manufacturers were tested on nasal polyp tissue sections. Two monoclonal

antibodies; one from R and D Systems and one from Santa Cruz and one polyclonal antibody from Calbiochem. The antibody chosen for the staining of tissue sections in the present piece of work was the polyclonal anti-CCR4 antibody from Calbiochem. Both the R and D Systems monoclonal antibody and the Calbiochem antibody, appeared to mark the same cells, as seen from staining of sequential sections, yet the clarity of staining with the Calbiochem antibody was much greater than the R and D Systems antibody. Alternatively, the use of the Santa Cruz antibody resulted in extensive non-specific staining, judged by the fact that even at a very high dilution factor, the entire tissue section stained positive for CCR4. This questions the work of Panina-Bordignon and colleagues who carried out all their CCR4 staining using the Santa Cruz antibody (Panina-Bordignon *et al.*, 2001). They found extensive CCR4 staining of T cells, even in tissues derived from sham-challenged subjects and also some positive staining of epithelial cells for CCR4 (Panina-Bordignon *et al.*, 2001).

The ability of the immune system to mount an effective and rapid immune response is dependent upon the generation and persistence of memory cells. Cells that have acquired a memory phenotype have less stringent requirements for activation than do naïve cells, such as responsiveness to lower concentrations of antigen, (Rogers *et al.*, 2000), reduced reliance on co-stimulation than naïve cells and the ability to be activated by antigen presented to them on APC other than DC (Croft *et al.*, 1994). These cells retain their memory phenotype and remain in the circulating pool of lymphocytes. Upon detection of the initial inciting antigen, they begin to re-express certain markers of memory, activation and homing. There is no clear cut definition of either a memory or an activated cell and the presence of any one surface marker merely identifies a cell as a memory cell candidate. A combination of markers distinguishes a memory or activated cell from one which is naïve and has not yet encountered antigen. Figure 3.22 provides information on the expression of the markers, the cell types and the cell activation state.

A group of markers of memory, activation and homing markers was investigated on CD4+ T cells and additionally assessed for their co-expression with CCR4. Greater

percentages of co-expressing cells were also predicted to be found in the asthmatics. There were no obvious patterns found in the expression of the markers studied on CD4+ T cells. However, some observations were made for the co-expression of the markers with CCR4. The same trends were observed for all subjects of both patient categories.

Raised percentages of cells staining positive for CD45RO, CD44^{high}, CD49d, ICOS, and CD134 were expected to be found in the asthmatics in comparison to the healthy controls but this was not the case. The expression of certain markers is complicated further by increased intensity, switches in isoforms and also the loss of markers rather than their presence. For instance, the expression of CCR7 cannot distinguish between naïve cells and central memory cells; however, the absence of CCR7 identifies naïve cells that have been activated by antigen and effector memory cells. Hence, increased percentages of CCR7- cells were expected in the asthmatics. A lack of CD62L expression identifies effector memory cells in their activated state and activated derivatives of naïve cells. CD62L+ cells are naïve or non-activated effector or central memory cells; therefore, raised percentages of CD62L- cells were expected in the asthmatics. CD44 is a widely expressed cell adhesion molecule present on virtually all vertebrate cells (Ponta et al., 2003), stimulation induces the transition of CD44 from its low to high-affinity binding state. Thus CD44^{high} cells were expected to be found in higher percentages in the asthmatics. Finally, evidence suggests that activation causes a conformational change in CD49d from a low to a high-affinity state and cell percentages staining as CD49d^{high} were expected to be raised in asthmatics in comparison to healthy controls.

The expression of CLA showed that the large majority of CLA+ cells were CCR4^{high}. CLA is expressed on a glycoform of P-selectin glycoprotein-1 (PSGL-1) and is a ligand for CD62E (E-selectin), preferentially expressed on skin endothelium (Berg et al., 1991) and is involved in the trafficking of T cells to inflamed skin. The pattern of expression of CLA on CCR4+ CD4 T cells is identical to those generated by Andrew et al who have also shown by flow cytometric analysis that the large majority of CLA+

cells are CCR4^{high} (Andrew *et al.*, 2001). Published work has demonstrated that CLA has no role in asthma. Ainslie and colleagues determined the percentage of lymphocytes from healthy patients that adhered to bronchial endothelium in sections taken from patients undergoing lung resection. When sections of bronchus were treated with a blocking antibody to CD62E there was no reduction in cell adherence to the endothelium (Ainslie *et al.*, 2002). However, asthmatics were not included in these studies. Picker and colleagues looked at the expression of homing markers on memory/effector T cells in both the skin and the airways between asthmatics and healthy patients and found that the cells in the airways were CLA negative and this was the case in both patient groups (Picker *et al.*, 1994). The CLA positive cells in both healthy and asthmatic patients may be circulating memory cells which would be recruited into the skin following any form of skin damage. The existing data implies that CLA+ cells would be raised in patients with some form of contact allergy. It is therefore possible, that the apparent isolated population of 4 asthmatics with a percentage expression of CLA above the median, are more allergic and develop contact hypersensitivity in response to one or more agents.

CCR7 and CD49d show a similar pattern to that of CLA. In line with the literature, there were no differences between the healthy and the asthmatic patients in terms of CCR7 expression. This is supported by Kallinich and co-workers who found no differences in the expression of CCR7 on peripheral blood T cells between healthy and asthmatic patients. (Kallinich *et al.*, 2005). CCR7 plays a role in helping T cells and DC migrate into the T cell zones of lymphoid organs in order to effectively initiate an immune response. Following activation of the cell, CCR7 is lost from the cell surface. The findings generated in the current work imply that the CCR7- CCR4^{high} cells are activated effector T cells. Comparable, is the trend seen for CD49d, as this is also in relation to the CCR4^{high} cells however, the results were opposite to what was expected. The CCR4^{high} cells express CD49d at a lower fluorescence intensity. CD49d is the α4β1 member of the VLA integrin subfamily and it is one of a number of endothelial/lymphocyte markers involved in endothelial adhesion and activation events, culminating in the migration of cells from high endothelial venules, into

tissues. The participation of CD49d and its function in asthma has been extensively researched and has resulted in the development of a CD49d antagonist which is currently involved in clinical trials. Xu *et al* found that CD49d was implicated in lymphocyte homing to bronchus-associated lymphoid tissue (BALT) with a model using lymph node lymphocytes that were removed from NOD mice, stained for tracking purposes and treated with a blocking antibody to CD49d, before being transferred to a NOD recipient. They showed that blocking the interaction between CD49d and VCAM-1 significantly blocked lymphocyte homing to BALT but not mesenteric or peripheral lymph nodes and peyers patches (Xu *et al.*, 2003). Abraham and colleagues investigated the role of CD49d in experimental allergic asthma in sheep. They discovered that treating sheep with a tight binding inhibitor of CD49d after allergen challenge, protected them against the early (EAR) and late airway response (LAR) and the subsequent airway hyperresponsiveness (AHR) and even if the inhibitor was administered after the sheep were allowed to generate the early airway response, both the LAR and the AHR were still abolished (Abraham *et al.*, 2000). The results generated on the expression of CD49d in the current study, where the CCR4^{high} cells are expressing CD49d with lower fluorescence intensity, perhaps also of a lower affinity/different variant could suggest that the CCR4^{high} cells may have less stringent requirements for firm adhesion to endothelial cells. Additionally, the expression of high levels of this marker on highly activated CCR4+ cells could have been replaced by a different surface marker that aids endothelial adhesion for these, possibly chronically activated cells. Another possible reason for the trend could be that an altered conformation of CD49d on the CCR4^{high} cells may have led to a weaker binding affinity of the antibody used for flow cytometry. Inconsistent with the findings of CCR7 are the results for CD62L. Based on the pattern seen for CCR7 and a loss of expression on CCR4^{high} cells, a similar pattern would have been expected for CD62L. CD62L (L-selectin) is known to mediate tethering and rolling of lymphocytes to HEV of peripheral lymph nodes and endothelium at sites of inflammation and upon activation of the cells, the surface bound CD62L is shed. Its role in tethering lymphocytes to the bronchial endothelium has been investigated. Ainslie *et al* found that blocking of CD62L resulted in a 50% inhibition of lymphocyte/endothelial

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binding (Ainslie *et al.*, 2002). Findings by Yamashita demonstrate a significant difference in the expression of CD62L on peripheral blood CD4+ memory T cells in atopic asthmatics in comparison to healthy control patients (Yamashita and Tanno, 1998). Also its role in cell homing to the inflamed airways in a murine model of asthma has been demonstrated. Keramidaris and co-workers demonstrated, using sensitised/challenged mice, that the migration of activated lymphocytes from CD62L deficient mice into inflamed lungs was markedly reduced in comparison to WT (wild-type) mice (Keramidaris *et al.*, 2001). The results generated in the present piece of work did not show any differences between the asthmatics and the healthy patients, and a noticeable lack of CD62L, on the CCR4^{high} cells, matching that seen for CCR7 was not found. Although the findings of Yamashita confirmed differences in the peripheral blood, their results might have not been genuine and a reason for this could have been their very small patient numbers. Therefore, a possible reason for the absence of a population matching the one seen for CCR7, could be that the expression of CD62L is one which is rapidly modulated and hence may be upregulated on the activated cell surface on re-entering the peripheral circulation. However, increased patient numbers might have affected the results and had more patients been tested then a more conclusive and significant finding may have ensued.

The expression of CD45RO confirms the findings of previous research demonstrating that the majority of CCR4 expressing cells are of the memory phenotype (Imai *et al.*, 1999, Yamamoto *et al.*, 2000). T cells expressing the CD45RA isoform are newly formed T cells that have not yet encountered antigen. Upon activation the RA isoform is modified to RO (Akbar *et al.*, 1991). In addition to CD45RO, CD44 is another reliable marker of memory. CD44^{high} cells are defined as activated/memory cells. CD44 is a widely expressed cell adhesion molecule present on virtually all vertebrate cells (Ponta *et al.*, 2003). It serves many functions such as participation in the adhesion of lymphocytes to endothelium (DeGrendele *et al.*, 1996), hematopoeisis and tumor metastasis (Lesley *et al.*, 1993). Multiple components of the extracellular matrix, namely hyaluronan (HA), collagen, laminin and fibronectin bind to CD44 on CD4 T cells, implicating CD44 in numerous processes involved in health and disease

(Ponta *et al.*, 2003). The primary ligand for CD44 is HA. CD44 exists in multiple isoforms, the significance of which is still poorly understood. CD44^{high} cells are defined as activated/memory cells. In the present study, as expected, a greater percentage of lymphocytes were CD4+ CD45RO+ than CD4+ CD45RA+. Also as expected, almost all CCR4+ cells were CD45RO+ and negative for CD45RA. The majority of T cells in the airways of asthmatics have been found to be of the memory phenotype. For example in a murine model of pulmonary inflammation using sensitised mice that had been subjected to 2 challenges with OVA, Garlisi and co-workers saw that T cells comprised the large majority of lymphocytes in the BAL, 75% of which were of the CD4 subset. Additionally they found that almost all of the CD4+ cells were positive for CD44 and were CD45RB^{low}, characteristic of memory cells (Garlisi *et al.*, 1995). As for human asthma, in the lung interstitium greater than 90% of T cells were shown to express a memory phenotype (Marathias *et al.*, 1991). In the present study, slightly higher percentages of CD4+ CD45RO+ rather than RA+ cells, were found in both asthmatic and healthy controls but the distribution of percentage expression was wider for both CD4+ CD45RO+ and CCR4+ CD45RO+ in the asthmatics. Increased patient numbers and an investigation of those with more severe asthma would have helped to further characterise any differences between the two patient groups. In accordance with the results from the present study, which demonstrates minimal differences in the mean percentage of RO and RA expressing CD4+ cells, are those from Saltini and colleagues who identified equal proportions of CD45RA and CD45RO in the peripheral blood of healthy controls (Saltini *et al.*, 1990). A possible reason for this could be the result of some form of homeostatic mechanism. Even though raised numbers of RO+ T cells have been identified in asthmatic airways, any differences in the peripheral blood of both healthy and asthmatic patients may be small or negligible and could be due to adjustments in the immune system, in order to regulate and maintain a consistent ratio of circulating naïve to memory cells so as not to compromise the host defence against infection.

Although characterised as an additional marker of memory, the literature surrounding CD44 is less well defined. In the present study, all CD4+ T cells expressed CD44, as

was expected, and these all stained at the same fluorescence intensity. There was a small CD4- population which stained positive for CD44 but this was of lower fluorescence intensity. Based on the fact that there is a substantial proportion of CD45RO+ T cells within the CD4+ population, it can be concluded that these cells with constitutive expression of CD44 are not all memory/activated cells. Upon activation, cells change from expressing low levels of CD44 to high levels and it is accepted, unlike other transient changes, that this is maintained on memory cells. To further confuse matters, in addition to cells expressing different levels of CD44, they also express different isoforms. On attempting to detect memory cells, the combination of CD44 with CD45RO is widely used and in this way memory cells are more reliably distinguished. Hadley and colleagues used the BrdU staining method in order to follow T cell fate and discovered that upon stimulation with superantigen, many BrdU+ T cells persisted for several months in a CD44^{low} state and they suggested that the cells had either divided without up-regulation of CD44 or downregulated CD44 rapidly after activation (Hadley *et al.*, 1997). However, this is unlikely to be the case in the present study due to what appears to be the absence of any CD44^{high} cells at all. It is possible that activation of T cells resulted in a conformational change in what would then be the CD44^{high} molecule, to which the antibody used for flow cytometry has a weaker binding affinity and this has resulted in fluorescence intensity akin to that of the constitutively expressing cells. Based on the literature, there is evidence that CD8+ cells have an increased tendency to retain the CD44^{high} phenotype (Hadley *et al.*, 1997), but on investigation of the data generated in this study, this is not the case. All CD8+ cells also stain with the same fluorescence intensity. However a population of CD44^{high} cells were identified and this was localised to monocytes but throughout this FACS study, monocytes stained positive for almost all the markers to varying degrees and it is likely that this staining was non-specific. It has been demonstrated that CD44 expression increases following engagement of CD134 which is consistent with a memory phenotype and a distinct lack of CD134 in this study makes this topic worthy of further investigation.

The expression of ICOS and CD134 was absent in all patients examined. Both ICOS and CD134 were expected to be found at higher percentages in the asthmatics. ICOS is a co-stimulatory molecule related to CD28 and CTLA-4 and it receives a CD28-independent costimulatory signal through a novel member of the B7 (CD80/CD86) family, called B7h (Swallow *et al.*, 1999), expressed on antigen presenting cells such as DC, macrophages and B cells. ICOS expression is found in vitro 24-48 hours after activation of naïve T cells (Coyle *et al.*, 2000) and displays a sustained expression on T cells of the memory/effector phenotype (Sporici and Perin, 2001). CD134 also serves as a co-stimulatory molecule, belonging to the TNF receptor family and is primarily expressed on T cells 12-48 hours after stimulation (Gramaglia *et al.*, 2000). It has been shown to be rapidly expressed within 4 hours, on re-activated memory/effector cells (Gramaglia *et al.*, 1998). It receives a signal through engagement by CD134L, which is also up-regulated on the activation (Flynn *et al.*, 1998) of DC (Ohshima *et al.*, 1997) and B cells (Stuber *et al.*, 1995). It can modulate CD4+ T cell function by way of enhanced proliferation (Gramaglia *et al.*, 2000) and cytokine production (Salek-Ardakani *et al.*, 2003). Evidence exists that implicates both these costimulatory molecules in favouring the generation of Th2 responses. Salek-Ardakani and colleagues investigated the importance of CD134 in lung inflammation by blocking the CD134-CD134L interactions in a murine model of asthma. They found that the lungs from anti-CD134 treated mice presented with normal bronchial epithelium but a much reduced cellular infiltrate in comparison to a control antibody and in addition, the concentration of Th2 cytokines was markedly reduced in BAL (Salek-Ardakani *et al.*, 2003). The capacity of CD134 in combination with CD28 in promoting IL-4 production also implicates it in Th2 cell responses (Ohshima *et al.*, 1998). Similar pieces of research exist for ICOS, for instance, using a murine model of asthma, Beier and colleagues demonstrated that the transfer of OVA stimulated, ICOS enriched T cells from OVA-specific TCR transgenic mice to normal BALB/c recipients resulted in increased airway cellular infiltrates, consisting mainly of eosinophils and IgE positive B cells (Beier *et al.*, 2004). Additionally, they noted that isolated ICOS expressing T cells were enriched in the Th2 cytokines IL-4, IL-5, IL-13 and IL-10. Some attention has focused on the potential of ICOS to be multifunctional. The varying levels of

ICOS expression has been investigated, which resulted in isolated populations of cells with characteristic cytokine production. The above findings were in support of those generated in a previous study by Lohning and colleagues. They demonstrated this by the use of flow cytometry on T cells in secondary lymphoid organs of mice. They showed that ICOS^{low} cells were found to be loosely associated with the early cytokines interleukin IL-2, IL-3, IL-6, and interferon IFNγ. The majority of cells were ICOS^{medium} and were tightly associated with production of IL-4, IL-5, and IL-13, and these cells exhibited potent inflammatory effects in vivo. However, ICOS^{high} T cells were linked with IL-10 production (Lohning *et al.*, 2003). Research has also found that both ICOS and CD134 make contributions to the differentiation of T cells into their respective subsets in general and not solely Th2 cells, however, it seems that the general consensus is that they favour a Th2-response outcome. A possible explanation for the apparent lack of both ICOS and CD134 in the present piece of work could be as a result of the staining procedure. Periods of incubation on ice and centrifugation may have led to conformational changes preventing antibody binding or down-regulation/internalisation of the markers.

In order to check whether the group of asthmatic subjects had a greater propensity to express CCR4 or CRTH2 relative to the healthy control group of subjects, isolated PBMC were cultured for 48h either with or without the polyclonal mitogen, PHA. When the expression of CCR4 and CRTH2 were assessed, there were no differences in the percentage increases in expression for either CCR4 or CRTH2 with or without treatment with PHA between healthy control subjects and mild asthmatic subjects. There is evidence which shows that CCR4 is one of a number of chemokine receptors which are upregulated on stimulation with polyclonal mitogens (D'Ambrosio *et al.*, 1998) as opposed to the likes of CCR1, CCR2, CCR3, CCR5 and CXCR3 (Sallusto *et al.*, 1997, Loetscher *et al.*, 1998, Loetscher *et al.*, 1996, Qin *et al.*, 1998) which are downregulated. However, no differences in the upregulation of CCR4 or CRTH2 were found between the healthy and the asthmatic subjects in the current study.

Figure 3.1

Figure 3.1 Expression of CRTH2 on CD4+ T cells. Top panels show isotype control for CRTH2 and representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.2

Figure 3.2 Expression of CCR4 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.3
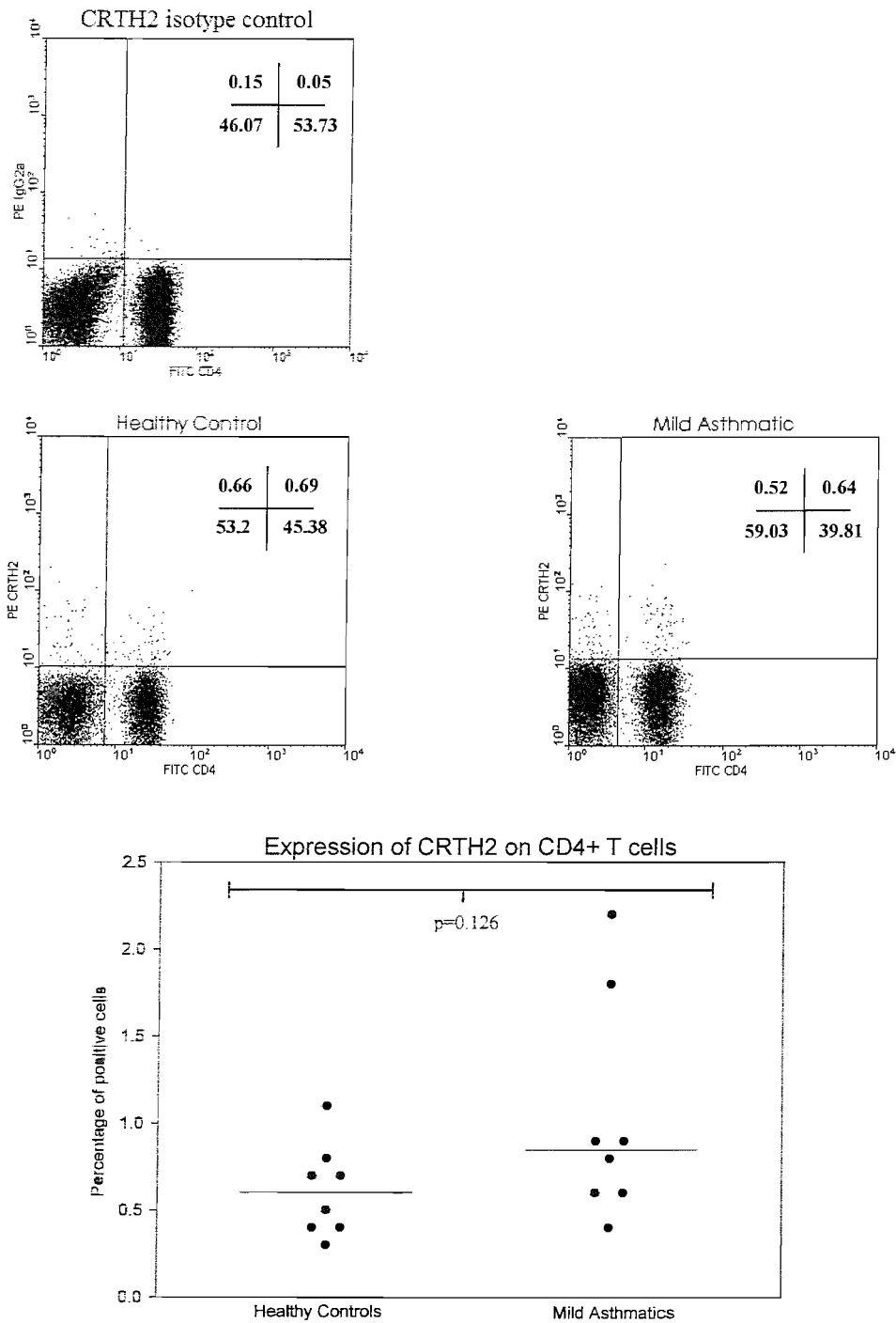

Figure 3.3 Expression of ICOS on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.4

Figure 3.4 Coexpression of CCR4 and ICOS on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
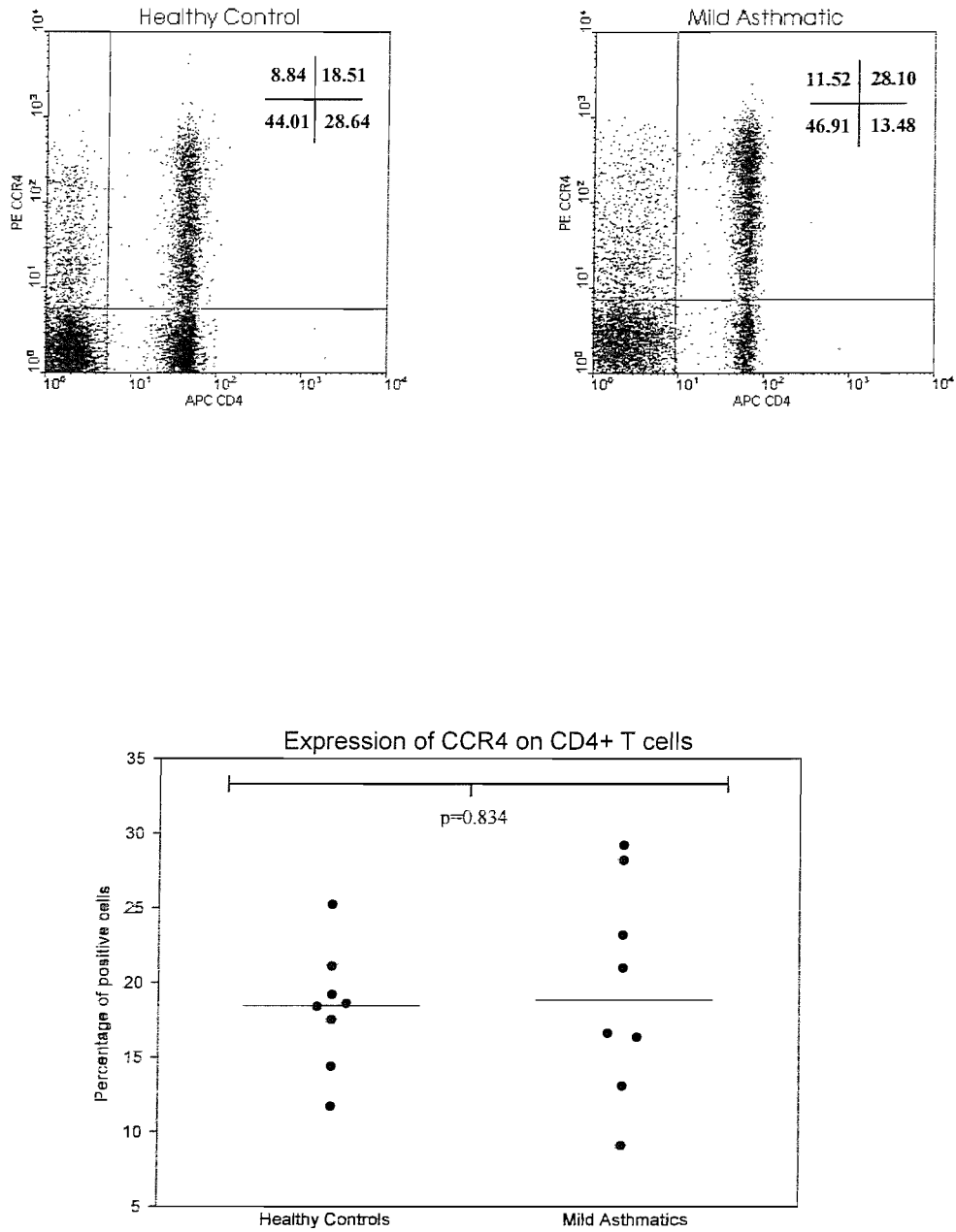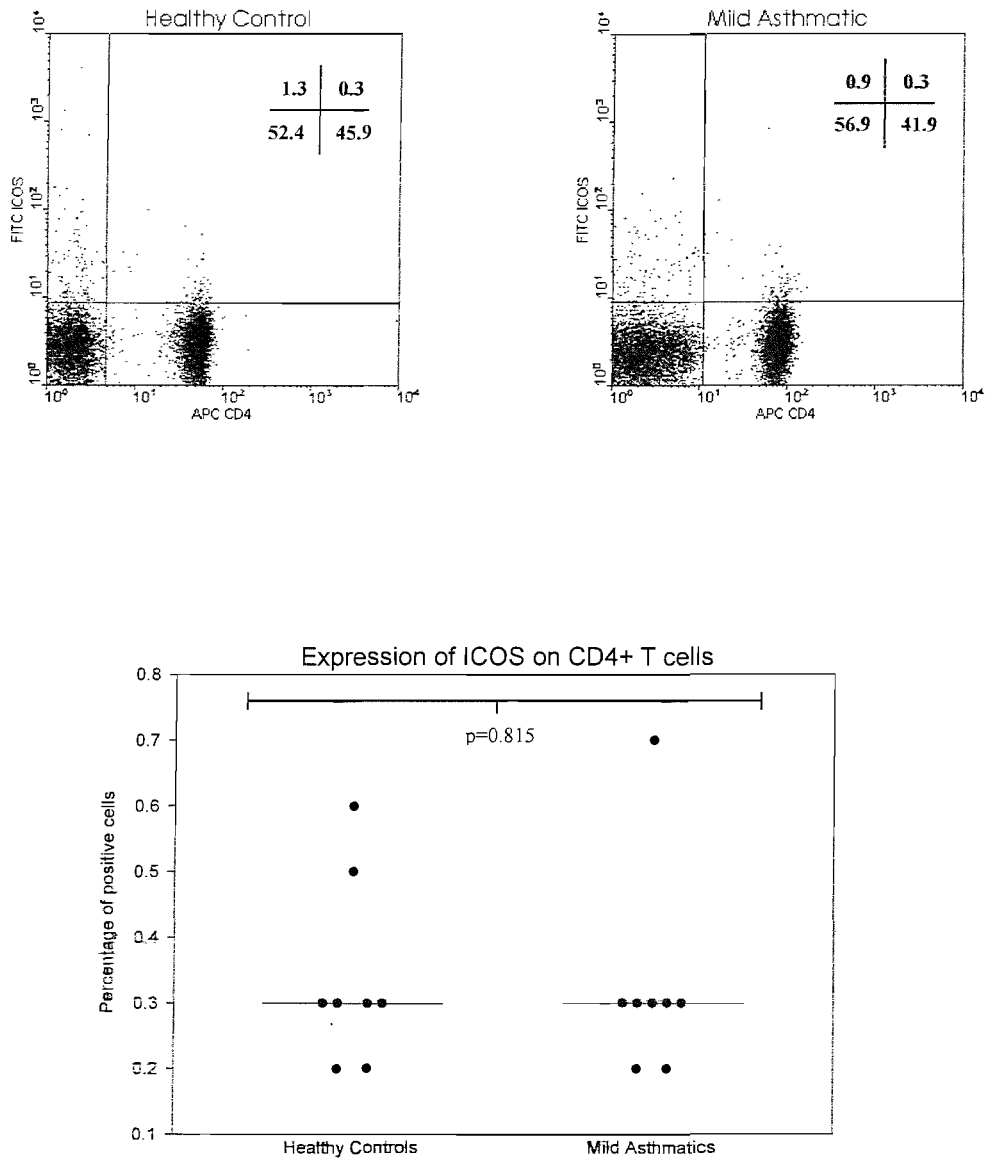
Figure 3.5

Figure 3.5 Expression of CLA on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.6

Figure 3.6 Coexpression of CCR4 and CLA on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

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Figure 3.7

Figure 3.7 Percentages of CD62L-CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
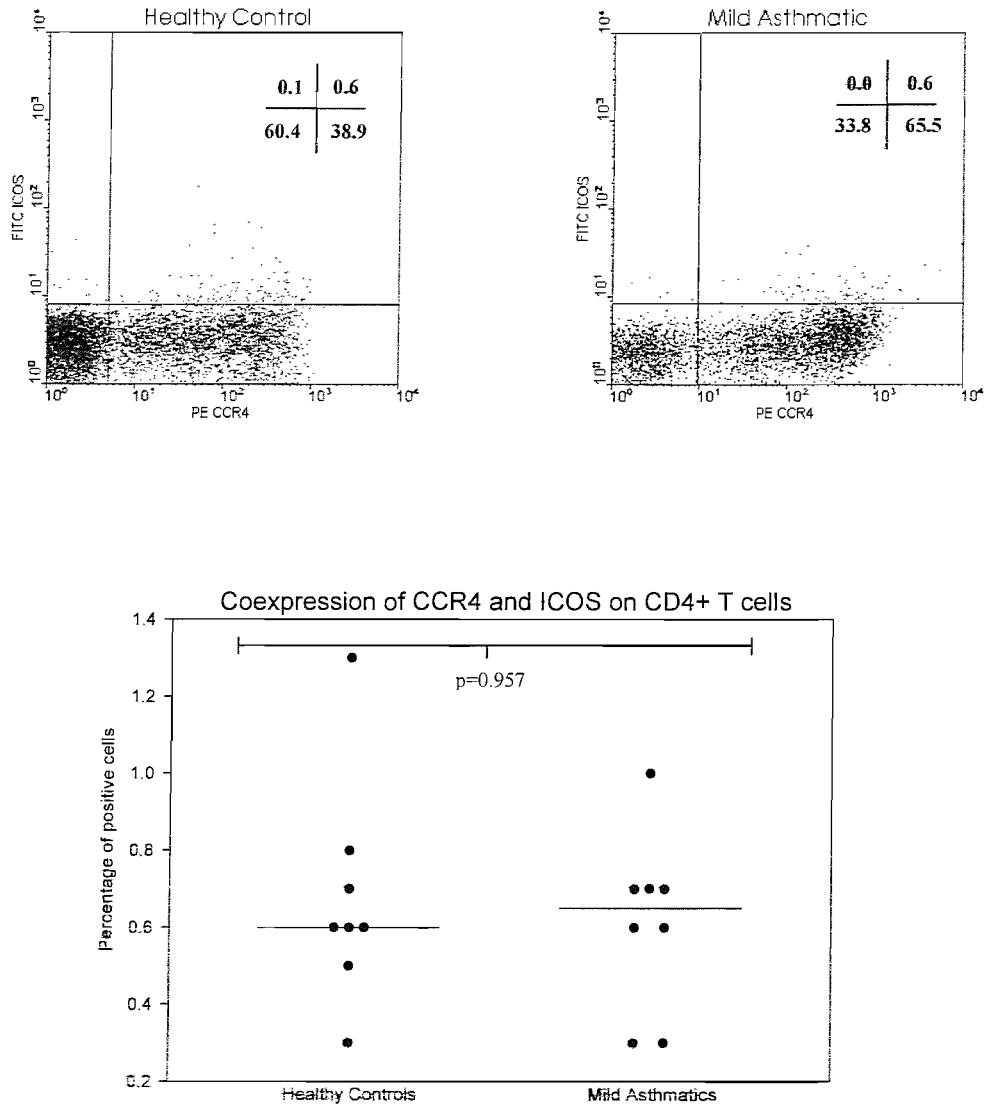
Figure 3.8

Figure 3.8 Percentages of CCR4+CD62L-CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.9

Figure 3.9 Expression of CD44 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
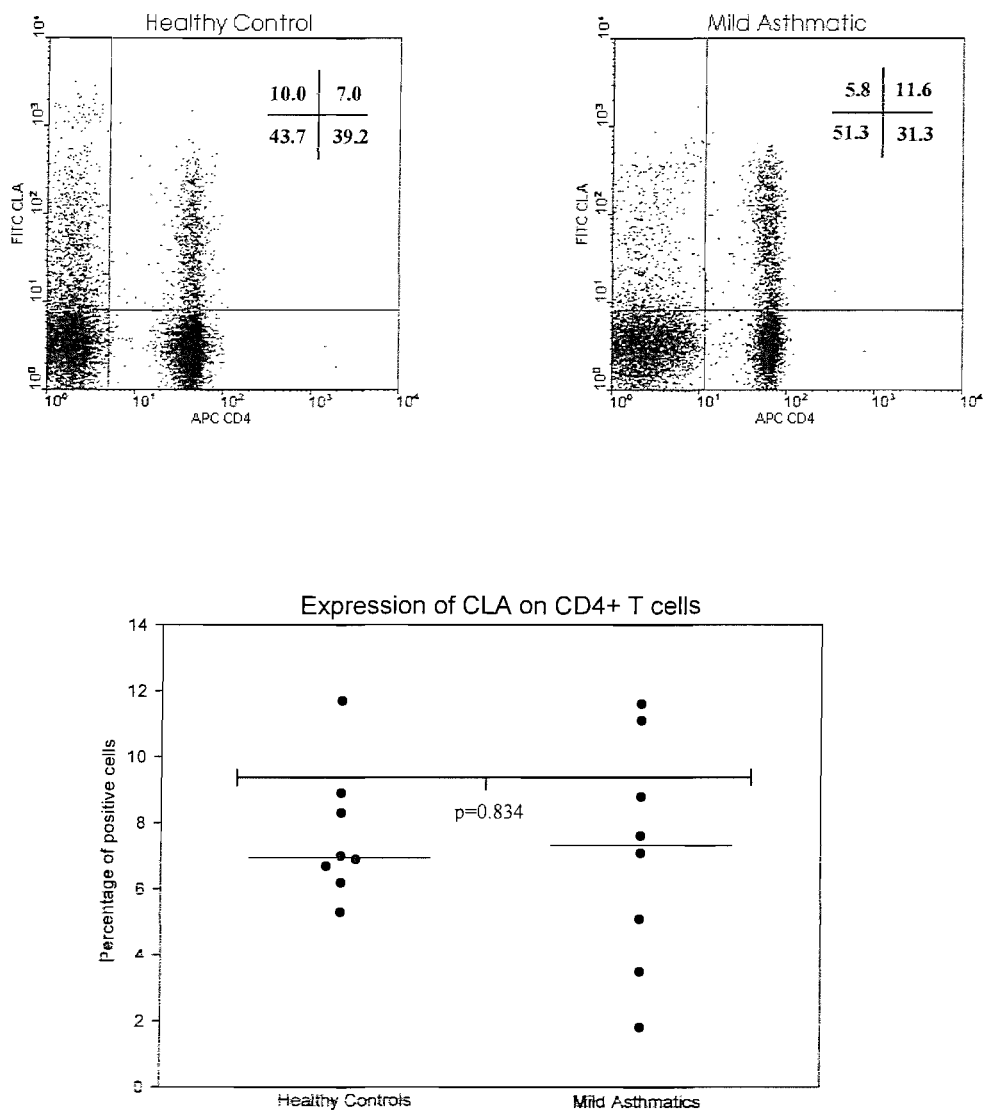
Figure 3.10

Figure 3.10 Coexpression of CCR4 and CD44 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
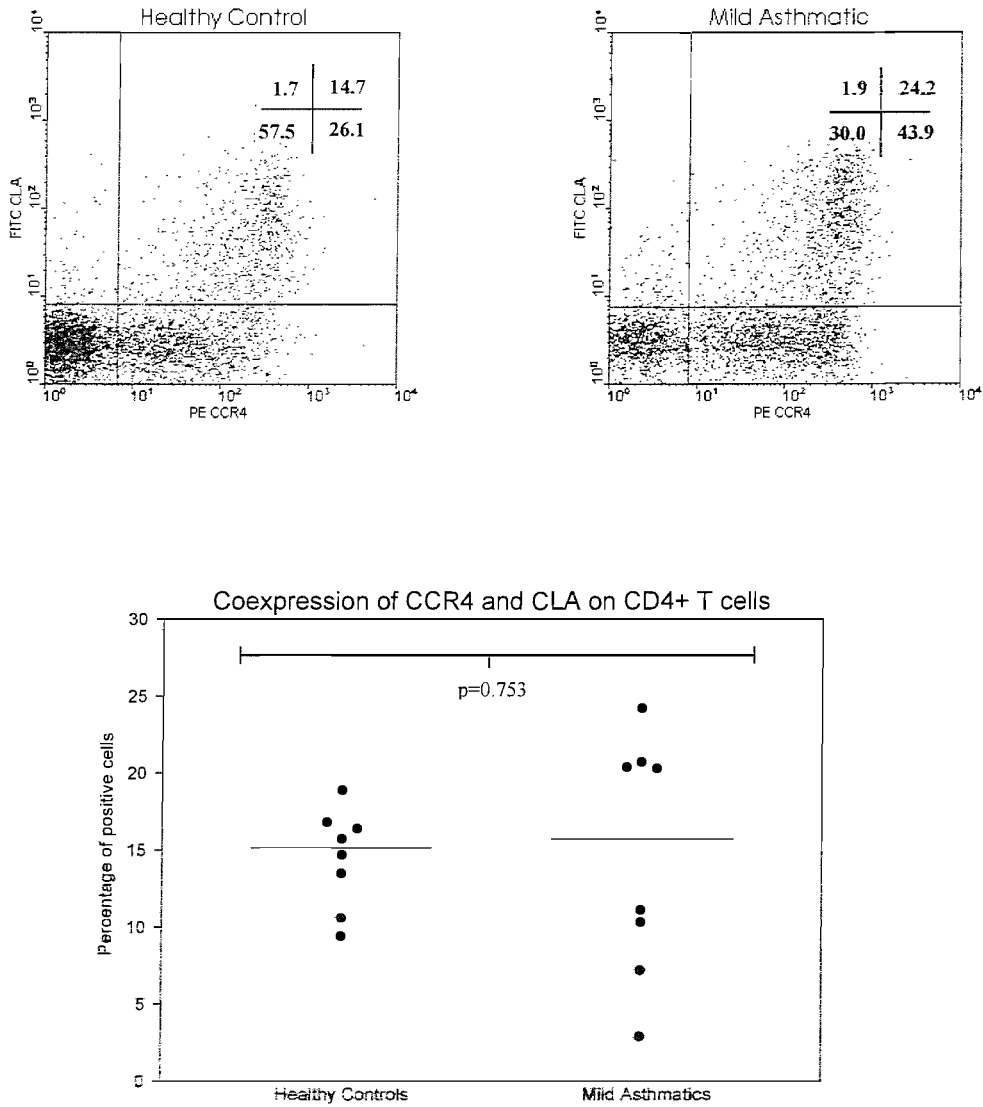
Figure 3.11

Expression of CD45RA on CD4+ T cells

Figure 3.11 Expression of CD45RA on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
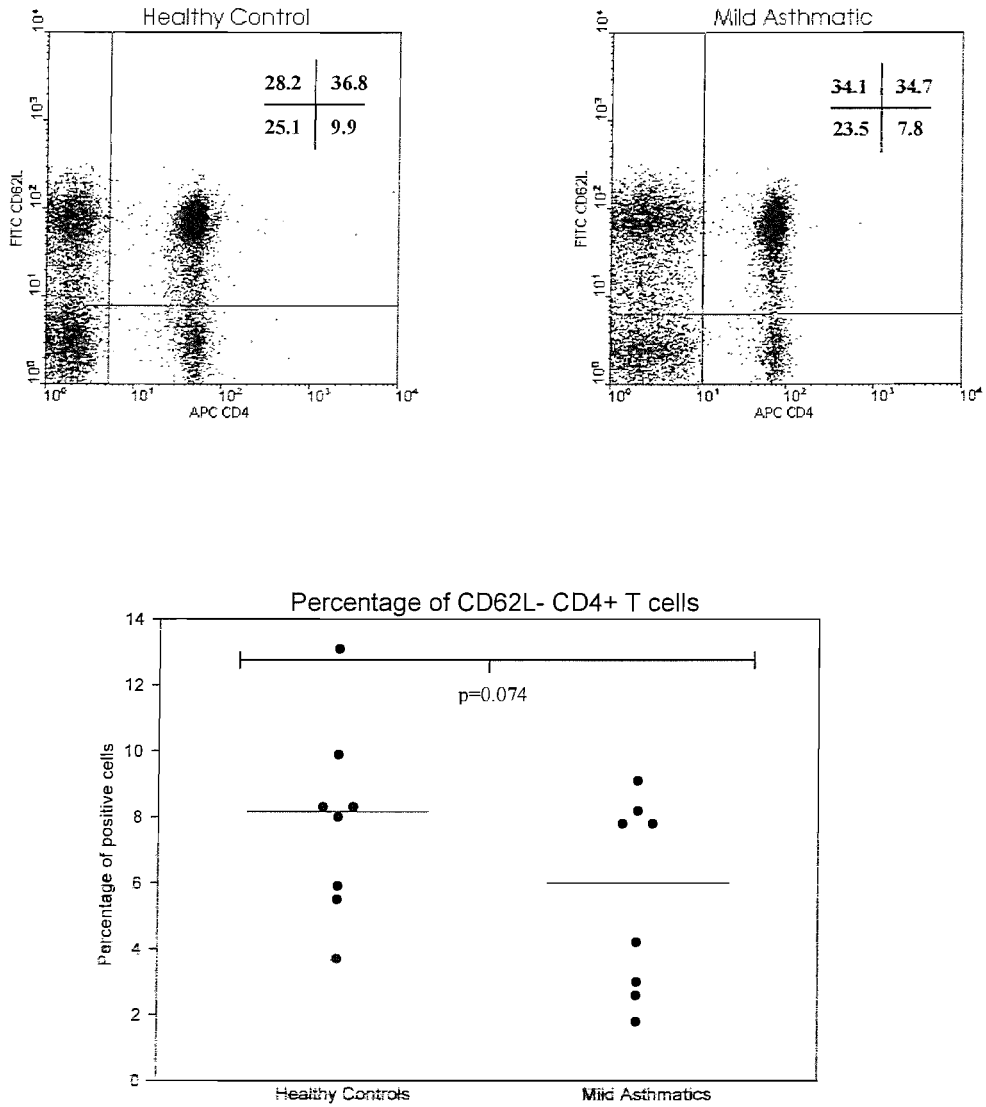
Figure 3.12

Healthy Control

31.5	2.5
30.4	35.6

Mild Asthmatic

14.9	1.9
19.8	63.4

Coexpression of CCR4 and CD45RA on CD4+ T cells

p=0.292

Figure 3.12 Coexpression of CCR4 and CD45RA on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
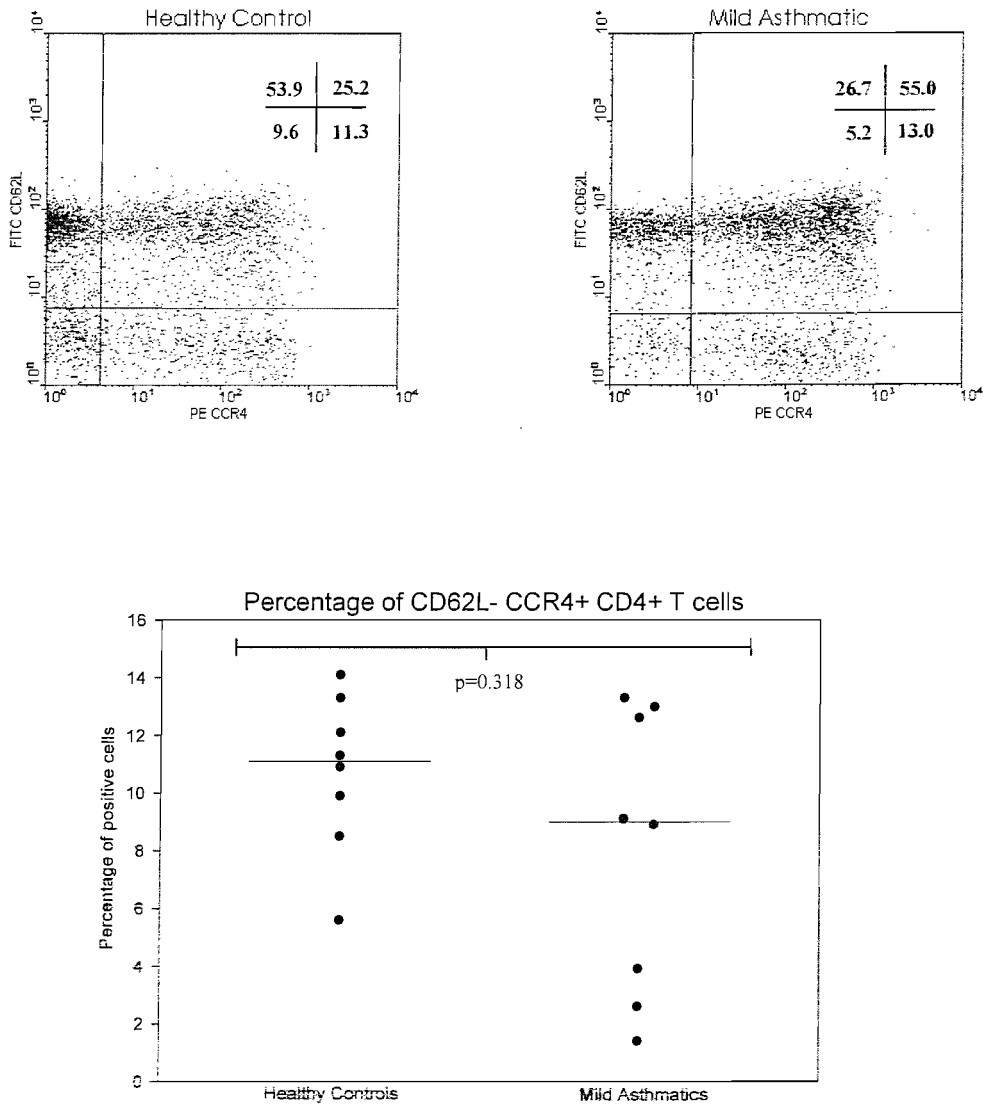
Figure 3.13

Figure 3.13 Expression of CD45RO on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

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Figure 3.14

Healthy Control

18.7	30.1
45.8	5.4

Mild Asthmatic

11.0	58.9
22.0	8.1

Coexpression of CCR4 and CD45RO on CD4+ T cells

p=0.6

Figure 3.14 Coexpression of CCR4 and CD45RO on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
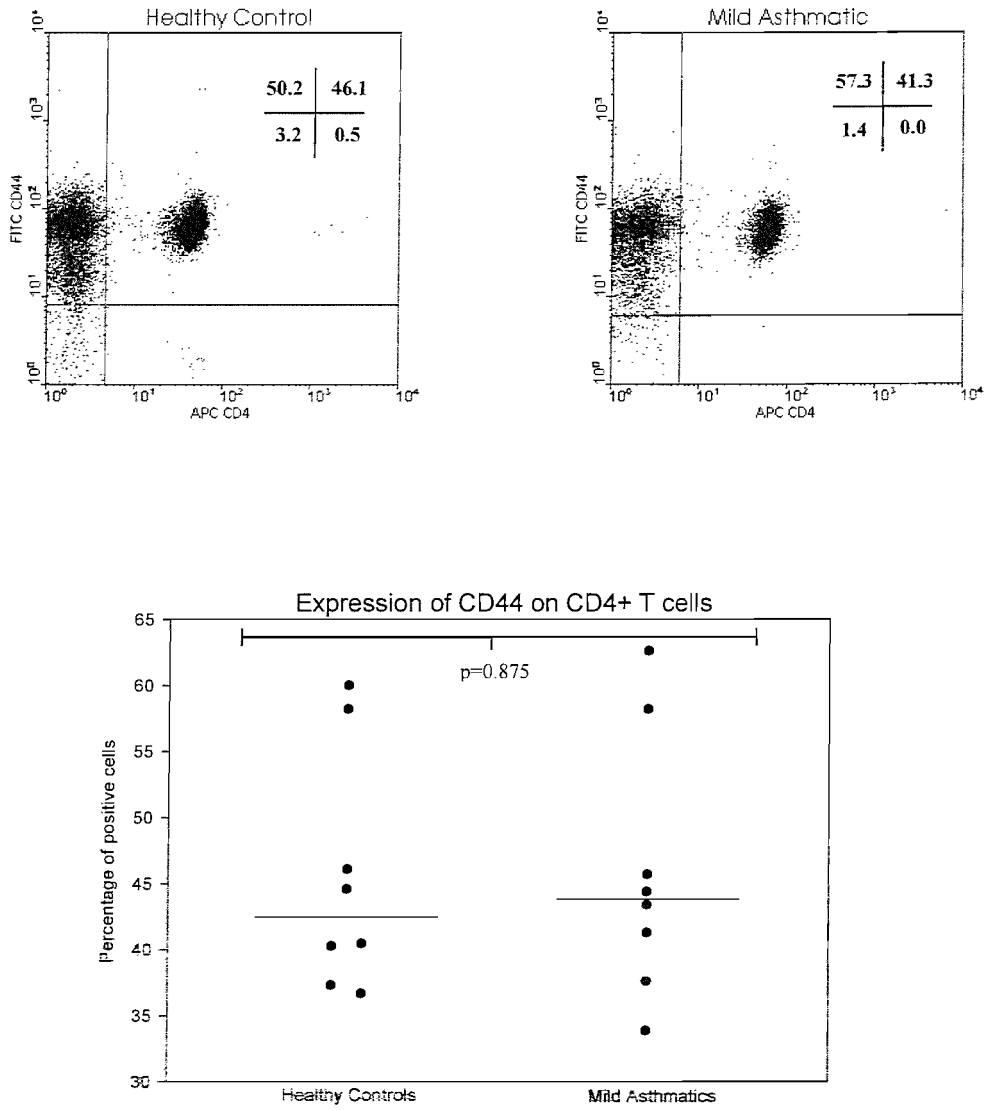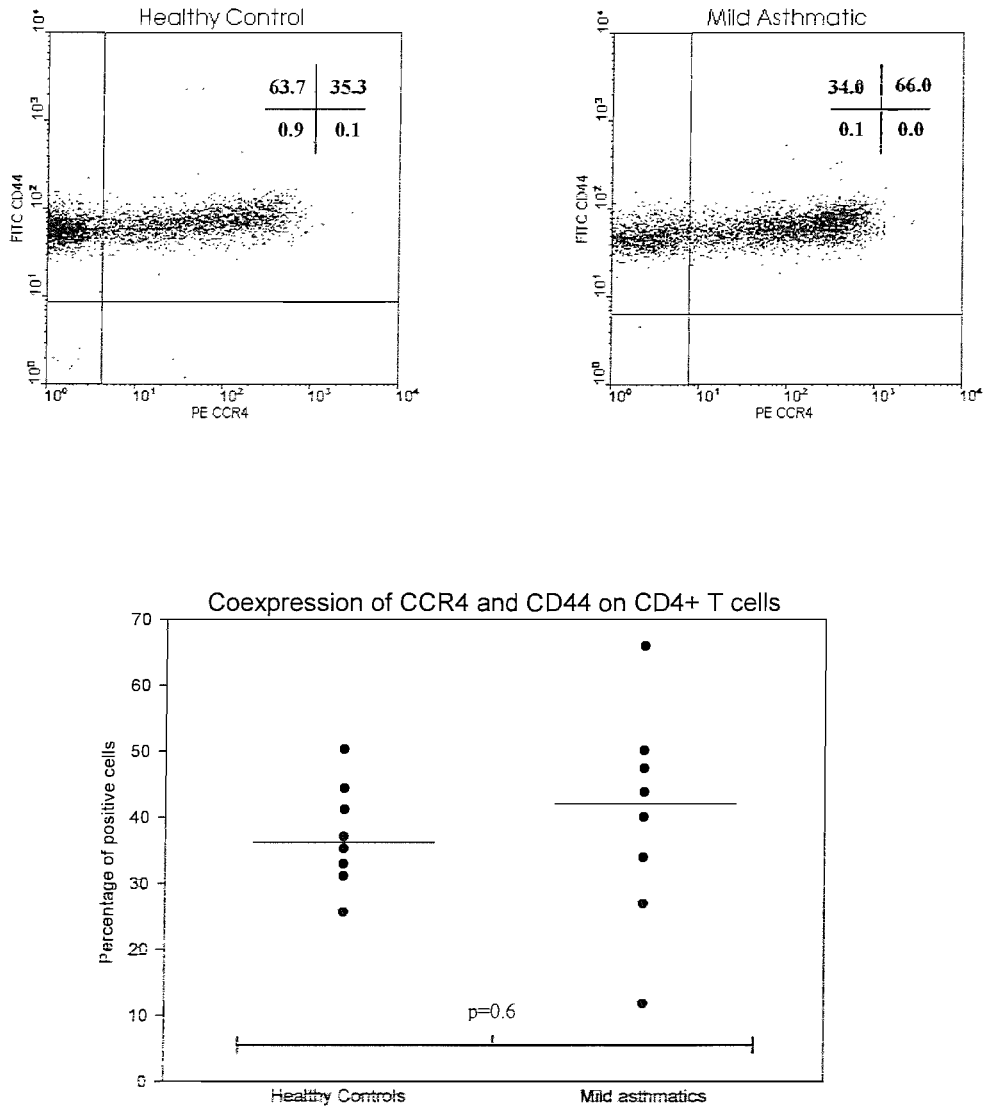
Figure 3.15

Healthy Control

0.2	0.3
52.3	47.1

FITC CD134

APC CD4

Mild Asthmatic

0.1	0.1
56.1	43.7

FITC CD134

APC CD4

Expression of CD134 on CD4+ T cells

p=0.523

Percentage of positive cells
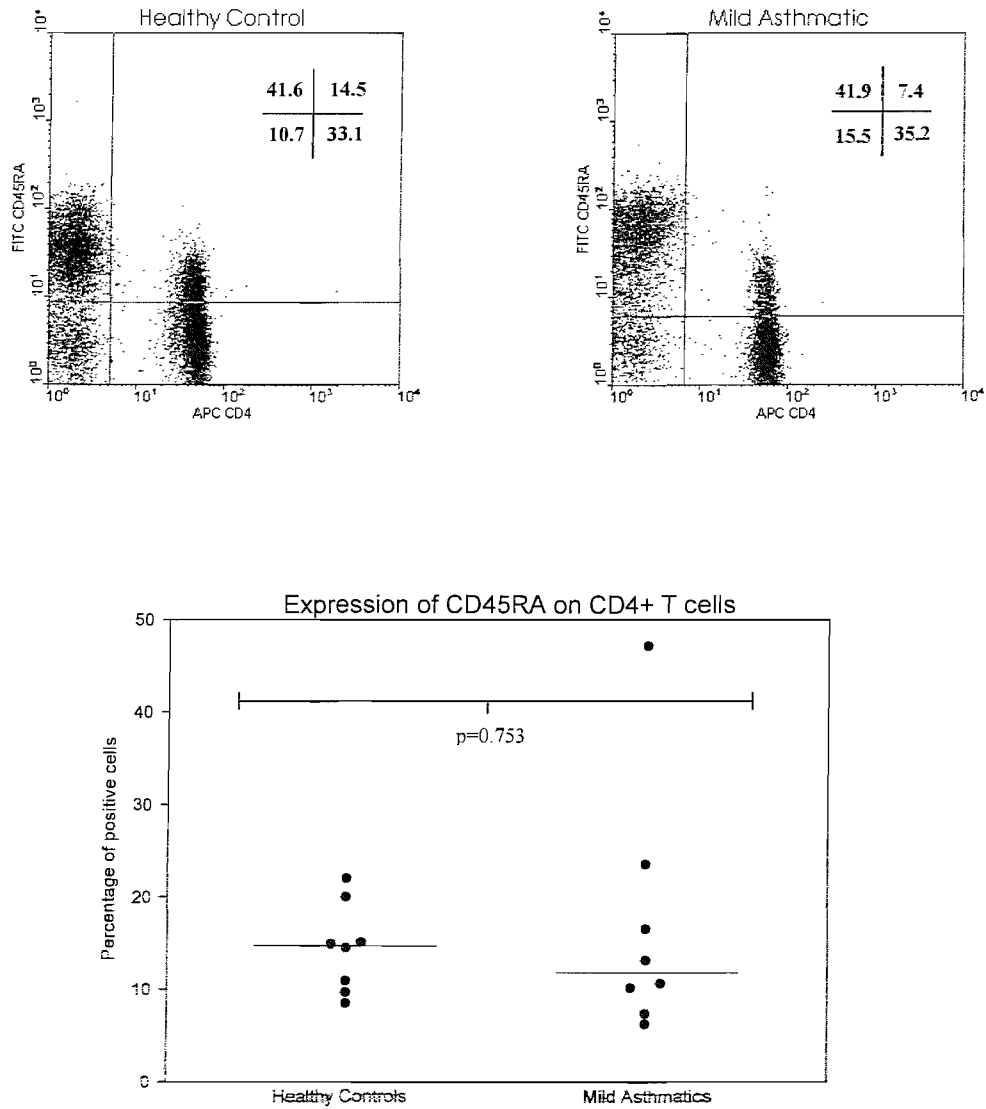
Healthy Controls   Mild Asthmatics

Figure 3.15 Expression of CD134 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

90
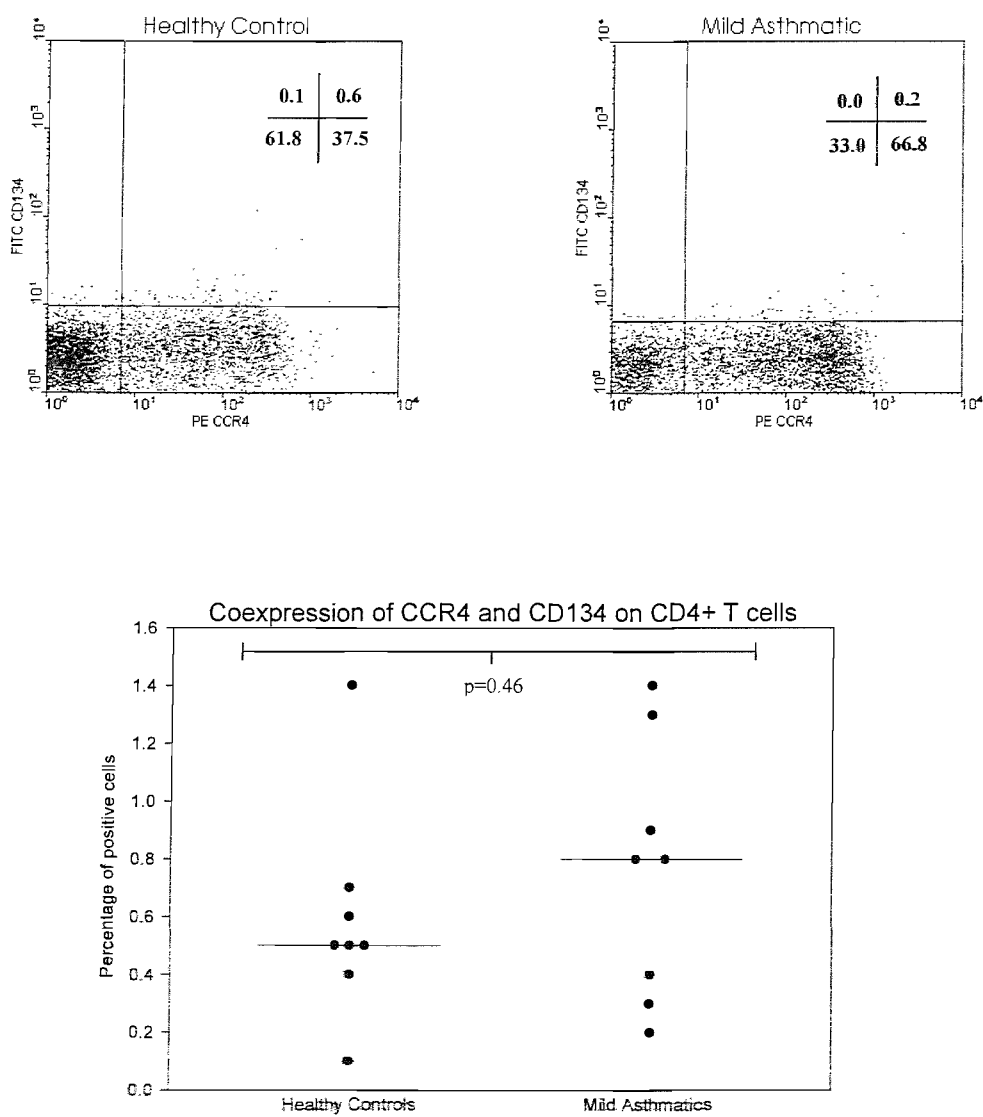
Figure 3.16

Figure 3.16 Coexpression of CCR4 and CD134 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.17
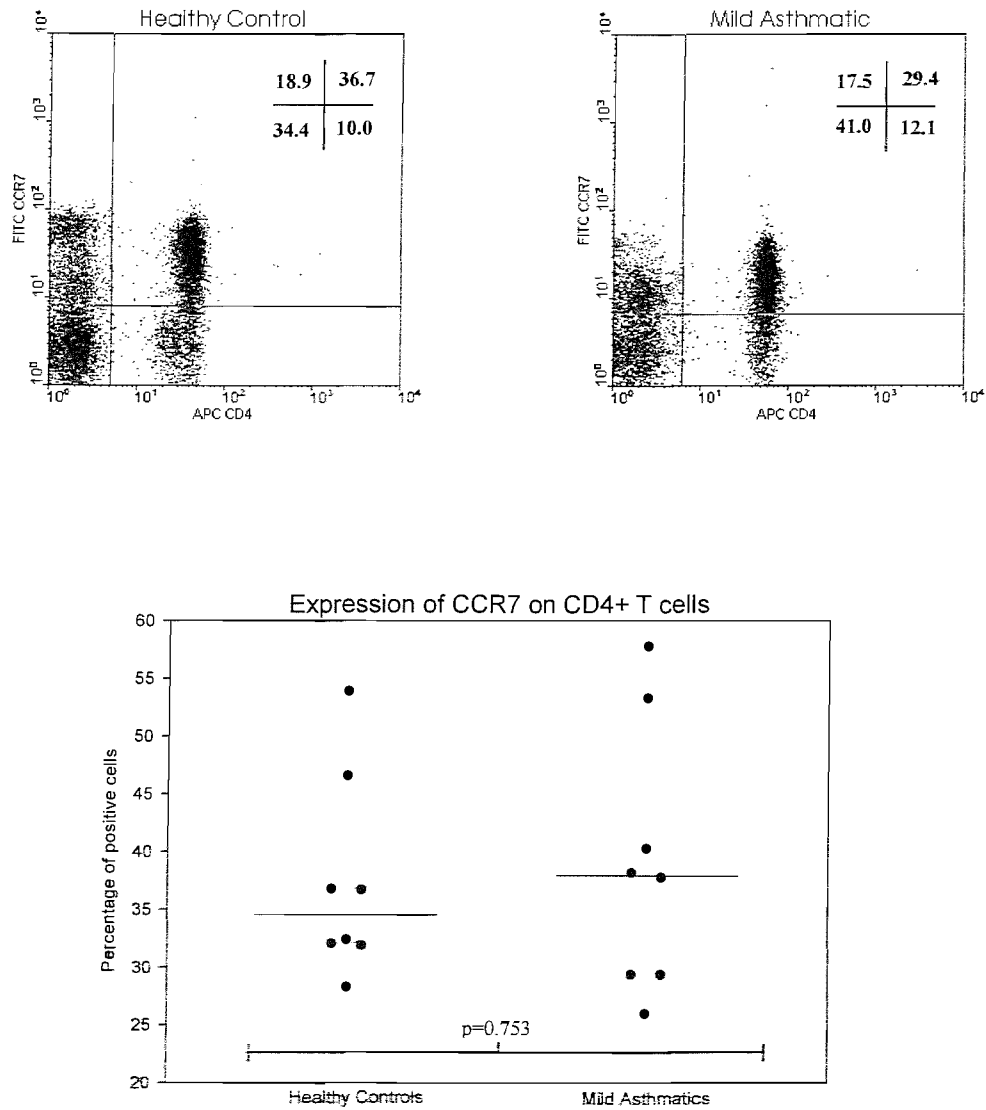
Expression of CCR7 on CD4+ T cells

Figure 3.17 Expression of CCR7 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
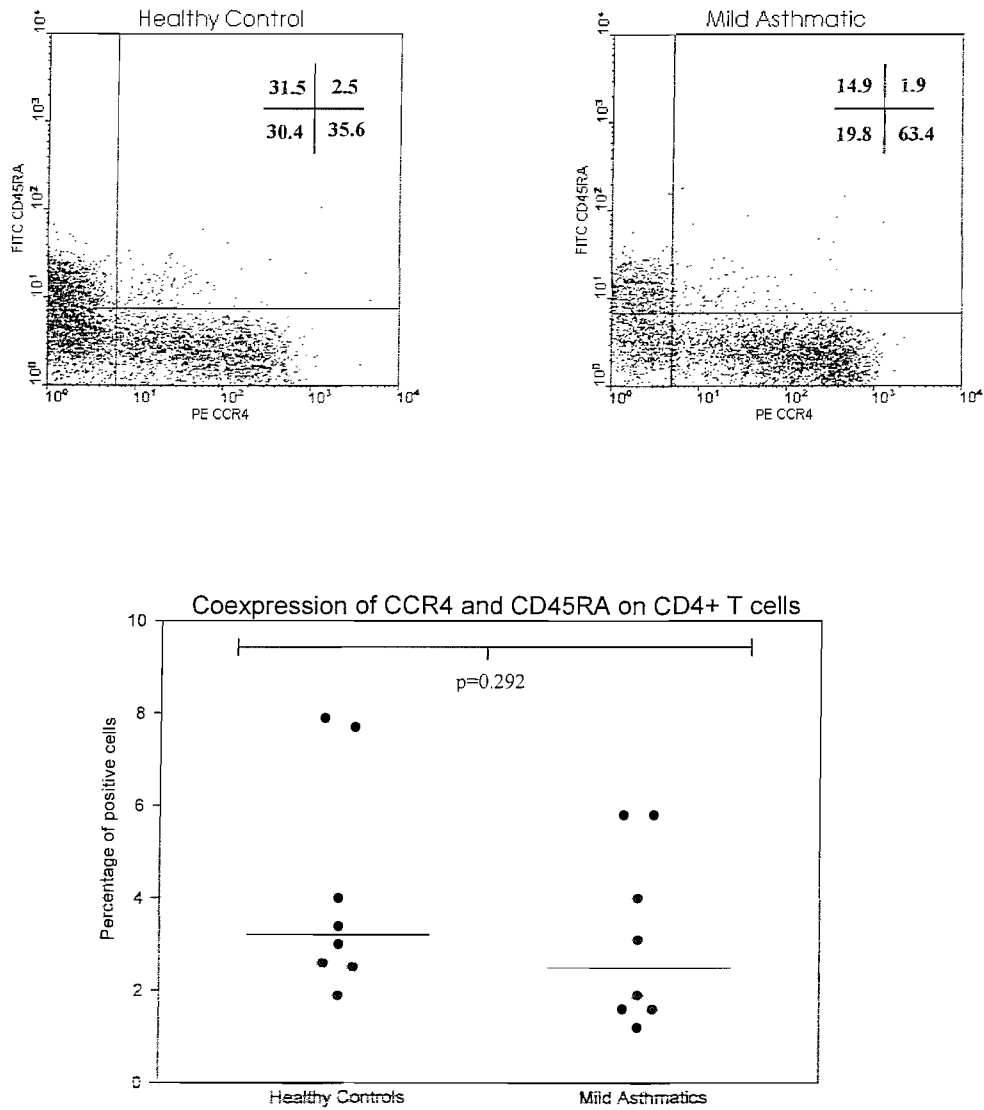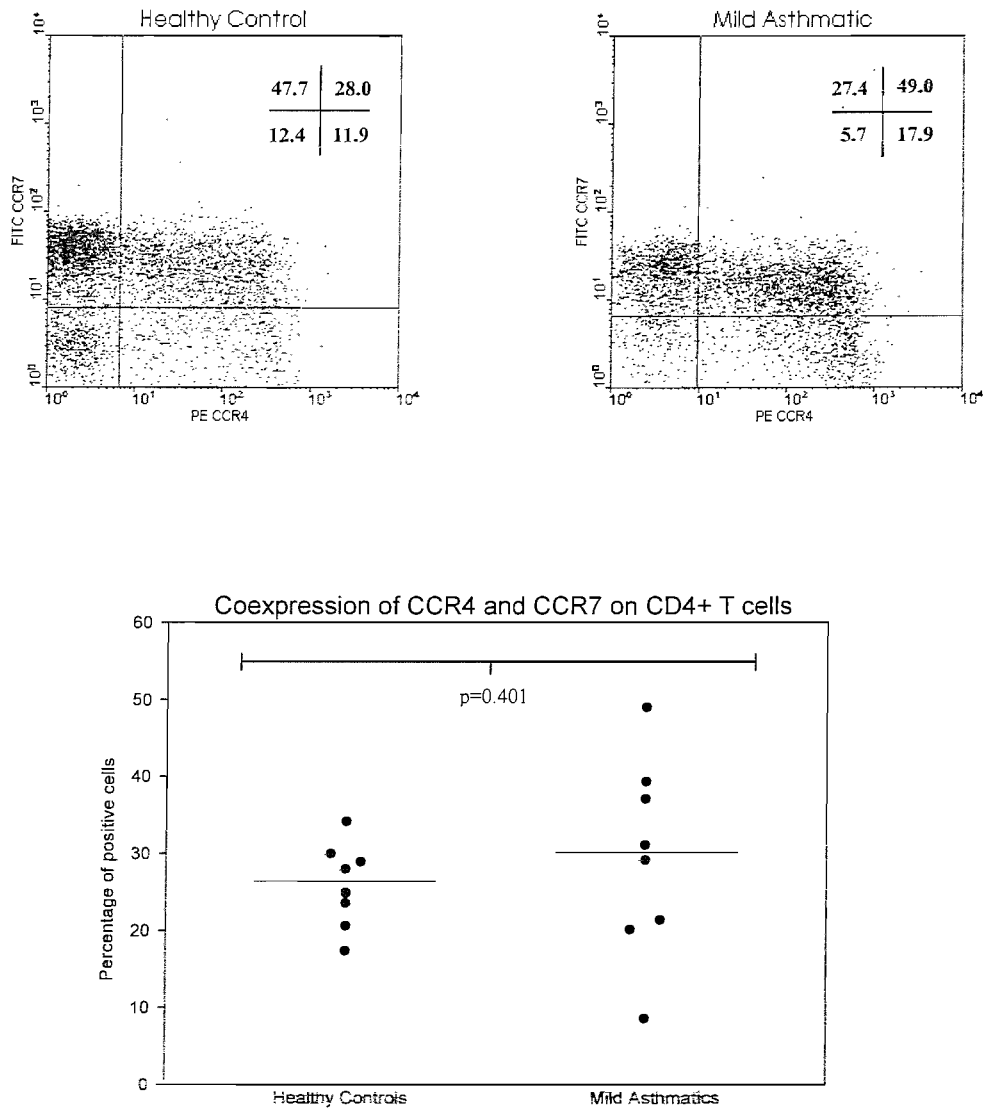
Figure 3.18

Figure 3.18 Coexpression of CCR4 and CCR7 on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.
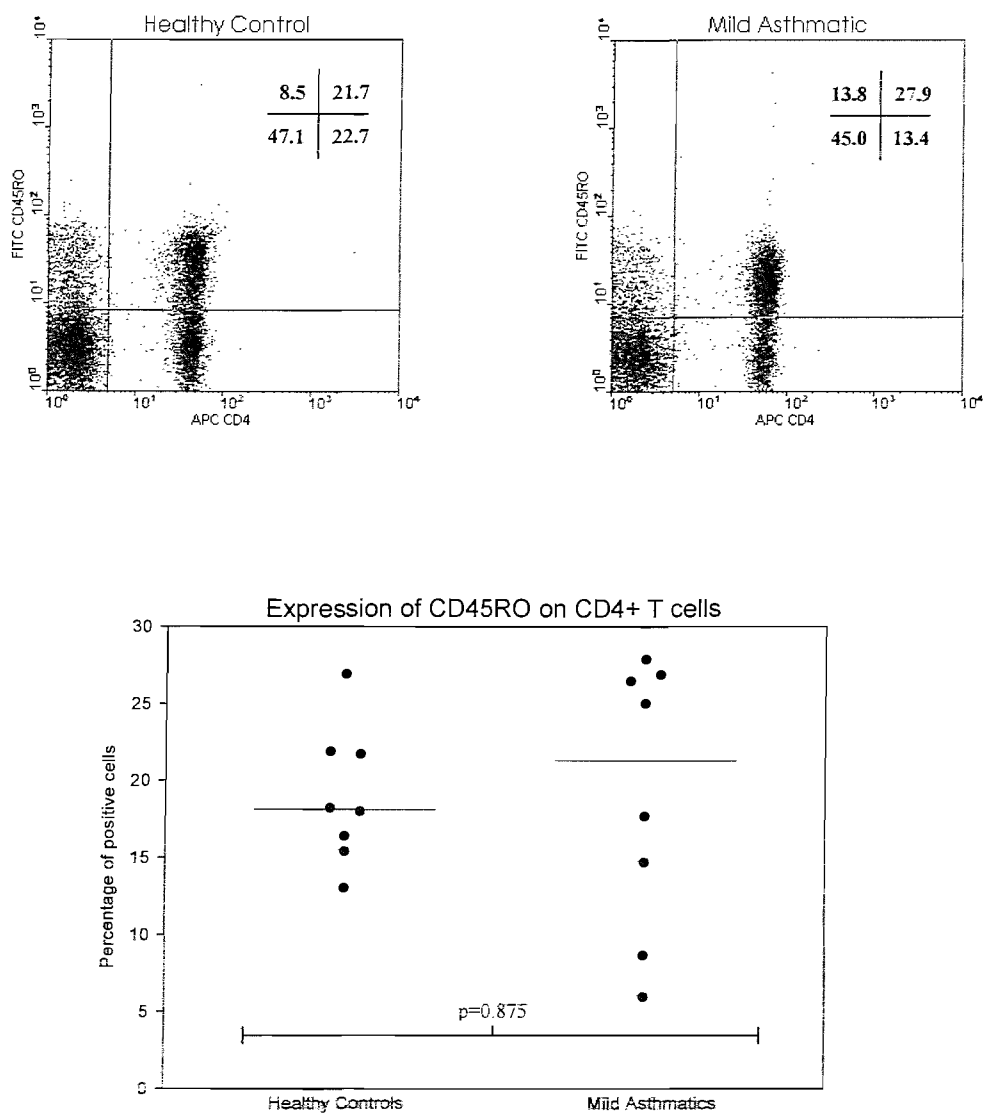
Figure 3.19
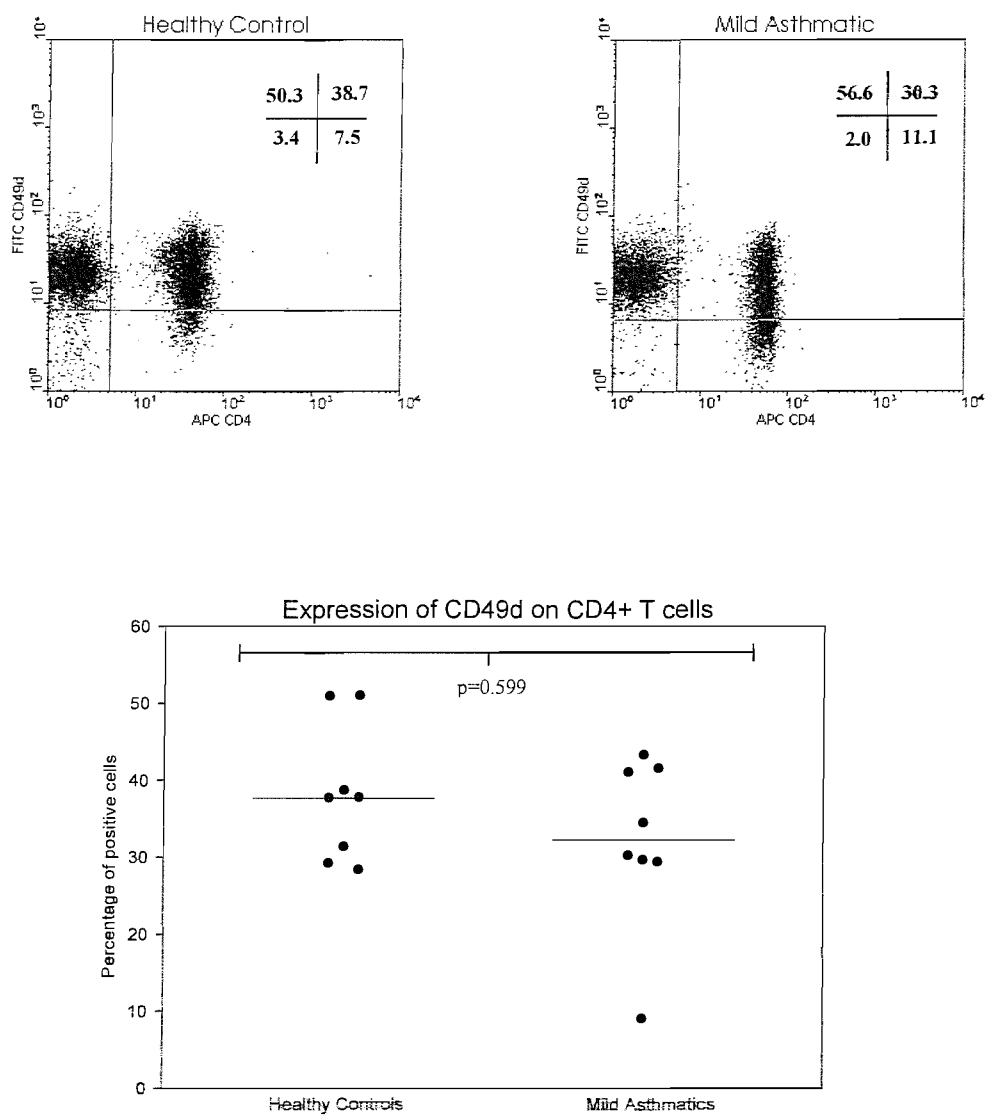
Figure 3.19 Expression of CD49d on CD4+ T cells. Top panels show representative results of flow cytometric analysis of a healthy control subject (left) and mild asthmatic (right). Bottom panel shows individual data, medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control subjects.

Figure 3.20
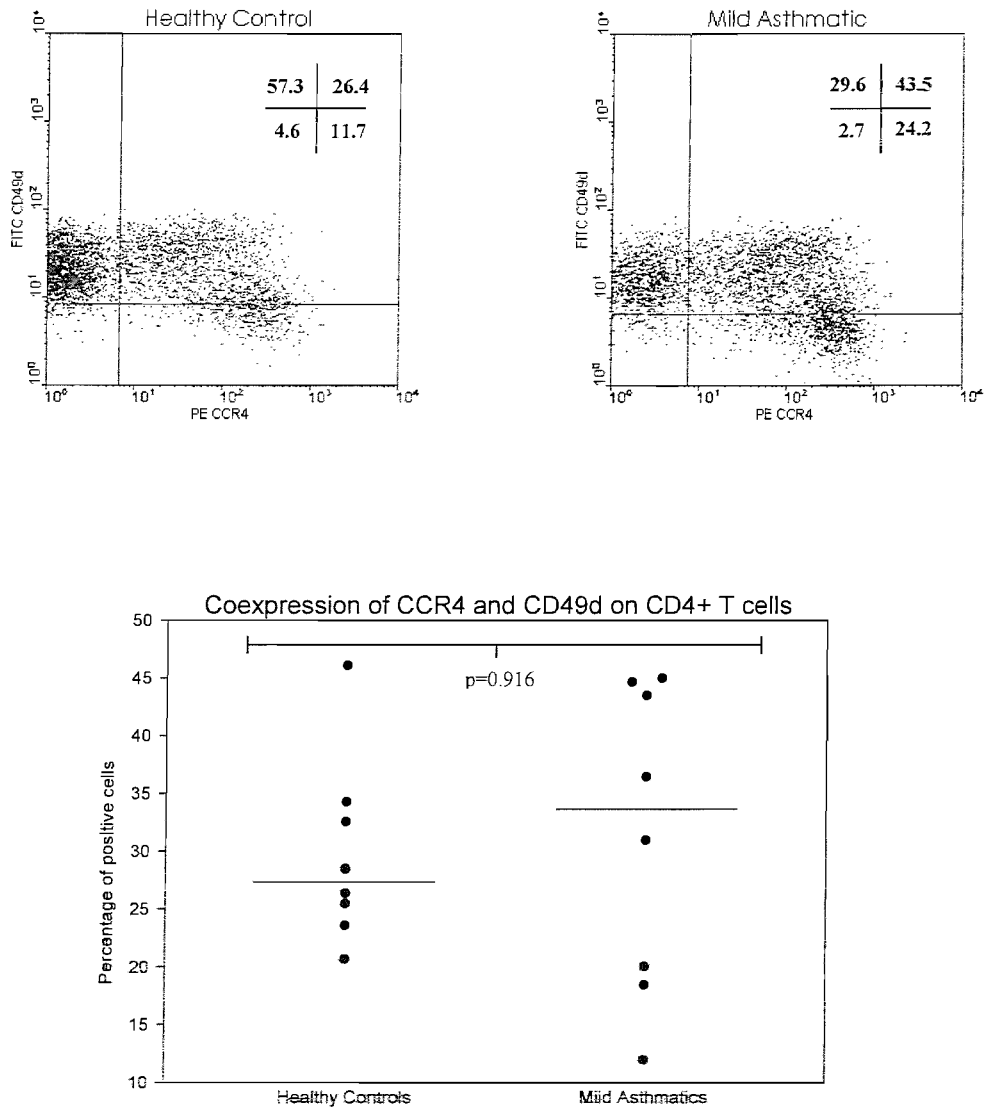
Figure 3.20 Coexpression of CCR4 and CD49d on CD4+ T cells. Top panels
show representative results of flow cytometric analysis of a healthy control
subject (left) and mild asthmatic (right). Bottom panel shows individual data,
medians (horizontal bar) and statistics for 8 asthmatics and 8 healthy control
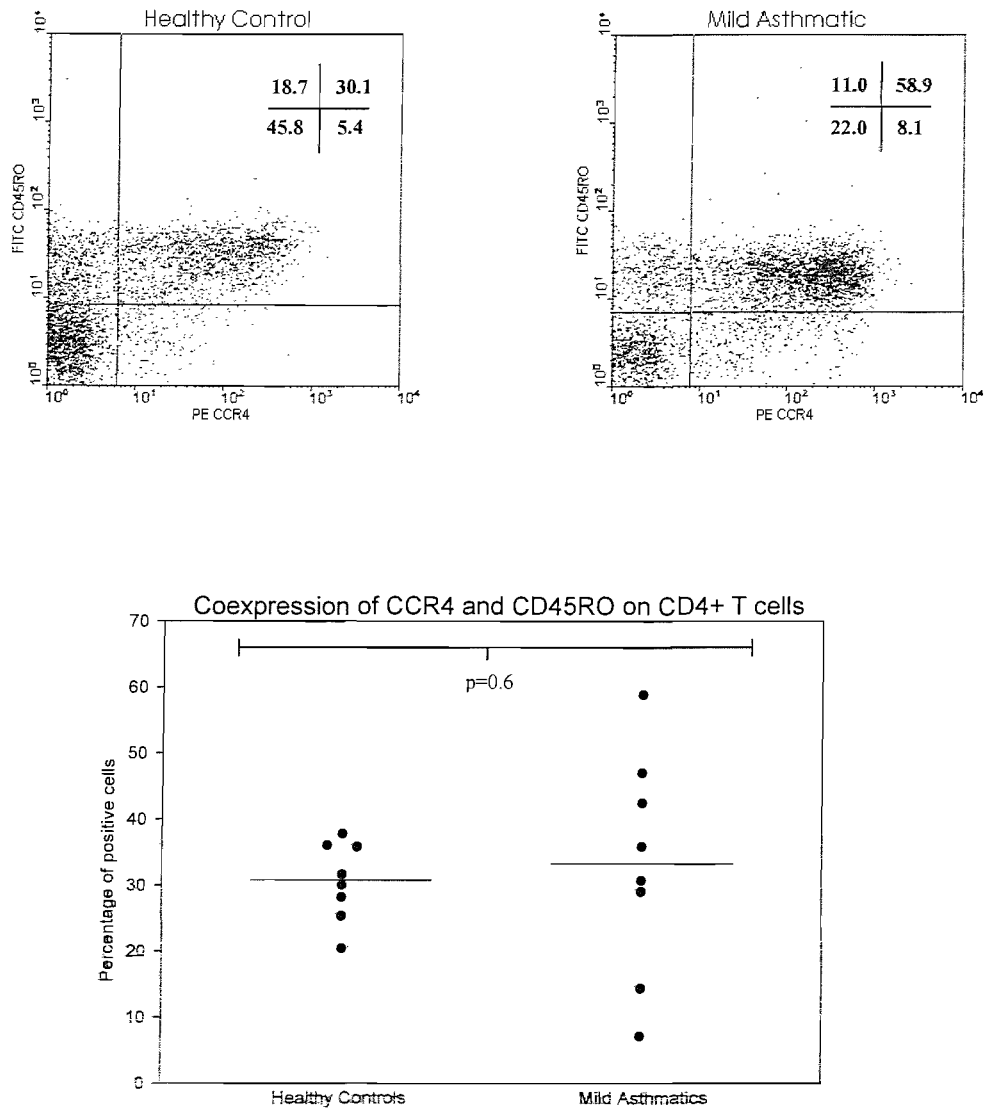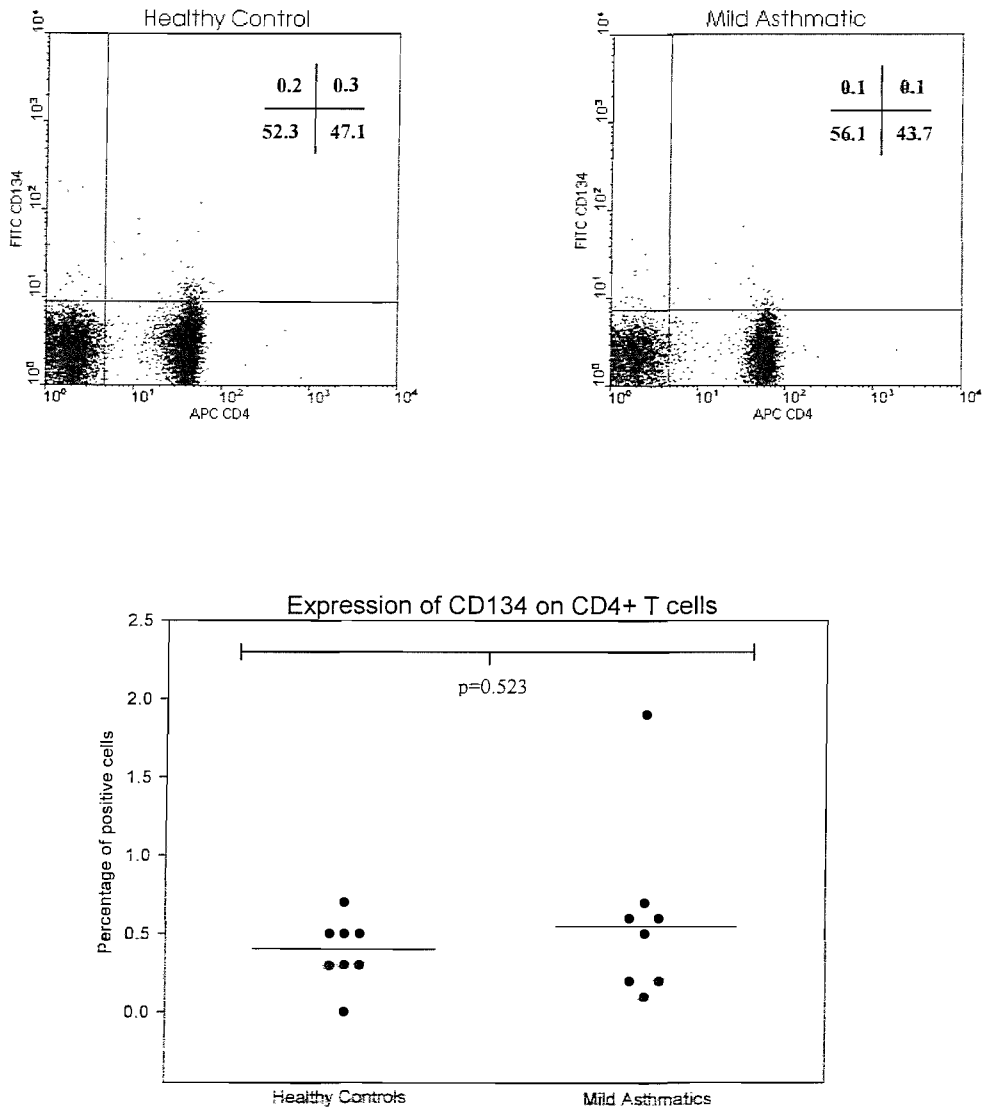subjects.

Figure 3.21
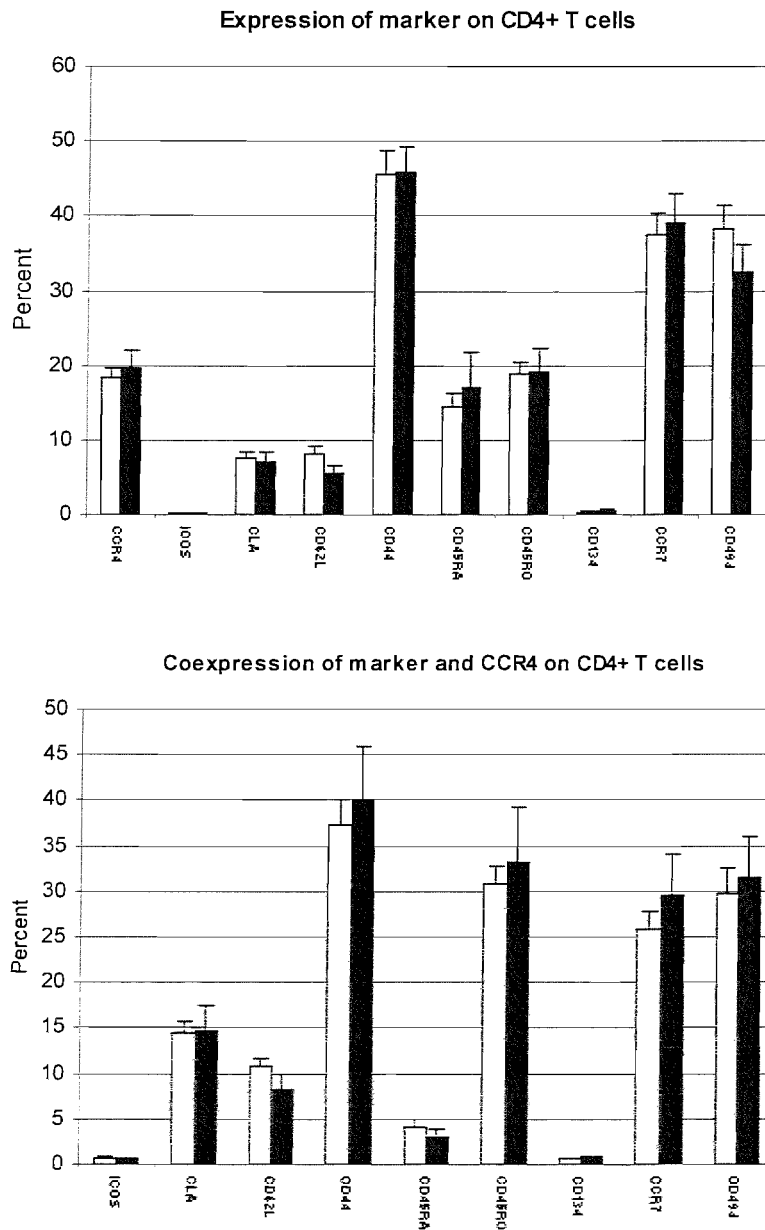
Figure 3.21 Expression of surface markers on CD4+ T cells. Top panel shows mean expression of markers for 8 healthy controls (white) and 8 asthmatic subjects (black). Bottom panel shows mean expression of surface markers on CCR4 and marker positive CD4+ T cells for 8 healthy controls (white) and 8 asthmatic subjects (black). Error bars represent 1 SD

Figure 3.22
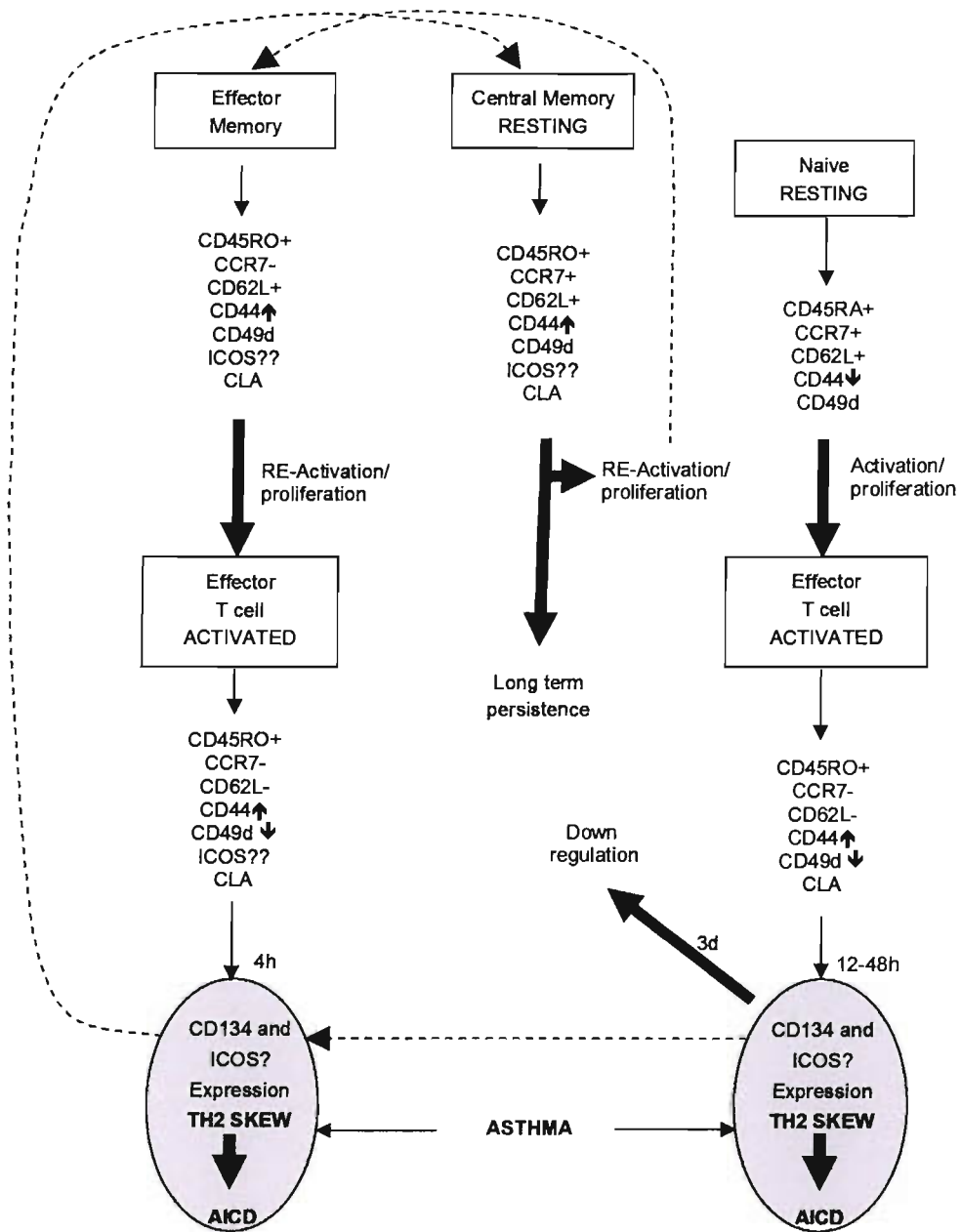
Figure 3.22 Diagram illustrating the expression of the markers studies, on which cell types and the activation state of the cell.

Figure 3.23

Changes in CCR4 and CRTH2 expression on CD4+ T cells with / without PHA stimulation n=9
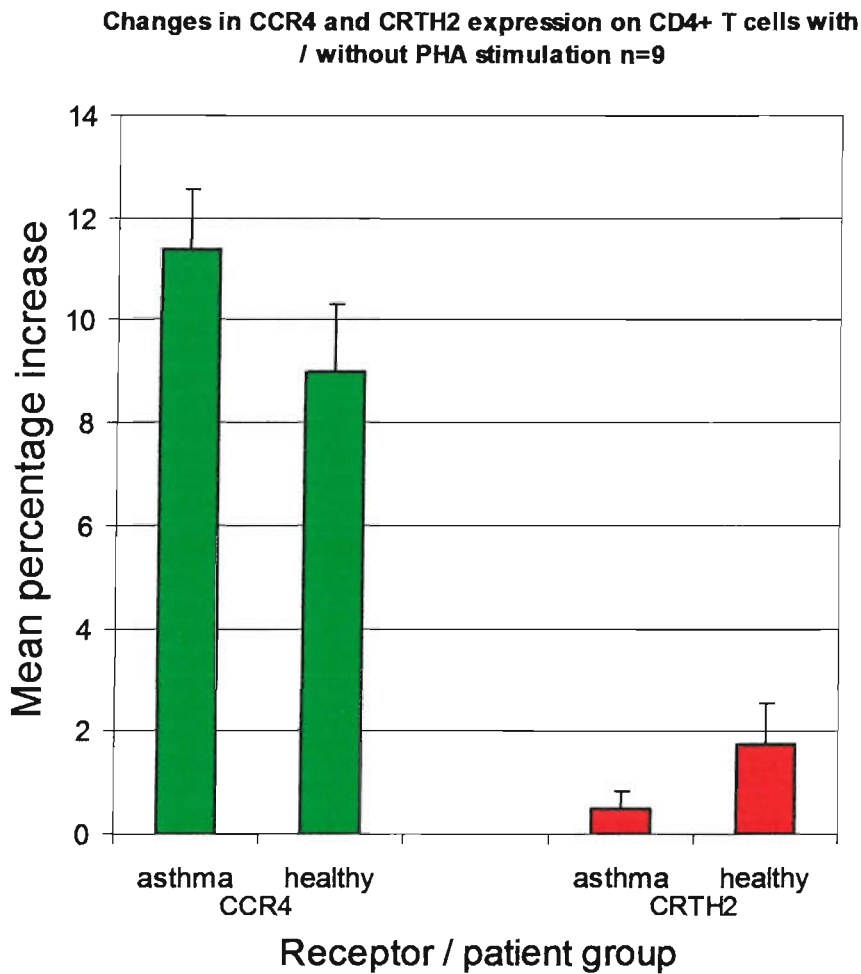
Figure 3.23   Expression of CCR4 and CRTH2 on CD4+ T after 48h culture with or without addition of PHA. Data show the mean percentage increase in CCR4 expression after PHA stimulation. in asthmatics (left green bar) and healthy subjects (right green bar) and CRTH2 (red bars) in asthmatics (left red bar) and healthy subjects (right red bar).   Data represent the mean values of 9 healthy control and 9 asthmatic subjects. Bars represent 1 SD.

Figure 3.24
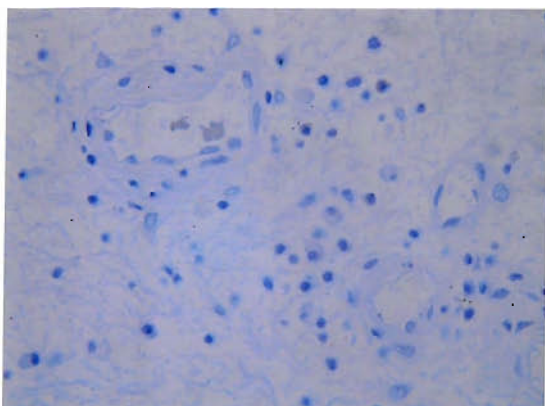
Figure 3.24a

Negative control TBS treated GMA embedded nasal polyp tissue section from one representative allergic rhinitic subject. Magnification x40.
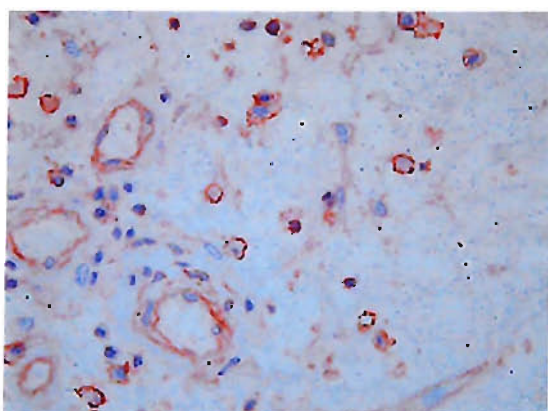
Figure 3.24b

Positive control epidermal growth factor receptor staining of a GMA embedded nasal polyp section from one representative allergic rhinitic subject. Magnification x 40.
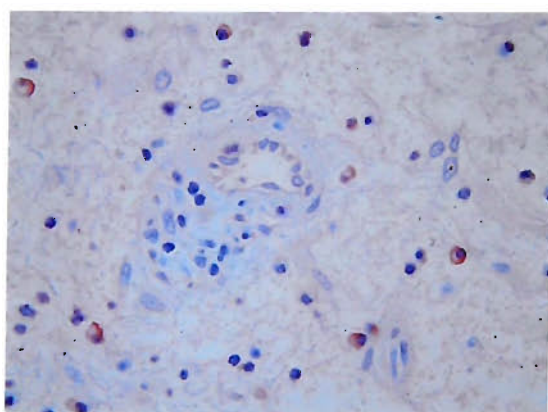
Figure 3.24c

Classical ring staining of CCR4+ve cells of a GMA embedded nasal polyp section from one representative allergic rhinitic subject. Used as a positive control for the staining of bronchial biopsy sections. Magnification x40.

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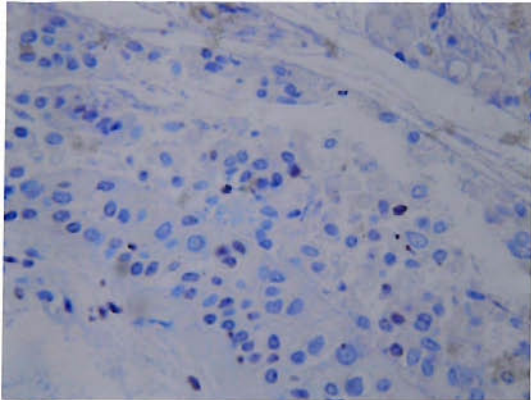
Figure 3.24d

Isotype control for CD3 (mouse IgG1) negative control staining of a GMA embedded bronchial biopsy section from one representative mild asthmatic subject. Magnification x40.
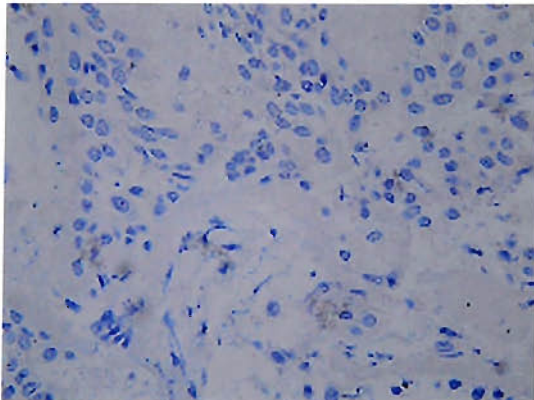
Figure 3.24e

Isotype control for CCR4 (Goat IgG) negative control staining of a GMA embedded bronchial biopsy section from one representative mild asthmatic subject. Magnification x40.
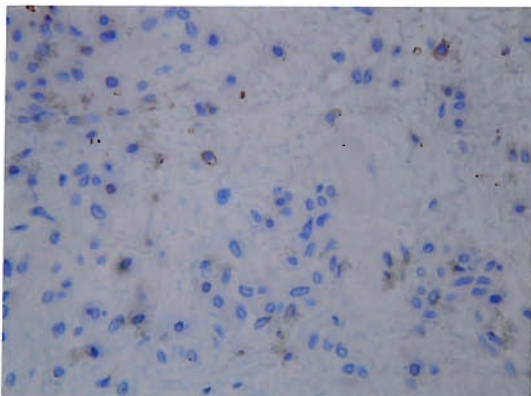
Figure 3.24f

Staining for CD3 of a GMA embedded bronchial biopsy section from one representative mild asthmatic subject. Magnification x40.
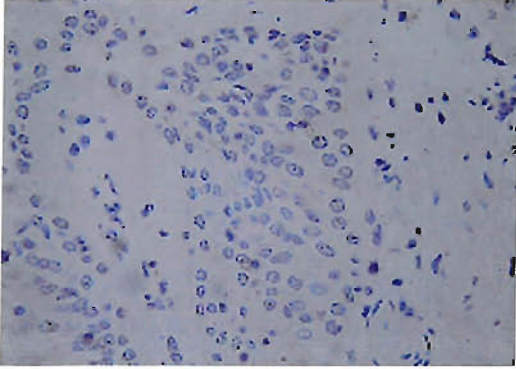
Figure 3.24g

Staining for CCR4 of a GMA embedded bronchial biopsy section from one representative mild asthmatic subject. Magnification x40.

Chapter 4

Results

Cytokine and chemokine

production by bronchial mucosal tissue

in response to *ex vivo* stimulation with allergen

4.1 Introduction

Inflammation is a hallmark of asthma. The inflammatory infiltrate comprises eosinophils, activated lymphocytes, activated macrophages and partly degranulated mast cells and basophils. A considerable amount of research has focused on CD4+ T cells as being pivotal in the orchestration of the allergic asthmatic response through the production of Th2 cytokines. An association between the expression of lymphocyte Th2 cytokines and asthma has been demonstrated and these cytokines can promote activation, migration and infiltration of the above cell types. Allergen can trigger the release of an array of cytokines produced either directly by Th2 cells or by other cell types on which Th2 cytokines act.

Mast cells, basophils, eosinophils, T cells, DC, alveolar macrophages and airway epithelial cells can produce an array of chemoattractants that promote accumulation of inflammatory cells in the lung, a number of which are upregulated after allergen challenge. Not only are chemokines important in asthma and allergy for their ability to regulate leukocyte recruitment, but they also assist in cellular activation, inflammatory mediator release, promotion of inflammatory Th2 responses and regulation of IgE synthesis (Gangur and Oppenheim, 2000).

It is now widely accepted that the Th2 cytokines, including IL-4, IL-5 and IL-13, account for many of the pathophysiological manifestations of allergy and asthma (Ngoc *et al.*, 2005). T cells themselves produce chemokines and Th2 cell-derived cytokines act on other cell types to produce chemokines. Thus the chemokines CCL11, CCL5, CCL2, CCL8, CCL7 and CCL13 act through interactions with CCR3; CCL17 and CCL22 with CCR4; and PGD_2 with CRTH2; these may all be important in drawing inflammatory cells into the airway in asthma due to the expression of these receptors proposed to be preferentially or selectively expressed on cell types important in asthma.

The aim of the experiments shown in this chapter was to determine whether a range of cytokines and chemokines are produced in increased amounts by the bronchial mucosa

in response to stimulation with allergen.   Studies employing allergen challenge *in vivo* have demonstrated cytokines and chemokines whose production can be detected either at the mRNA level by PCR (Bentley *et al.*, 1993, Prieto *et al.*, 2001) or *in* situ hybridisation (Bentley *et al.*, 1993) or by quantifying the proteins (Liu *et al.*, 2004, Holgate *et al.*, 1997). These studies have involved allergen challenge delivered either by inhalation of an aerosol of allergen solution (Bentley *et al.*, 1993, Holgate *et al.*, 1997), resulting in the stimulation of the whole lung, or by instillation of allergen solution into the airways via the fibreoptic bronchoscope, resulting in local challenge of the segment of the lung in which the bronchoscope has been wedged (Holgate *et al.*, 1997).

In this study the bronchial explant model (Dent *et al.*, 2002, Hidi *et al.*, 2000) was used to study the chemotactic activity that is generated by inflammatory and structural cells in the bronchial mucosa. This model enables airway mucosal tissue, obtained by bronchoscopy, to be challenged with allergen *ex vivo* and the supernatants to be analysed for chemotactic activity and cytokine levels (Hidi *et al.*, 2000). Using this model, Hidi *et al* found that stimulation of mucosal tissue from mild asthmatics with allergen to which the donors are sensitive generates significant chemotactic activity when supernatants from the explants are used in chemotaxis experiments which use the modified Boyden chamber (Hidi *et al.*, 2000). A significant, but not entire part of this activity was due to IL-16 and the activity could be inhibited significantly, by blockade of the CD28/B7 co-stimulatory pathway using a CTLA4-Ig fusion protein, suggesting that the generation of T cell chemotactic activity required T cell action (Hidi *et al.*, 2000). The objective of the present study was to extend the findings by quantifying an array of other cytokines and chemokines that are potentially relevant in allergen-induced responses including the cytokines IL-2, IL-4, IL-5, IL-10, IL-13, IFNγ and TNFα and chemokines: CCL2, CCL4, CCL5, CCL11, CCL17, CCL19, CCL20 and CCL22. The fact that the measured responses in the explant model are dependent solely on the cells residing in the airways at the time of challenge was considered. It is likely that a large proportion of the inflammatory mediator response (including

cytokines) is contributed to by additional inflammatory cells which migrate into the airways in the hours that follow allergen challenge.

Methods

Briefly, 8 bronchial biopsies were obtained by fibreoptic bronchoscopy. The biopsies were placed into culture in AIM-V medium in the presence or absence of house dust mite extract as a specific stimulus for allergen-specific T cells. After 24 hr culture at 37°C, supernatants were harvested and cytokine and chemokine concentrations were determined using two assays.

For the chemokines, standards and biopsy supernatants were added to wells pre-coated with a capture antibodies followed by addition of biotinylated reagents and the plate was read after the addition of a substrate solution. For the measurement of cytokines, luminex beads were coupled to anti-cytokine antibodies. Standards and biopsy supernatants were added with the desired concentration of a bead/capture antibody cocktail. Detection antibodies were added to each well followed by streptavidin PE, and the assay was read using a Bioplex machine.

The concentrations of chemokines and cytokines measured in the supernatants were corrected for the weight of the biopsies and results expressed as pg/mg of bronchial mucosal tissue. The results discussed and figures shown were produced from data generated by 2 multiplex ELISAs: one for chemokines and one for cytokines.

4.2 Results

4.2.1 IL-2

Significance was reached when comparing the IL-2 levels between the challenged and unchallenged supernatants for the healthy controls (p=0.015) (Fig 4.1, Table 4.1) and also when comparing IL-2 production in the unchallenged samples between the healthy and asthmatic subjects (p=0.001).

4.2.2 IL-4

Very low levels of IL-4 were produced per milligram of biopsy tissue in both healthy controls and asthmatics (Fig 4.2). However, there was an overall significant increase in the production of IL-4 (Table 4.1) after challenge for the asthmatics (p=0.015).

4.2.3 IL-5

The difference in the amounts of IL-5 detected 1) in the asthmatics with and without allergen challenge and 2) between the challenged samples of healthy controls and asthmatics were very noticeable (Fig 4.3) and highly significant (Table 4.1) with p values of <0.001 for both. The levels produced for the healthy patients from samples treated without allergen were very low and showed no significant increase with allergen stimulation.

4.2.4 IL-10

Whilst there appeared to be a trend for IL-10 production to be reduced in the challenged samples for the healthy controls this failed to reach significance (Fig 4.4). The levels produced in the presence of allergen were similar in the healthy and the asthmatic subjects.

4.2.5 IL-13

Very low levels of IL-13 were produced by the healthy controls and there was no increase with allergen challenge (Fig 4.5). The levels of IL-13 in the challenged samples of the asthmatics were higher than the healthy control challenged samples and this difference was significant (p=0.014) (Table 4.1).

4.2.6   TNFα

The levels of TNFα between healthy controls and asthmatics were similar and no trends could be seen (Fig 4.6).

4.2.7   IFNγ

IFNγ was not detected in any of the supernatants tested

4.2.8   CCL17

The differences between the healthy and asthmatic subjects for both the challenged and unchallenged samples were statistically significant showing that more CCL17 is spontaneously produced in the asthmatics than the healthy subjects (p=0.008) and that the asthmatics produce more CCL17 than the healthy controls do after allergen challenge (p=0.001) (Fig 4.7, Table 4.1).

4.2.9   CCL22

Levels of CCL22 produced by the healthy controls were similar both with and without challenge with allergen (Fig 4.8). Increases for CCL22 were seen upon challenge with allergen in 10 asthmatics patients. There was a significant difference in the CCL22 levels produced after challenge with allergen between the healthy and asthmatic subjects (p=0.004) (Table 4.1).

4.2.10   CCL2

All other cytokines and chemokines were diluted 1:5 in order for them to lie in the detection range of the ELISA, but CCL2 had to be diluted 1:25. There was a statistical significant difference found between the healthy and asthmatic samples in the challenged condition (p=0.013) (Fig 4.9, table 4.1). The majority of healthy control subjects produced lower amounts of CCL2 both before and after challenge than the asthmatic patients, with the exception of 2 outlying healthy control subjects.

4.2.11 CCL4

No differences were seen in the production of CCL4 between the unchallenged and challenged samples in both the healthy and asthmatic subjects (Fig 4.10). The levels of this chemokine detected were also no different between the healthy and asthmatic subjects either without or with allergen challenge.

4.2.12 CCL20

There were no differences between the concentrations of CCL20 with or without allergen treatment in the healthy control subjects (Fig 4.11). For the asthmatics, in supernatants from explants stimulated with allergen, concentrations of CCL20 produced were slightly higher in the asthmatics than the healthy controls and this difference was statistically significant ($p=0.013$) (Table 4.1).

4.2.13 CCL19

On the whole, the levels of CCL19 detected with and without allergen differed very little for both the healthy and the asthmatic patients, however the CCL19 detected in the challenged samples was significantly higher in the asthmatics as compared with the healthy controls ($p=0.04$) (Fig 4.12, Table 4.1).

4.2.14 CCL11

Low concentrations of CCL11 were detected. There were, however, significant differences between the asthmatic and the healthy subjects for both the unchallenged samples ($p=0.029$) and the challenged samples ($p=0.002$) (Fig 4.13, Table 4.1).

4.2.15 CCL5

A trend for decreased concentrations of CCL5 for the healthy subjects after allergen challenge was noticeable; however, this failed to reach significance (Fig 4.14, Table 4.1). It is in the samples from the challenged biopsies where the differences could be seen. The range of concentrations found in the asthmatics following challenge with allergen was more widely distributed but there were no differences between the two subject groups.

4.3 Discussion

The aim of the present study was to extend previous observations of significant cytokine responses when bronchial tissue from atopic asthmatics is challenged *ex vivo* with allergen. The analyses of supernatants from experiments for healthy control subjects and mild, steroid-naïve atopic asthmatics have been completed. While many of the changes noted failed to reach significance, some trends were seen and a more definitive conclusion would be reached with further experiments. The results that are worthy of particular note are the apparent reduction in IL-2 levels when tissues from both patient groups were challenged, the increase in IL-5 concentrations in the asthmatics, but not healthy controls, raised levels of spontaneous and allergen driven CCL17 production and the increased CCL22 in the allergen challenged samples of asthmatics in comparison to the allergen challenged samples of the healthy controls, lower levels of CCL2 and CCL11 in the allergen challenged samples of asthmatics than the allergen challenged healthy controls.

IL-2 is produced mainly by activated T cells (primarily Th0 and Th1), but it can also be produced by eosinophils and airway epithelial cells (Aoki *et al.*, 1997). It acts in an autocrine fashion, binding to the IL-2 receptor on T cells from which it was produced in order to enhance proliferation. Virchow and colleagues have previously reported increased levels of IL-2 in atopic asthmatics after segmental allergen challenge in comparison to the saline challenged site (Virchow *et al.*, 1995). In this study, IL-2 levels measured in supernatants of the unchallenged (control) biopsies from the control non-atopic subjects were higher when compared to the supernatants from the asthmatic subjects. This would be consistent with a predominant Th1 cytokine phenotype that has been consistently reported in the literature (Del Prete, 1992). However, interestingly, the concentrations of IL-2 decreased in both subject groups following allergen challenge. It is difficult to speculate but there are a number of explanations. This may be the result of cleavage of the α-subunit of the IL-2 receptor, CD25, by the cysteine protease Der p 1, a constituent of HDM allergen (Schulz *et al.*, 1998) which could have prevented the autocrine mechanism of action of IL-2, thus preventing its

further production. Another explanation could be that IL-2 is in fact consumed by the cells present in the biopsies upon allergen stimulation.

There is a complex interaction of numerous cell types within the airways, comprising eosinophils, activated T cells, mast cells, macrophages, epithelial cells, fibroblasts and smooth muscle cells. The aforementioned cells are a rich source of mediators that are active in the airways. Cytokines and chemokines act in concert, serving to amplify the inflammation and recruit more cells into the airways. Classical Th2-type cytokines such as IL-4, IL-13, IL-5 and IL-9, along with many other cytokines and chemokines have been detected in abundance in asthmatic airways, sputum and BAL. In the current study, significant differences between the two subject groups were found with the Th2-type cytokines IL-4 and IL-5. Surprisingly, IL-4 levels appeared also to increase slightly in the non-atopic control subjects in addition to the asthmatic which would point to an ability to generate a Th2 response. There were statistical differences within the patient groups but no significant differences were seen between the two patient groups.

The measured levels for both IL-5 and IL-13 were consistent with what is known about the role of these two Th2 cytokines in allergic disease. Whilst IL-13 has not been previously measured in the explant model, mRNA IL-5 levels have been found to increase when explants from atopic subjects were stimulated with allergen (Lordan *et al.*, 2001). Teran and colleagues have also identified increased levels of IL-5 in BAL fluid after allergen challenge in comparison to regions of the airways treated with saline only (Teran *et al.*, 1999a) and the data from the present study are in support of this. In the current study IL-5 levels increased significantly with challenge in the asthmatic subjects. IL-13 was found to be raised on the whole in the asthmatics after challenge but the levels between healthy and asthmatic subjects for the unchallenged samples were very similar. Other studies have shown the expression of mRNA for IL-13 in both atopic and non-atopic asthmatics and IL-4 in asthmatics following allergen challenge to be increased in quantities in bronchial biopsies (Humbert *et al.*, 1997, Ying *et al.*, 1997a). The results presented here are in support of this with

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statistically significant results for increased IL-4 production in the asthmatics after allergen challenge and a trend for production of more IL-13 in the asthmatics following allergen challenge, which in the current study, failed to reach significance. These findings support those of Kroegel and colleagues who demonstrated increases in IL-13 levels in asthmatics after segmental allergen challenge and their work showed that these levels increased in parallel with IL-4 (Kroegel *et al.*, 1996).

IL-4 is a key Th2 cytokine produced by Th2 cells, eosinophils, basophils and mast cells. It plays a major role in the development and progression of allergic/Th2 mediated responses, favours Th2 dominance through the inhibition of the Th1 response and exhibits overlapping, yet not identical effector properties with IL-13. IL-13 is produced by activated T cells (Fireman, 2003) and shares many of the functional properties of IL-4 (Izuhara and Arima, 2004, de Vries and Zurawski, 1995). It is a modulator of monocyte and B cell function (Minty *et al.*, 1993) and akin to IL-4 can influence the development of Th2 cells (McKenzie *et al.*, 1998).

The role of IL-5 is primarily in the development, differentiation, recruitment, activation and survival of eosinophils (Sanderson, 1988, Clutterbuck and Sanderson, 1988, Wang *et al.*, 1989, Lopez *et al.*, 1988, Sanderson, 1990). The cellular sources of IL-5 are T cells, mast cells, eosinophils and basophils (Yamaguchi *et al.*, 1988). The evidence for IL-5 being a key eosinophil factor was obtained using cytokine blocking antibodies or IL-5 knock-out mice. Using IL-5 deficient mice that had been chronically challenged with allergen, Leigh and co-workers found that the sustained airway hyperreactivity and aspects of airway remodelling seen in WT mice remained the same in IL-5 deficient mice (Leigh *et al.*, 2004), implying that these clinical characteristics of asthma are not reliant upon IL-5. In the same experiment, they found that both IL-4 and IL-13 deficient mice were protected from developing sustained airway hyperreactivity and aspects of airway remodelling (Leigh *et al.*, 2004). IL-4 has also been shown to play a role in the recruitment of eosinophils into the airways. Pauwels and colleagues found that IL-4 knock-out mice had an inability to develop an allergic eosinophilic airway infiltration (Pauwels *et al.*, 1997) and this has been

confirmed by Brusselle and colleagues (Brusselle *et al.*, 1994). Despite this, to date, no single cell derived factor has been isolated as being responsible for any individual aspects of asthma and the synergistic effects of cytokines and chemokines in the milieu in which they are produced contribute to the pathology of the disorder. Indeed, although eosinophil numbers can be dramatically reduced in both blood and sputum of atopic asthmatics after treatment with anti-IL-5 antibody (Leckie *et al.*, 2000), subsequent studies by Flood-Page *et al* demonstrated only 50% decreases in the number of eosinophils in bronchial biopsies of atopic mild asthmatics after 3 infusions of an anti-IL-5 antibody (Flood-Page *et al.*, 2003).

In the current study, no differences were seen in TNFα production between asthmatics and healthy controls. TNFα is a cytokine that acts synergistically and has immunomodulating properties, resulting in the enhancement of chemokine production and hence increased recruitment of cell types into the airways. Reports of TNFα synergising with IL-4 to increase the production of CCL11 exist and synergy with IFNγ has been shown to increase the production of CCL5 (Teran *et al.*, 1999b). In addition, Moore and colleagues found that human airway smooth muscle cells treated with IL-4 or IL-13 alone resulted in a 2-fold release in CCL11 in the supernatant. On the addition of TNFα alone there was a 4-fold increase in CCL11 release but TNFα in combination with IL-4 or IL-13 resulted in a much enhanced release of 10 or 20-fold respectively (Moore *et al.*, 2002). This confirms that these cytokines can serve to facilitate cellular migration. However, studies by Barnes and colleagues in which a blocking antibody against TNFα was applied in mild asthmatics have failed to show both efficacy of this treatment and a role for TNFα in mild disease (Barnes, 2002). This would be consistent with the findings in the current study that TNFα levels were not raised in the asthmatics and did not rise after stimulation with allergen. However, a subsequent study by Berry and colleagues showed that treatment with the soluble TNF receptor was able to significantly improve both asthma control and bronchial hyperresponsiveness in severe asthmatics, suggesting that TNFα might have a role in severe but not mild disease (Berry *et al.*, 2006). In support of this, Howarth and colleagues investigated the levels of TNFα in BAL of healthy subjects, mild asthmatics

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and severe asthmatics both by immunoassay and by gene expression in endobronchial biopsy specimens (Howarth et al., 2005). They discovered that TNFα levels in BAL fluid, TNFα gene expression and TNFα immunoreactive cells were increased in subjects with severe, corticosteroid dependent asthma (Howarth et al., 2005). Additionally, treatment with etanercept, which works through binding to both TNFα and TNFβ, thus preventing binding to cell surface receptors, improves asthma symptoms, lung function and bronchial hyperresopnsiveness (Howarth et al., 2005).

The main objective of the current study was to identify the chemotactic activity for T cells. Statistically significant differences in CCL17 and CCL22 production in the challenged samples of healthy and asthmatic subjects have been found. For CCL17, increased amounts were found in the unchallenged samples of the mild asthmatics compared with the healthy controls and there is a definite trend for increased CCL17 production at baseline levels and following allergen challenge in the asthmatics relative to the healthy controls. CCL22 was also produced in greater amounts in the asthmatics with allergen challenge and it was between the challenged samples of the asthmatics in comparison to the healthy controls where a significant difference was seen. The trends for CCL5 and CCL11 both followed a similar pattern. Lower concentrations of both chemokines were detected in the allergen challenged samples of healthy controls in comparison to both the unchallenged samples and the asthmatic challenged samples. The allergen challenged samples of the asthmatics, again for both chemokines, cover a wide range of concentrations.

CCL17 and CCL22 are both increased in BAL of asthmatics after segmental allergen challenge (Pilette et al., 2004) and CCL22 has also been found in increased concentrations in the BAL of steady state asthmatics at baseline levels in comparison to healthy controls (Lezcano-Meza et al., 2003). Additionally, both CCL5 and CCL2 have been found at baseline levels in BAL and the concentrations were the same in healthy controls and asthmatics. However, they were both detected in increased concentrations in the asthmatics 4 hours after segmental allergen challenge but the values then returned to baseline at 24 hours after allergen challenge (Holgate et al.,

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1997). An important point to consider in relation to the present study is the length of time the biopsies were cultured for. An increased length of culture may have resulted in some of the cytokines being released in greater magnitude at a later stage in the response which could have resulted in significant results for the asthmatics after allergen challenge in addition to significant differences in the levels produced by challenged biopsies between the healthy and asthmatic subjects.

Production and release of chemokines is attributable to the actions of cytokines. For example, using RT-PCR, Berin and colleagues found that treatment of a human bronchial epithelial cell line with IL-4 alone increased CCL17 production 2-fold. IFNγ caused a 3-fold increase, and TNFα alone caused an increase by 30 fold, whereas when TNFα and IL-4 were used in combination the amount of CCL17 produced was increased 3,250-fold (Berin *et al.*, 2001). The production of both CCL17 and CCL22 was found at increased concentrations in human PBMC supernatants after 72 hours culture with IL-4 and IL-13 (Pilette *et al.*, 2004). Although CCL11 has been detected in raised quantities in asthmatic sputum (Zeibecoglou *et al.*, 1999), BAL (Lamkhioued *et al.*, 1997) and mucosa (Ying *et al.*, 1997b), the cited research implies that it is not solely responsible for eosinophil recruitment and additionally it is thought to act in concert with other chemokines and/or cytokines. For example, the cooperation of CCL11 with IL-5 has been shown to be important for eosinophil accumulation (Collins *et al.*, 1995). Another cytokine recently shown to enhance CCL11 production is IL-9. Gounni and colleagues recently showed that stimulation of asthmatic airway smooth muscle cells resulted in the release of CCL11 but caused no release of CCL17 (Gounni *et al.*, 2004). The release of CCL5 seems to be regulated by different cytokines. John and co-workers demonstrated by mRNA detection, that the ability of human airway smooth muscle cells to produce CCL5 was enhanced by TNFα but not by IFNγ, and upon treatment with a combination of the two cytokines, mRNA quantities were increased in comparison to TNFα alone (John *et al.*, 1997). They also found that this increase was inhibited by treatment with IL-4, IL-10 and IL-13 (John *et al.*, 1997) and this has also been observed in a keratinocyte cell line (Xiao *et al.*, 2003). The above research suggests that, although known as a true Th1-type cytokine, IFNγ is not

redundant in asthma and although its effects are not direct and more often than not, it has been shown to lack enhancing properties, unless in combination with another factor, it must still play a role in perpetuating the immune response in asthma. However, work shows that this cytokine alone, does not accentuate the allergic response, but conversely is required for dampening down the Th2 response. For example, administration of 12 hourly injections of recombinant IFNγ for 24 hours before and after challenge with OVA in previously sensitised mice prevented the infiltration of both eosinophils and CD4 T cells into the trachea. In addition they found that administration of a single injection of anti-IFNγ antibody 24 hours before OVA challenge showed the opposite, with infiltration of eosinophils and CD4+ T cells into the trachea being increased by 60 and 53% respectively, relative to an IgG control (Iwamoto *et al.*, 1993). No IFNγ was detected in any of the patients tested. It was anticipated that more IFNγ would be detected in the healthy controls and the absence of IFNγ in the asthmatics was expected, on the basis that a lack of IFNγ in the asthmatics could partly explain the inappropriate Th2 response. This questions whether there had been any experimental error during the procedure of the assay.

There was a significant difference in the amounts of CCL2 detected in the challenged samples of healthy and asthmatic subjects. A number of studies have found raised levels in asthmatics and the findings of the present study are in support of this. Significant increases in the concentration of this chemokine in the BAL of allergic asthmatics in comparison to the healthy control subjects has been demonstrated (Jahnz-Rozyk *et al.*, 1997, Alam *et al.*, 1996, Folkard *et al.*, 1997). This cytokine was expected to be found in raised levels in the asthmatics in addition to after challenge. This was noted by Holgate and colleagues after segmental allergen challenge (Holgate *et al.*, 1997).

In conclusion, intriguing responses have been shown in the bronchial explant study so far (IL-5, CCL2, CCL17, CCL22, IL-13), some of which confirm previous observations. Other observations (IL-2, CCL11, CCL19, CCL20 and CCL5) were less expected and are worthy of further investigation. Increases in patient numbers would

be beneficial in order to strengthen the results and the analysis of the results using Principal Component Analysis may have given additional informative statistics.

Figure 4.1

Figure 4.1. Concentration of IL-2 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 19 mild asthmatics (bottom). The difference in the unchallenged samples between the healthy and asthmatic subjects was statistically significant with a value of p=0.001.

Figure 4.2

Figure 4.2. Concentration of IL-4 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 19 mild asthmatics (bottom).

Figure 4.3

Figure 4.3.   Concentration of IL-5 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 18 mild asthmatics (bottom). The difference in the challenged samples between the healthy and asthmatic subjects was statistically significant with a value of p=<0.001.

Figure 4.4

Figure 4.4.   Concentration of IL-10 in supernatant per milligram of
biopsy tissue for healthy controls and mild asthmatics. Panels show
individual data of both unchallenged (left) and allergen challenged (right)
samples for 14 healthy controls (top) and 18 mild asthmatics (bottom).

Figure 4.5

Figure 4.5. Concentration of IL-13 in supernatant per milligram of
biopsy tissue for healthy controls and mild asthmatics. Panels show
individual data of both unchallenged (left) and allergen challenged (right)
samples for 14 healthy controls (top) and 19 mild asthmatics (bottom). The
difference in the challenged samples between the healthy and asthmatic
subjects was statistically significant with a value of p=0.014.

Figure 4.6

Figure 4.6. Concentration of TNFα in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 9 mild asthmatics (bottom).

Figure 4.7

Figure 4.7.   Concentration of CCL17 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 18 mild asthmatics (bottom). The difference in the challenged samples between the healthy and asthmatic subjects was statistically significant with a value of p=0.001 and for the unchallenged samples between the healthy and asthmatic subjects p=0.008.

Figure 4.8

Figure 4.8.   Concentration of CCL22 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 17 mild asthmatics (bottom). The difference in the challenged samples between the healthy and asthmatic subjects was statistically significant with a value of p=0.004.
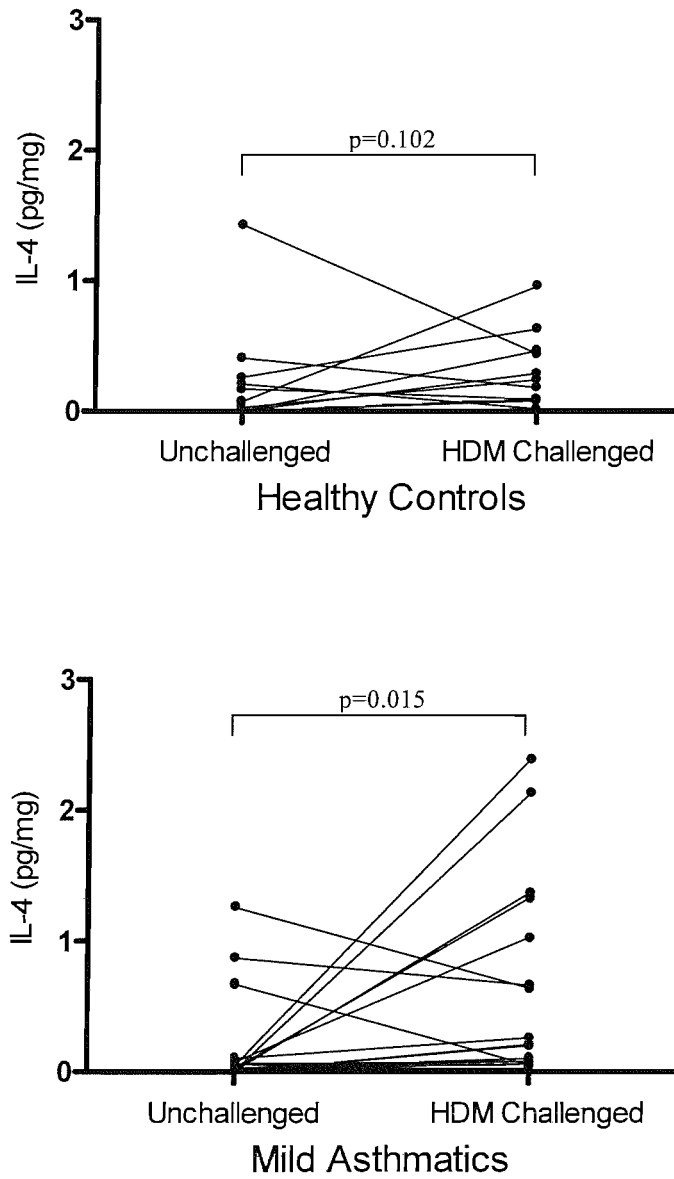
Figure 4.9

Figure 4.9. Concentration of CCL2 in supernatant per milligram of
biopsy tissue for healthy controls and mild asthmatics. Panels show
individual data of both unchallenged (left) and allergen challenged (right)
samples for 14 healthy controls (top) and 19 mild asthmatics (bottom). The
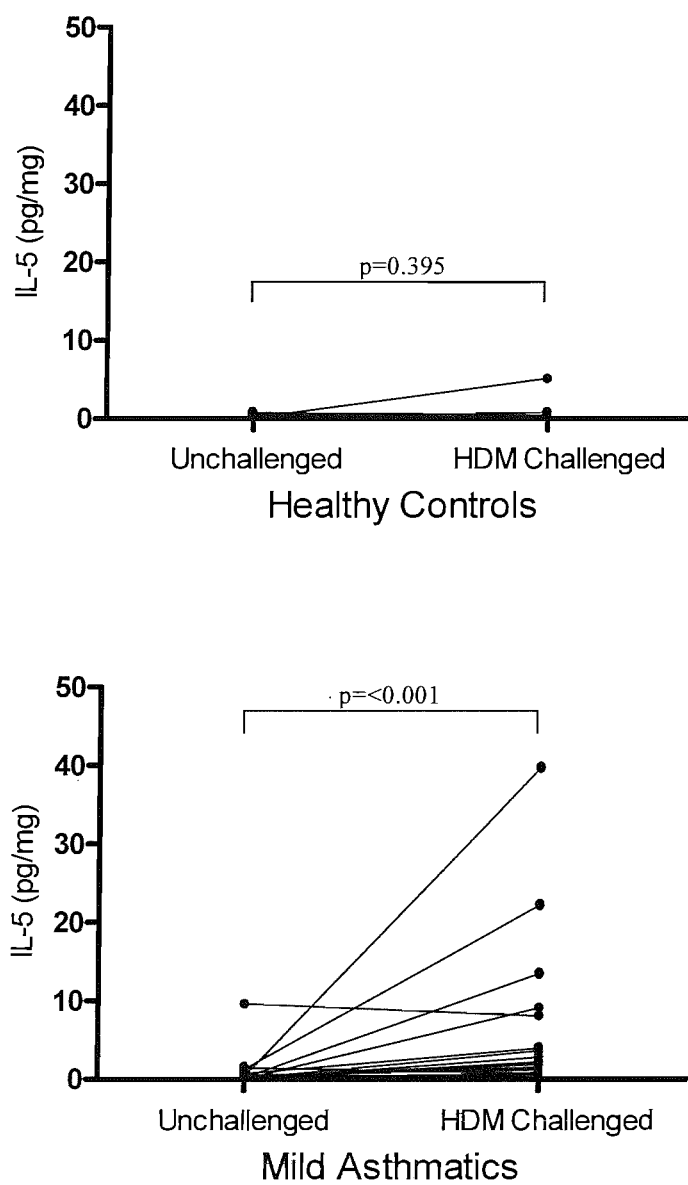difference in the challenged samples between the healthy and asthmatic
subjects was statistically significant with a value of p=0.013.

Figure 4.10
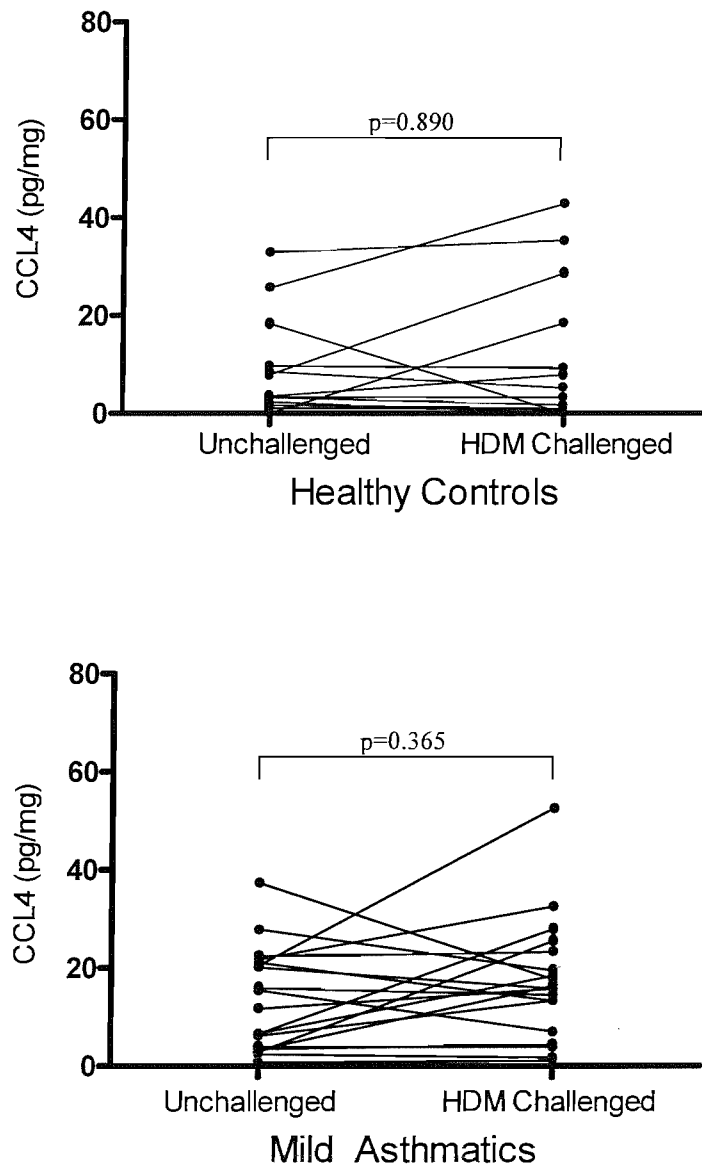
Figure 4.10. Concentration of CCL4 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 19 mild asthmatics (bottom).
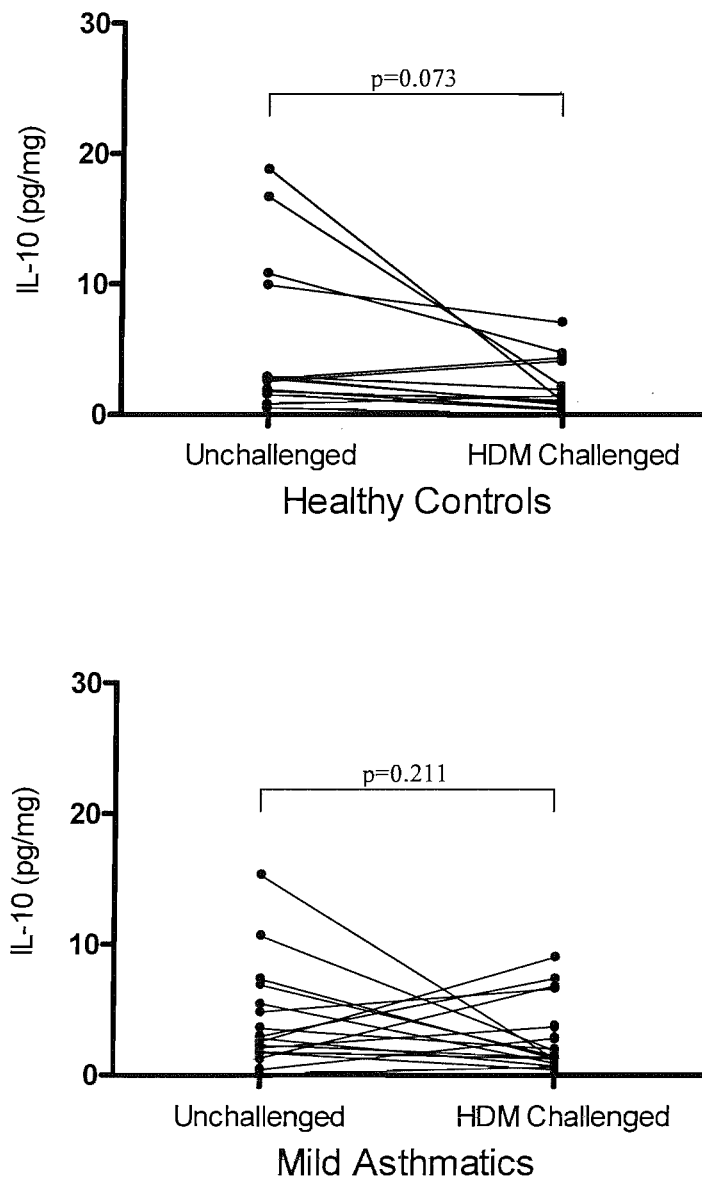
Figure 4.11
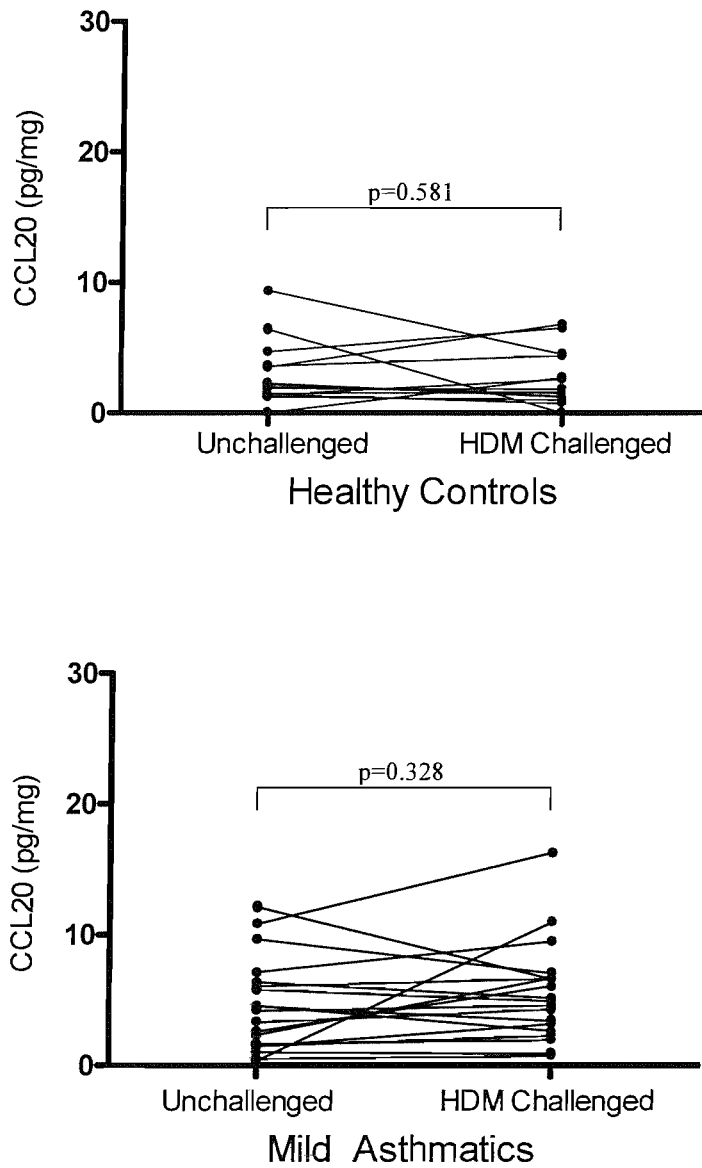
Figure 4.11.   Concentration of CCL20 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 19 mild asthmatics (bottom). The difference in the challenged samples between the healthy and asthmatic subjects was statistically significant with a value of p=0.013.
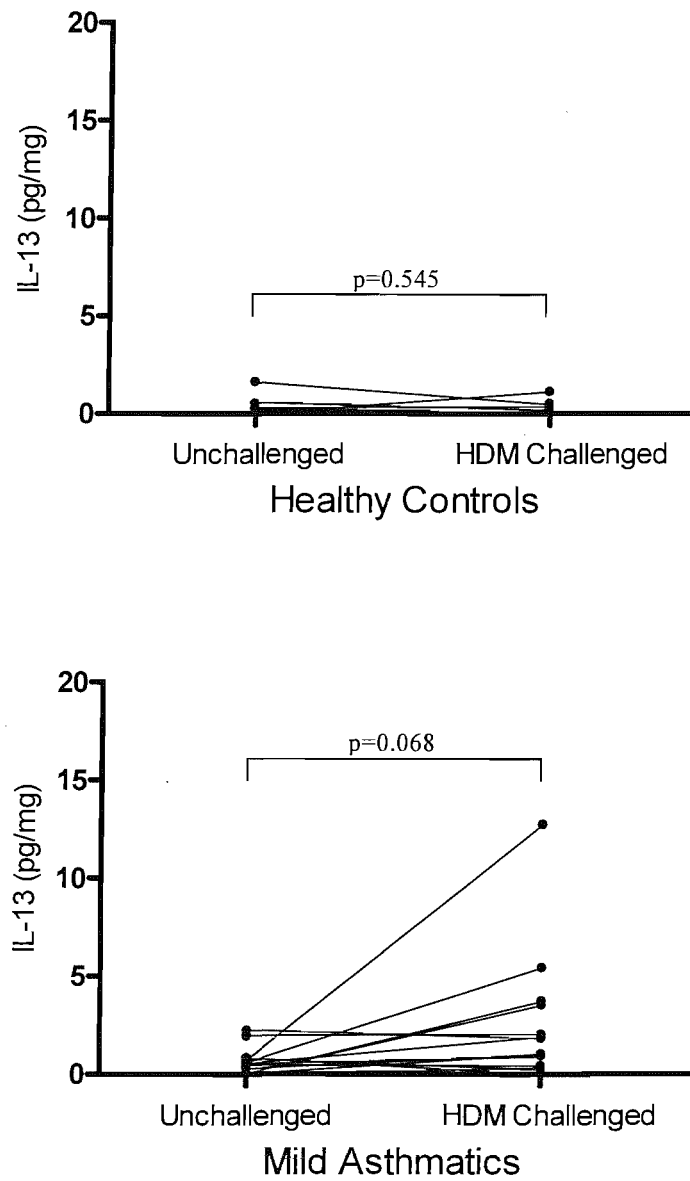
Figure 4.12
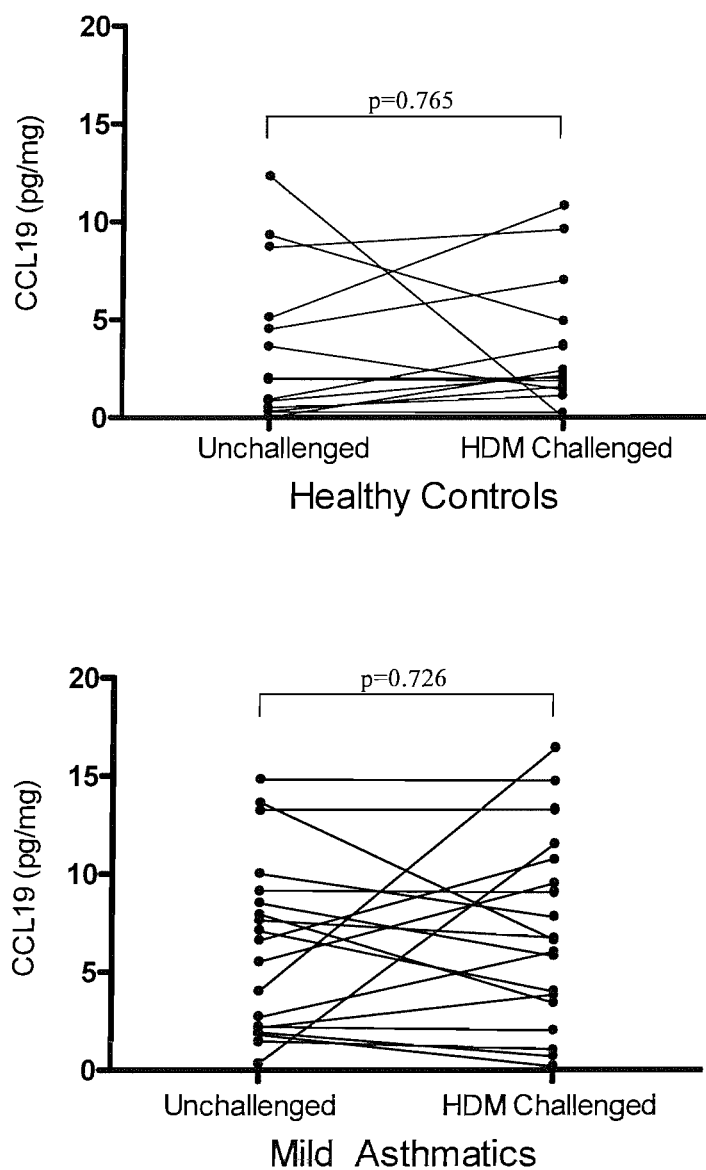
Figure 4.12. Concentration of CCL19 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 19 mild asthmatics (bottom). The difference in the challenged samples between the healthy and asthmatic subjects was statistically significant with a value of p=0.040.

Figure 4.13
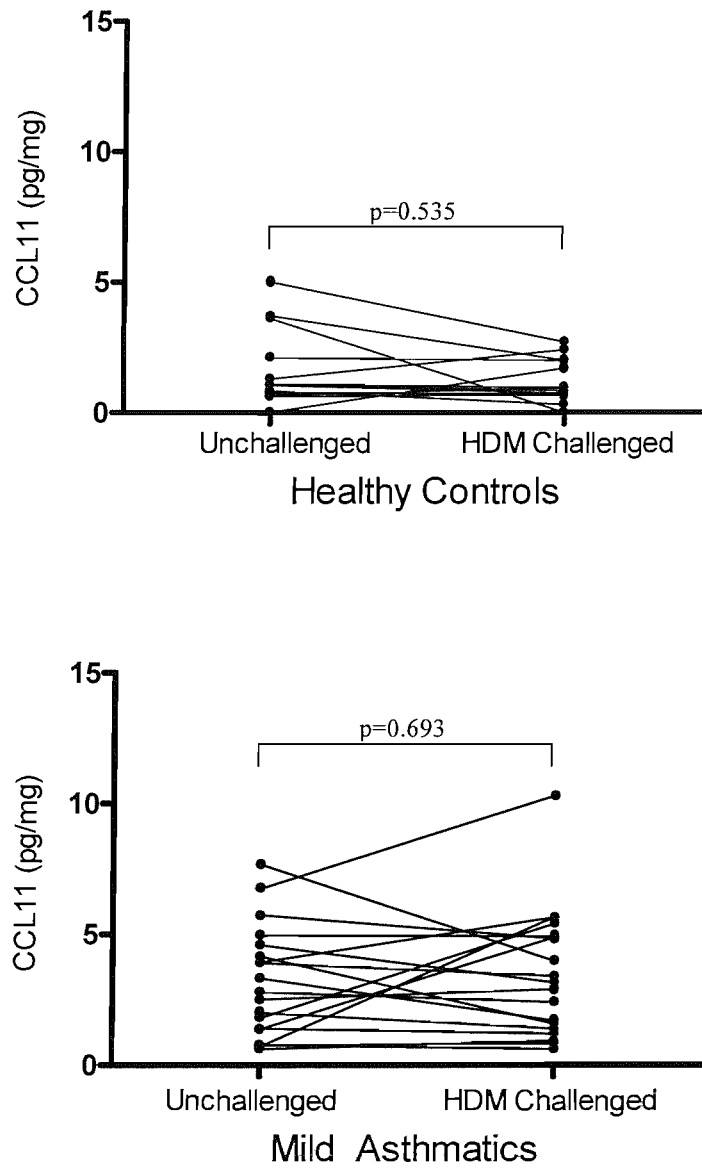
Figure 4.13. Concentration of CCL11 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 19 mild asthmatics (bottom). The difference in the challenged samples between the healthy and asthmatic 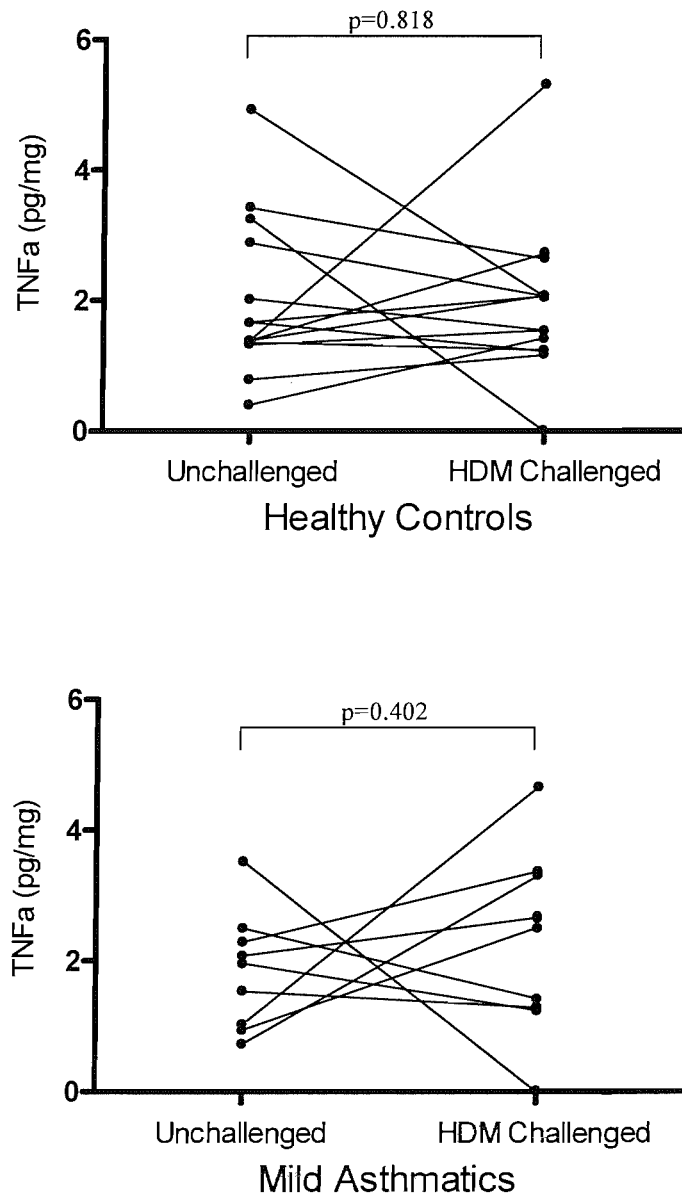subjects was statistically significant with a value of p=0.002 and for the unchallenged samples between the healthy and asthmatic subjects p=0.029.
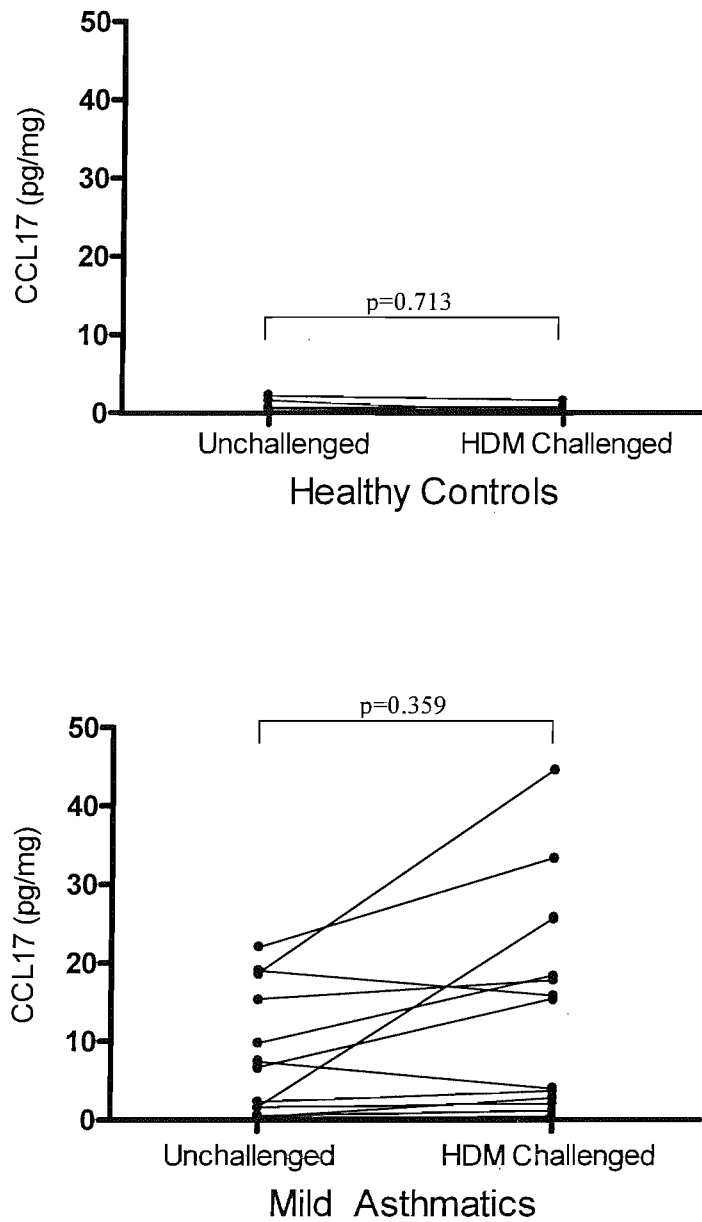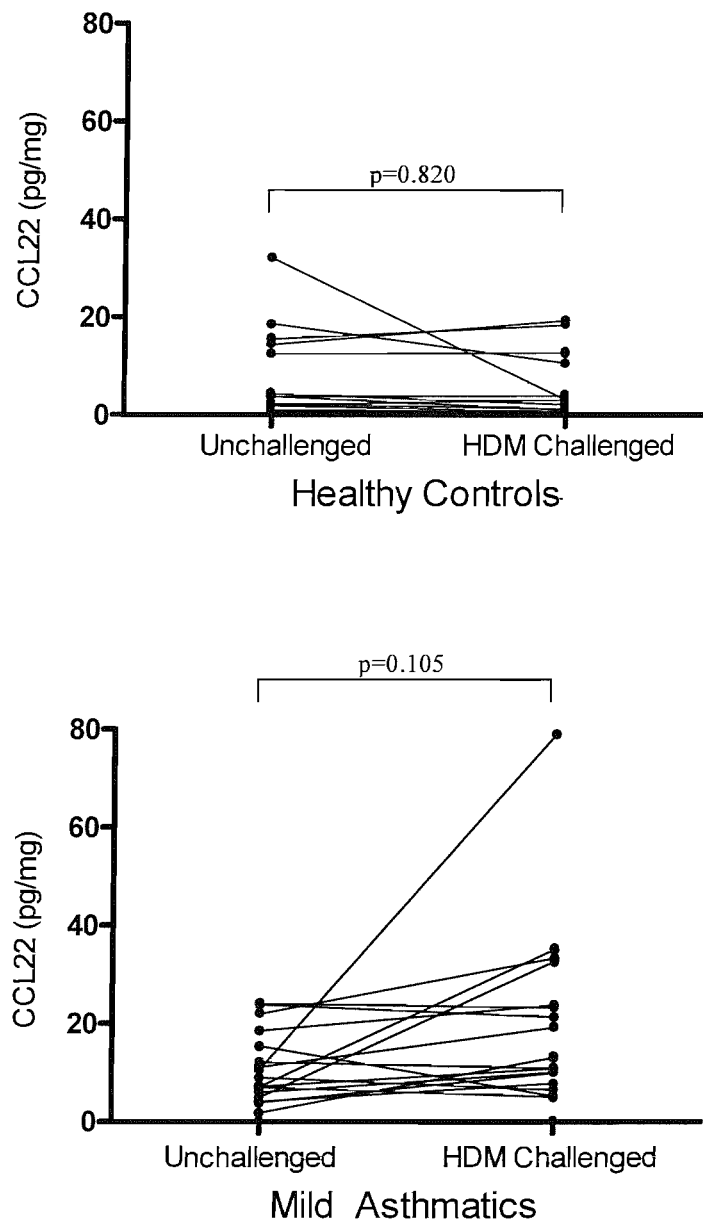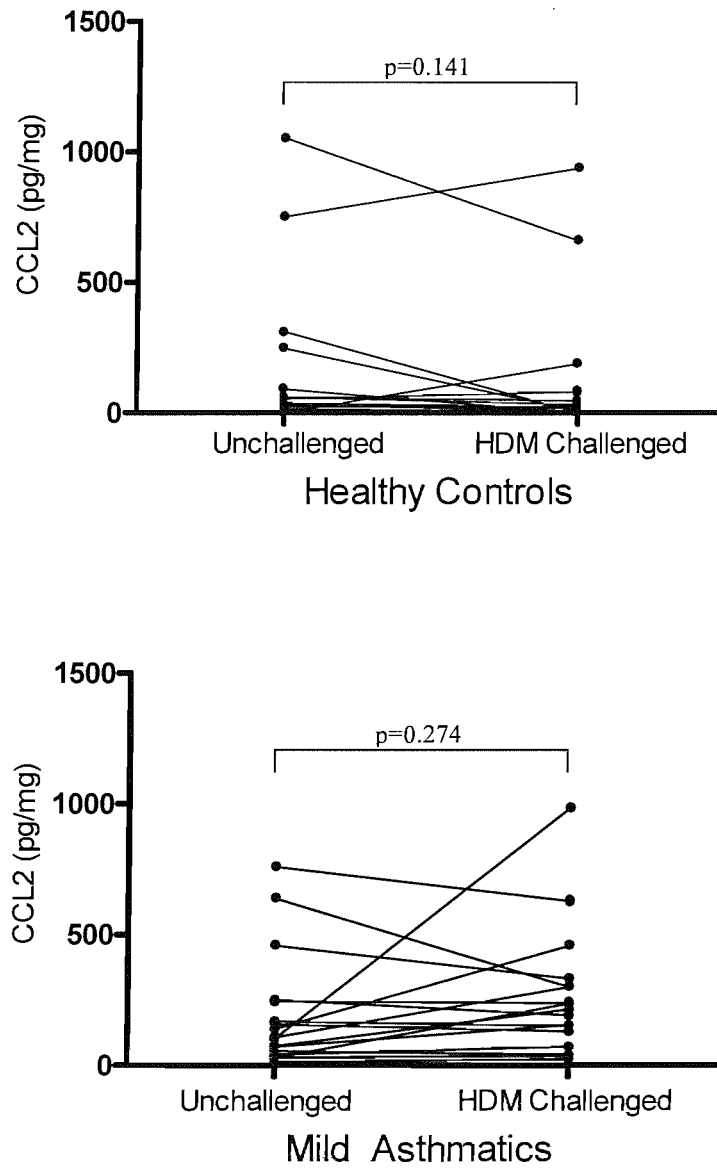
Figure 4.14
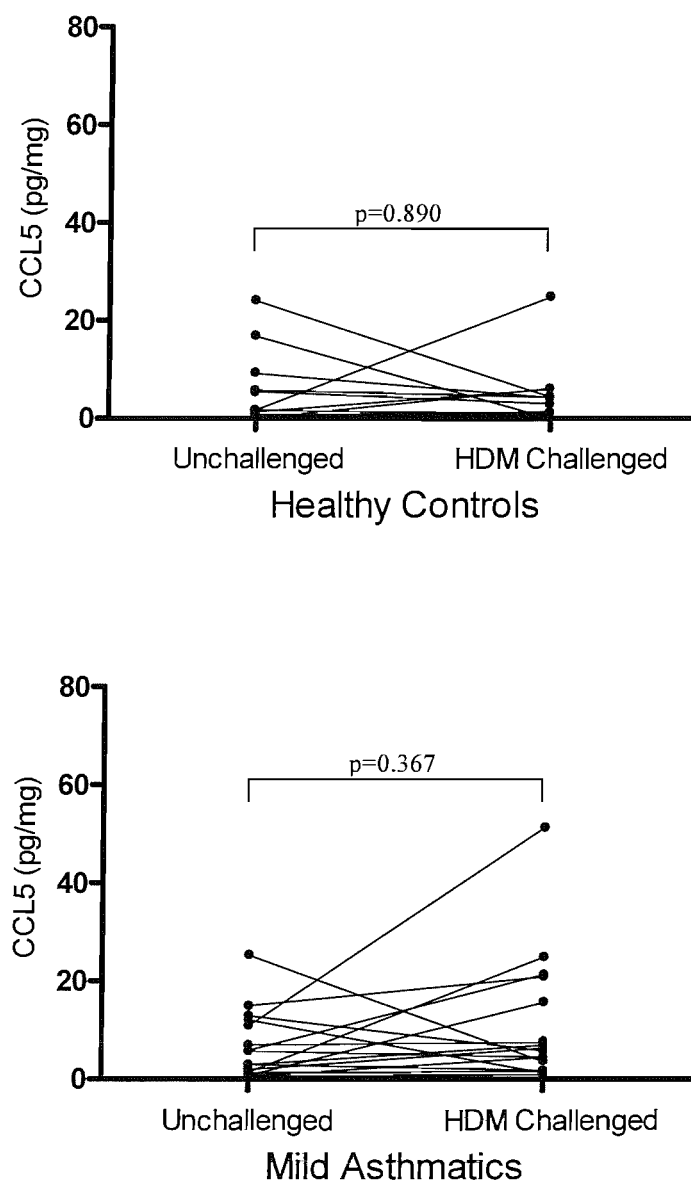
Figure 4.14. Concentration of CCL5 in supernatant per milligram of biopsy tissue for healthy controls and mild asthmatics. Panels show individual data of both unchallenged (left) and allergen challenged (right) samples for 14 healthy controls (top) and 18 mild asthmatics (bottom).

Table 4.1

Cytokine/ Chemokine	Statistical significance			
	HC unchallenged/ challenged	MA unchallenged/ challenged	HC unchallenged/ MA unchallenged	HC challenged/ MA challenged
IL-2	**0.015**	0.905	**0.001**	0.570
IL-4	0.102	**0.015**	0.866	0.488
IL-5	0.395	**<0.001**	1.000	**<0.001**
IL-10	0.073	0.211	0.676	0.582
IL-13	0.545	0.068	0.176	**0.014**
CCL17	0.713	0.359	**0.008**	**0.001**
CCL22	0.820	0.105	0.234	**0.004**
TNFa	0.818	0.402	0.875	0.468
CCL2	0.141	0.274	0.500	**0.013**
CCL4	0.890	0.365	0.135	0.074
CCL20	0.581	0.328	0.216	**0.013**
CCL19	0.765	0.726	0.051	**0.040**
CCL11	0.353	0.693	**0.029**	**0.002**
CCL5	0.890	0.367	0.425	0.091

Table 4.1. Table of statistics. Four different statistical values displayed for each of 14 cytokines/chemokines. Significant values are shown in bold type and are representative of significant increases in cytokine/chemokine production.

Chapter 5

Results

The role of CCR4 and its ligands CCL17 and CCL22 in

T cell chemotaxis in atopic asthma

5.1   Introduction

Recruitment of T cells into the asthmatic airways requires an interaction between adhesion molecules expressed on endothelial cells and epithelial cells, and their ligands as well as an interaction between chemokines and other chemoattractant factors (cytokines and lipid mediators). The chemoattractants produced locally in the airways serve to facilitate the recruitment of lymphocytes and other cell types by inducing their migration along a concentration gradient, to the site of inflammation.

The general migration of T cells is a well characterised phenomenon, with the identification of T cells displaying migratory responses towards several chemokines, cytokines and lipid mediators. Much of the published data has focused on PBMC or naïve T cells which have been treated with cytokines and anti-cytokine antibodies in order to polarise them either towards Th1 and Th2 (Austrup *et al.*, 1997, Colantonio *et al.*, 2002, Andrew *et al.*, 2001) prior to assessing expression of chemokine receptors and performing chemotaxis assays. Although advances have been made using single chemokines as chemotactic agents, to date, the combined effect of a cocktail of chemokines on T cell recruitment has been limited. Research has demonstrated enhanced chemotaxis of CCR4 transfected T cells when combinations of recombinant chemokines and cytokines have been used as the chemoattractant (Sebastiani *et al.*, 2005) and such synergism may exist for native chemokines and cytokines produced locally in the airways. To what extent these observations are related to what happens *in vivo* is unclear. Recent research conducted in Southampton has investigated the role for cytokines IL-16 and CCL5 (RANTES) in mild atopic asthma using a bronchial explant model (Hidi *et al.*, 2000, Dent *et al.*, 2002). This has shown that significant, but not all of the activity is due to IL-16, a well known chemoattractant for CD4+ T cells, with less dependency on CCL5. In view of extensive reports of other cytokines and chemokines in BAL, it is entirely plausible that other cytokines/chemokines could be equally important.

The majority of T cells migrating into the airways after allergen challenge in mild atopic asthmatics have been shown to express CCR4 (Panina-Bordignon *et al.*, 2001),

thus implicating CCL17 and CCL22 in the recruitment process. As a result of findings of this nature, much attention has been focused on the proposed inhibitory effects of blocking of CCR4 (Conroy *et al.*, 2003, Chvatchko *et al.*, 2000) or its ligands CCL17 and CCL22 (Lloyd *et al.*, 2000) and the effect this could have on both T cell migration and any downstream effects on airway inflammation and the ensuing bronchial hyperresponsiveness.

The aims of the experiments reported in this chapter were to evaluate the contribution of the CCR4 chemokine receptor to the chemotactic activity that has now been repeatedly demonstrated in the explant model and to thereby shed more light on the relative role of CCR4 in mild asthma. To this extent the bronchial explant model was used to generate allergen-induced chemotactic activity and to evaluate its specificity for CCR4 using 2 responder cell types that bear CCR4 on their surface: 1) the CCRF-CEM cell line that exclusively expresses CCR4, and 2) T cells polarised towards the Th2 phenotype using IL-4 and anti-IFNγ, which have previously been shown to express significant amounts of CCR4. Both of these cell types have been shown previously to respond to the CCR4 ligands CCL17 and CCL22. At the same time the expression of other receptors was investigated, in addition to CCR4, namely, CRTH2, CCR2, CCR3, CCR5, CCR7, CCR8 and CXCR3 on both CCRF-CEM cells and the PBMC throughout the duration of their polarisation. In selected experiments, a specific blocking molecule to CCR4 was used, provided by Glaxo SmithKline. This molecule had been developed by researchers at Glaxo SmithKline and characterised for its specificity of inhibition of responsiveness to CCR4 ligands.

Methods

Cell surface expression of chemokine receptors was performed by standard flow cytometry as described in chapter 2. The results were expressed as the percentages of cells staining positively as appropriate.

CCRF-CEM cells and polarised PBMC were used in the chemotaxis assays. CCRF-CEM cells, a cell line expressing high levels of CCR4 was purchased from European

Collection of Cell Cultures (Salisbury, UK). Th2 polarised cells were obtained using standard protocols as described in detail in chapter 2. Briefly, PBMC were isolated from peripheral blood, using standard procedures (chapter 2). In order to maximise CCR4 expression, PBMC were subjected to a 14 day culture protocol to polarise the cells towards either a Th1 or Th2 phenotype. To polarise cells towards the Th2 phenotype, they were treated on day 0 and day 8 with PHA (1µg/ml), αIFNγ (5µg/ml) and IL-4 (50ng/ml) and on day 4 and 12 with IL-2 (10ng/ml). The changes in CCR4 expression were monitored at baseline, day 7 and day 13.

The different cell types (CEM cells or polarised cells) were stained with the fluorescent dye, Calcein (Molecular Probes, Netherlands) and fluorescence values indicative of migration were obtained by analysing the migrated cells in the bottom wells of the chemotaxis plates as a measure of chemotactic activity.

Calcein stained cells were adjusted to the desired density and recombinant chemokines and/or biopsy supernatants were diluted and placed into the lower wells of the chemotaxis plate and a membrane filter was fitted securely on top. Calcein stained polarised cells and/or CCRF-CEM cells were pipetted on top of the filter directly above each chemokine solution/biopsy supernatant containing well. The plate was incubated for 3 hours and read using a fluorescence plate reader. Cell migration was then expressed as an index of chemotaxis (the fluorescence value of cells migrating to the chemoattractant divided by the fluorescence value of cells migrating spontaneously).

5.2 Results

Firstly, the expression of a panel of chemokine receptors on the CCRF-CEM cell line was investigated.

5.2.1 Surface expression of CCR4 on the CCRF-CEM T cell line

The flow cytometry dot plot displays the forward and side-scatter characteristics of the CEM cell line (Fig 5.1). There were no sub-populations of cells and the majority of the cells were larger than blood derived PBMC. The histogram indicates the percentage of CCR4 expression on the cell line (Fig 5.1) and the majority of the cells (96.61%) were positive for CCR4.

5.2.2 Expression of 8 chemokine receptors on the CCRF-CEM T cells line

Although the description of the cell line was such that it had high levels of expression of CCR4, the presence/absence of other chemokine receptors was unknown to the manufacturers. To this end, flow cytometry was used to check for the expression of a panel of chemokine receptors.

The percentage expression of CCR4 on the cell line was 98.96 and these CCR4 positive cells were all positive for CD4 (Fig 5.2). There was only non-specific background staining seen for chemokine receptors other than CCR4.

5.2.3 Chemotaxis of CCRF-CEM cells to 6 recombinant chemokines

It was anticipated that the CEM cells would migrate only to CCR4 ligands CCL17 and CCL22 and in order to confirm this, the chemotaxis of CEM cells to 6 recombinant chemokines was investigated.

CCL22 induced a typical bell-shaped chemotactic response, with the concentration shown to induce the greatest CEM cell migration being 4nM. The chemotactic indices reflect the migratory responses of the cells with the maximum point of migration, for the three experiments shown (Fig 5.3) being 51.12, 105.7 and 24.99. Chemotaxis to

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CCL17 was also shown but to a lesser extent than that of CCL22: in 2 experiments (Fig 5.3 a and c), the maximal amount of migration to CCL17 was observed at concentrations three times higher than the concentration of CCL22. There was no CEM cell migration to CCL5, CXCL10, CCL2 or PGD_2.

5.2.4   Inhibition of CCL22 mediated chemotaxis of CCRF-CEM cells with a
            CCR4 blocking molecule

Before applying the CCR4 blocking molecule to the supernatants of the bronchial explants, the specificity and potency of this antagonist was investigated. Chemotaxis to a constant CCL22 concentration of 3.7nM was inhibited by the blocking molecule in a dose-dependent manner (Fig 5.4), with an IC_{50} of 26.1nM.

5.2.5   Chemotaxis of CCRF-CEM cells to bronchial biopsy supernatants from
            6 healthy controls and 6 mild asthmatics

Having demonstrated that CEM cells were responding in a typical bell-shaped dose-response manner to the CCR4 ligands CCL17 and CCL22, and having demonstrated that allergen challenge induced increased release of both these chemokines *ex vivo* in the explant model (chapter 4), the next aim was to determine whether the chemotactic activity in the supernatants was specific for CCR4.

There was no migration of CEM cells when exposed to bronchial biopsy supernatants from either healthy control or asthmatic subjects using either the supernatants from unstimulated or allergen-stimulated explants (Fig 5.5). In control experiments CCRF-CEM cells were seen to migrate well to CCL22, with a chemotactic index of 5.26, indicating that the experimental conditions of the chemotaxis assay were appropriate.

5.2.6   Expression of 8 chemokine receptors on PBMC at baseline levels in healthy control and asthmatic subjects

Having failed to demonstrate that the supernatants of the bronchial biopsy explants have significant chemotactic activity for the cells which express only CCR4 (the CEM cell line), PBMC polarised towards the Th2 phenotype were assessed for migration to the biopsy supernatants. Flow cytometry was used to assess chemokine receptor expression.

The data presented includes polarisation experiments using PBMC from 3 mild atopic asthmatics and 3 healthy non-atopic individuals. The median percentage expression of CCR4 on PBMC at baseline in healthy controls and asthmatics was 15.51% and 14.47% respectively (Fig 5.6). There were also no differences in the expression of CRTH2, CCR2, CCR3, CCR5, CCR7, CCR8 and CXCR3 between the two subject groups.

5.2.7   Expression of 8 chemokine receptors on PBMC after 1 week of Th1 polarising culture conditions in healthy controls and mild asthmatics

After one week of polarisation treatments using cells from 3 asthmatic and 3 healthy non-atopic donors, the median percentage expression of CCR4 on cells polarised towards the Th1 phenotype for the healthy and asthmatic subjects were 15.18 and 30.81% respectively. This was an increase of 16.34 and 7.43% from baseline levels for the asthmatic and healthy controls respectively. There were little changes in the expression of CRTH2, CCR2, CCR3 and CCR8 which were expressed on only a very small percentage of cells at baseline and week 1 in both the healthy and asthmatic subjects. The median percentage expression of CCR7 for the healthy and asthmatic subjects were 31.93 and 25.5% respectively and these values were slightly lower than baseline levels, as was the expression of CCR5.

5.2.8   Expression of 8 chemokine receptors on PBMC after 1 week of Th2
            polarising culture conditions in healthy controls and mild asthmatics

The median expression of CCR4 for the cells polarised towards the Th2 phenotype was higher than both the levels at baseline and the expression on the Th1 polarised cells. The median percentage expression was 73.28 and 36.23% for the asthmatic and healthy subjects respectively. There were little changes in the expression of CRTH2, CCR2, CCR3, CCR5, CCR8. CXCR3 was higher in the asthmatics.

5.2.9   Expression of 8 chemokine receptors on PBMC after 2 weeks of Th1
            polarising culture conditions in healthy controls and mild asthmatics

Median percentage expression of CCR4 was 12.8 and 7.52% in the asthmatic and healthy control subjects respectively and these values were slightly lower that those at week 1. CRTH2, CCR2, CCR3 and CCR8 were expressed on a very small percentage of cells in both subject groups. CCR5 was expressed on a higher percentage of cells in the asthmatics and the expression of CCR7 was the same for the asthmatics and the healthy controls.

5.2.10   Expression of 8 chemokine receptors on PBMC after 2 weeks of Th2
            polarising culture conditions in healthy controls and mild asthmatics

The median expression of CCR4 in the healthy and asthmatic subjects for these cells was 70.45 and 61.6% respectively which was greatly increased from baseline and also the Th1 polarised cells. The expression of CRTH2 was higher in the healthy controls. CCR2, CCR3, CCR5 and CCR8 were found at very low levels and were similar in the two subject groups. The expression of CXCR3 was higher than those of the Th1 polarised cells in both the asthmatic and healthy subjects and the same trend was seen for CCR7.

5.2.11   Chemotactic responses of Th1 and Th2 polarised cells from 2 healthy
              donors to CCL22

Two experiments using Th1 and Th2 polarised cells obtained from PBMC of 2 healthy
donors were performed. As anticipated, Th2 polarised cells migrated better towards
CCL22, with a peak chemotactic index of 2.3, than Th1 cells which failed to migrate.
The difference in the chemotactic responses of Th1 and Th2 cells was a reflection of
their expression of CCR4 (Fig 5.11) which was higher in both subjects within the Th2
polarised population. For subject 1, 31.77% and 7.21% percent of total cells were
CCR4+ in the Th2 and Th1 populations respectively. In subject 2, the difference was
even more striking; the percentage of CCR4+ cells in this donor was 8.13% in the Th1
population and 68.11% in the Th2 population. Interestingly, even though subject 2 had
a higher proportion of CCR4+ cells, the chemotactic response to CCL22 were similar
to those of subject 1, with maximal migration being seen at 11.11nM.

5.2.12   Chemotaxis of Th2 polarised cells from healthy control subjects to 6
              recombinant chemokines

Having demonstrated good chemotactic responses of polarised cells to CCL22, a
known and potent ligand for CCR4, an assessment of the polarised cells in respect of
chemotacic responsiveness to other ligands followed.

The Th2 polarised cells from 3 healthy control subjects migrated substantially to
CCL22 with maximal migration of the Th2 cells seen at concentrations of
approximately 3nM and chemotactic indices of 10.49, 10.09 and 9.65 for subjects a-c
respectively (Fig 5.12). The chemotactic index was also greater than 1 for CCL17 as
expected, but was also greater than 1 in one subject for CCL2. No migratory activity
to CCL5, CXCL10 and PGD_2 was noted.

5.2.13   Chemotaxis of Th2 polarised cells from mild asthmatic subjects to 6
              recombinant chemokines

The Th2 polarised cells from the asthmatic subjects also migrated predominantly to
CCL22 however the chemotactic index at the highest point of migration was

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approximately 1.30, 3.86 and 4.91 for subjects a-c respectively. This was lower than the indices for the healthy subjects. The concentration of CCL22 which induced the most migration was approximately 3nM in each patient and was the same as that of the healthy patients (Fig 5.13).

5.2.14   Chemotaxis of Th2 polarised cells from healthy controls and asthmatics
             to biopsy supernatants from 6 healthy controls and 6 asthmatics

Having demonstrated that the polarised cells, expressing CCR4, migrate well in response to CCL22, two sets of experiments were performed using explant supernatants as the source of chemotactic activity. As responder cells, in these experiments Th2 polarised cells from two subjects were used; a mild asthmatic subject and a healthy non-atopic subject.

The Th2 polarised cells from both the healthy and asthmatic subjects did not migrate to the healthy control and asthmatic biopsy supernatants respectively (Fig 5.14). Migration of the Th2 cells from both patients was induced by CCL22 and this migration was blocked, partially by the GSK CCR4 blocking molecule.

5.2 Discussion

Chemokines and their receptors are important ligand-receptor pairs which serve to mediate cell trafficking. In asthma and allergic responses specific receptors and their corresponding ligands have been found to be important for the migration of T cells into the airways. For this chapter, the aim was to determine the extent to which CCR4, the receptor for CCL17 and CCL22, expressed preferentially on Th2 cells, is responsible for cell migration into the airways of allergic asthmatic subjects.

Using a T cell line and PBMC, which had been polarised towards the Th2 phenotype, these cell types were characterized with respect to their chemokine receptor expression and their migration to recombinant chemokines. Having found both these cell types had a high percentage of CCR4 expression and responded well to recombinant CCL22 in a typical bell-shaped dose-response manner which could be inhibited by a CCR4 antagonist, they were used in chemotaxis assays, where the supernatants derived from the culture of bronchial biopsies were used as the chemoattractant stimuli. Supernatants from the culture of healthy control and mild asthmatic bronchial biopsies that had been treated either with or without HDM allergen were used and the extent to which CCR4 was responsible for migration was determined by blocking cell migration with a CCR4 blocking molecule. In a series of 6 experiments using supernatants from healthy control subjects, no chemotactic responses to the bronchial biopsy supernatants with either the CCRF-CEM cell line or the T cells polarised *ex vivo* with IL-4 and anti-IFNγ were observed. The data so far, therefore concludes one of two things: 1) that the chemotactic activity, generated when asthmatic tissue is stimulated with allergen, is not specific for either CCR4 or indeed Th2 T cells or 2) that the model, i.e. the use of a T cell line and Th2 cells polarised *ex vivo* is not an appropriate model to test T cell chemotactic activity.

In the first series of experiments, the CCRF-CEM cell line appeared to be a good model for the objectives of this chapter due to the large majority of cells expressing high levels of CCR4. Flow cytometry demonstrated that almost all the cells were positive for CCR4. This finding was in accordance with Cronshaw and colleagues

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who demonstrated the ability of CCL22 to activate phosphoinositide 3-kinase and induce chemotaxis of the CEM T cell line to CCL22 (Cronshaw et al., 2004). These authors found that CEM cell chemotaxis to CCL22 resulted in a classic bell-shaped dose-response curve which is in line with the results found in the present study. The optimal concentration of CCL22 in their study was 1nM (Cronshaw et al., 2004) which is slightly lower than that seen in the current study in which 3nM concentrations of CCL22 induced the greatest degree of cell migration. This is, however, not a marked difference and could be due to the fact that Cronshaw and colleagues selected chambers with a pore size of 5μm whilst the pore size chosen for the present piece of work was 3μm. In their study, Sebastini and colleagues demonstrated that 10nM was the optimal CCL22 concentration for inducing Th2 cell chemotaxis and the use of 1nM resulted in no cellular migration (Sebastiani et al., 2005). In agreement with Cronshaw and co-workers, these authors used a pore size of 5μm.

Having failed to see significant migration to explant supernatants of the CCRF-CEM cell line, polarised T cells were used as the source of responder cells. Initial investigation by flow cytometry of the expression of CCR4 and the other 7 chemokine receptors on the surface of PBMC that were polarised into Th2 cells, showed evident differences in receptor expression when compared with the CCRF-CEM cell line. The cells derived in culture of PBMC for 14 days expressed CCR4, CCR5, CCR7, CXCR3 and CRTH2 to varying degrees, whereas the CCRF-CEM cells expressed only CCR4, suggesting that these Th2 cells, armed with a panel of chemokine receptors, might migrate better to biopsy supernatants than the CEM cells. Previous studies have investigated chemokine receptor expression on Th1 and Th2 polarised cells. For instance, D'Ambrosio and colleagues generated Th1 and Th2 cell clones from cord blood lymphocytes, following the same method as that used in the present study (D'Ambrosio et al., 1998). Using northern blot analysis, they found that there was a large difference in CCR4 mRNA expression between the Th1 and the Th2 cells, where no appreciable mRNA expression for CCR4 was found in the Th1 cells in comparison to the Th2 cells (D'Ambrosio et al., 1998). In the present study, differences in the expression of CCR4 between the two cell subtypes were noted for both healthy and

asthmatic subjects but Th1 cells still expressed appreciable amounts of CCR4. With regard to CCR5, D'Ambrosio and colleagues found that mRNA was much greater in the Th1 cells.

The results on the expression of CXCR3 in the present study did not appear to differ to the same extent as those observed by D'ambrosio and colleagues, who found a greater difference between the Th1 and the Th2 generated cells. However, the data generated by Annunziato and co-workers on the expression of CXCR3 shows that after stimulation, CXCR3 expression was raised to high levels, irrespective of whether the cells had been conditioned with IL-4 or IL-12 (Annunziato *et al.*, 1999) and these results are similar to those presented here. Akin to the present study, D'Ambrosio *et al* found the expression of CCR3 and CCR8 to be negligible in both cell subtypes. (D'Ambrosio *et al.*, 1998). Although Colantonio and colleagues showed a drop in CCR7 expression on both Th1 and Th2 cells (Colantonio *et al.*, 2002), different results were shown by Burgstahler and co-workers who reported the opposite, in that CCR7 was up-regulated upon antigen stimulation (Burgstahler *et al.*, 1995) and the present work supports this finding. It is worth considering that the down-regulation and/or up-regulation of receptors on the polarised Th2 cells might have rendered the cells unresponsive to any synergistic effects that the different combinations of chemokines found in the biopsy supernatants might have had and this could be an explanation for the lack of cell migration to these supernatants.

Many research groups have investigated the combined effects of one or more chemokines on cell migration in order to discover to what extent individual chemokines are responsible for the migration of different cell types. However, much of the published work has involved the use of recombinant chemokines. In this respect, the culturing of bronchial biopsies *ex vivo* using the explant model is a very valuable tool. The explant model is useful for studying the effects of allergen on bronchial tissue *ex vivo*, reflecting closely the *in vivo* situation and offering several advantages over experimental *in vivo* challenge in asthmatic volunteers using inhalation of instillation of allergen via the bronchoscope. Firstly, repeat

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bronchoscopy to study the effect of allergen in the airways is not required. Secondly, tissue responses of more severe asthmatics, which cannot be challenged with allergen, can be studied. Third, problems of dilution of secreted mediators during BAL are avoided. Fourth, the produced cytokines are not consumed by in-coming inflammatory cells. Finally, and most importantly, compounds which cannot be applied *in vivo*, such as those used in this study, can be used to test concepts of immunoregulation and provide valuable information for drug development.

The explant model has provided evidence of increased production of IL-4, IL-5, IL-13, CCL17, CCL22 and a reduced production of IL-10. However, one consideration that has to be taken into account is the absolute concentration of the chemoattractants that can be detected in the explant and how the activity of these native proteins compares with recombinant chemokines. Cell migration in the present study was found to be optimal at a CCL22 concentration of approximately 3nM and the equivalent concentrations of CCL22 in the unchallenged and challenged supernatants were considerably lesser, with the mean values of supernatants being 32.12pg/mg without and 20.47pg/mg with allergen. The lowest concentration in the large majority of experiments, at which cell migration could be detected was 1nM. It is conceivable, therefore, that a higher concentration of CCL22 is required to induce cell migration *in vitro* that that found *in vivo* or in the bronchial biopsy supernatants produced *ex vivo*. In additon to the expression of numerous receptors on cells that circulate in the blood, *in vitro* cell recruitment may be dependent on the presence of multiple chemokines and other cell types. Indeed, a recent study has reported that there is synergy of CCL22 with CXCL10. This work by Sebastini and colleagues involved an investigation of the chemotactic responses of polarised Th1 and Th2 cells. They showed that when CXCL10 was added together with CCL22, the number of migrating cells increased markedly in both Th1 and Th2 generated cells in comparison to CCL22 alone (Sebastiani *et al.*, 2005). In addition, the blocking of CXCR3 on T cell lines expressing CXCR3 and CCR4 still resulted in enhanced chemotaxis when the two chemokines were combined in comparison to CCL22 alone. This suggested that the synergistic action of the two chemokines was mediated via CCR4 and not CXCR3

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(Sebastiani *et al.*, 2005). Most surprising was the revelation that it was not only CXCL10 that synergised with CCL22; an additional 20 different chemokines showed the same synergistic action, by increasing the efficacy and/or potency of CCL22 (Sebastiani *et al.*, 2005).

The efficacy of the blocking molecule kindly supplied by Glaxo SmithKline was proven by performing chemotaxis assays using the CEM T cell line. A constant CCL22 concentration of 3nM was used and cell migration was blocked in a dose dependent manner with cell migration being completely abolished at blocking molecule concentrations from 2.5µM and above. However, this experiment only demonstrated that the blocking molecule inhibited the migration of a cell line shown to express only CCR4 out of a panel of 7 other receptors but it did not determine the extent to which the molecule was specific for CCR4. Although the blocking molecule was at its most effective at concentrations of 2.5µM and above, a decision was made before these experiments were performed, to use the blocking molecule at a concentration no higher than 10nM due to questions raised by scientists from Glaxo SmithKline regarding its specificity for CCR4 alone. Whilst the issue of specificity of this blocker remains open, this is of secondary importance since no chemotactic activity of either the CEM cell line or the Th2 polarised cells was seen in response to the bronchial biopsy supernatants, so there was nothing to block.

It also must be considered that CCR4 may not be an important chemokine receptor. Two pieces of literature have provided evidence to show that this in fact may be the case. Firstly, the blocking of CCR4 has resulted in negligible changes in CCR4+ve cell recruitment into the lungs of allergen challenged guinea pigs (Conroy *et al.*, 2003). Ova sensitised guinea pigs were challenged with allergen and then treated with either an anti-CCR4 blocking antibody or a control antibody. Using flow cytometry they found that there were no differences in numbers of T cells recovered from BALF with or without the blocking antibody. Additionally, Chvatchko *et al* demonstrated that cellular recruitment and induction of airway hyperreactivity was unaltered in CCR4 deficient mice, generated by gene targeting (Chvatchko *et al.*, 2000). They used an

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OVA-induced mouse model of airway inflammation. They concluded that CCR4-/-
and CCR4+/+ mice did not differ with regards to bronchial hyperresponsiveness and
OVA-induced eosinophilia in BALF and lung tissue (Chvatchko *et al.*, 2000). During
their study, they polarised cells from mouse lymph nodes and spleens towards the Th1
and Th2 phenotype and found that cells generated from CCR4-/- mice failed to
respond to CCL22 whereas those from CCR4+/+ mice showed a strong chemotactic
response (Chvatchko *et al.*, 2000). This is in line with the results presented in this
chapter. However, these two studies do cast scepticism on the role that CCR4 plays.

In the present study, Th2 cells migrated to CCL22 and migration was blocked, albeit to
a small extent, using the Glaxo SmithKline blocking molecule. However, there was no
significant migration of either the CEM cell line or the Th2 polarised cells to the
bronchial biopsy supernatants.

The vast amount of literature on the transient changes in chemokine receptor
expression after cell stimulation demonstrates that the results between research groups
differ quite substantially and chemotaxis of the cells towards recombinant chemokines
would be likely to reflect this. Despite the lack of migration assays carried out in
pieces of work involving extensive studies of chemokine receptor expression, those
research groups who do document the chemotactic responses of polarised cells have
failed to do so in an *ex vivo/in vivo* environment. It is therefore plausible that the
failure of the cells to respond to bronchial biopsy supernatants as shown in the current
chapter, yet detection of a migratory response to CCL22, could lie in the artificial
polarisation of the cells. This may have rendered them unresponsive to the cocktail of
native chemokines that were detected in the biopsy supernatants, yet permitted them to
respond to recombinant CCL22. In essence; the sensitivity of the cells to chemokines
may have been affected by the harsh and extreme polarisation conditions. This 14 day
treatment will have resulted in the cells being committed to a phenotype that is
permanently activated and this may have resulted in decreased sensitivity of the cells.
The highest value obtained for CCL22 from the analysis of the biopsy supernatants,
before taking biopsy weight into account was 888pg/ml which is 27-thousand times

less than that of recombinant CCL22 required to induce the migration of Th2 polarised cells so it was very unlikely that the cells would migrate to the biopsy supernatants anyway. Another important point to note is that of patient variability. Due to the length of culture and the sheer cell numbers needed per assay, it was not possible to use the same batch of cells or indeed those from the same patient so this will have resulted in different migration profiles of the cells for each experiment.

Finally, the cells polarised *in vitro* may behave very differently from cells polarised *in vivo*. Asthma is a disease where Th2 cells are thought to play a major role (Robinson *et al.*, 1992, Castro *et al.*, 2000). Therefore, it is reasonable to hypothesise that the increase in Th2 cells within the asthmatic airways may result, at least partly, because of a selective recruitment of Th2 cells polarised elsewhere before migration into the airways. An alternative explanation for the Th2 accumulation lies in local, mucosa-based polarisation of naïve, or indeed memory T cells specific for allergen. The exact frequency of allergen specific T cells in the airways is unknown but in the circulation it is estimated to be 1:100,000, which is thought to be lower that the ratio in the airways.

In summary, it has not been shown that the chemotactic activity for T cells, demonstrated previously using unfractionated and unpolarised T cell populations as responder cells is dependent on CCR4. Future studies will need to address the relevance of using T cells stimulated and polarised *ex vivo* with allergen (i.e. allergen driven T cell lines or clones) as these could possibly behave in a manner that is more akin to cells that are polarised by allergen *in vitro*.

Figure 5.1
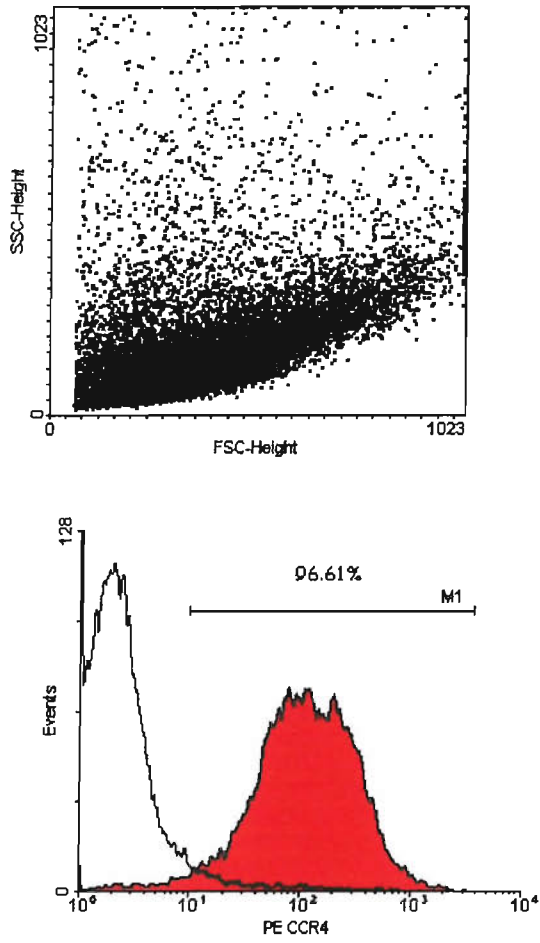
Figure 5.1   Upper panel shows the forward and side scatter plot for CCRF-CEM cells. Lower panel shows the percentage expression of CCR4 on CCRF-CEM cells.

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Figure 5.2

Percentage chemokine receptor expression on CCRF-CEM cells
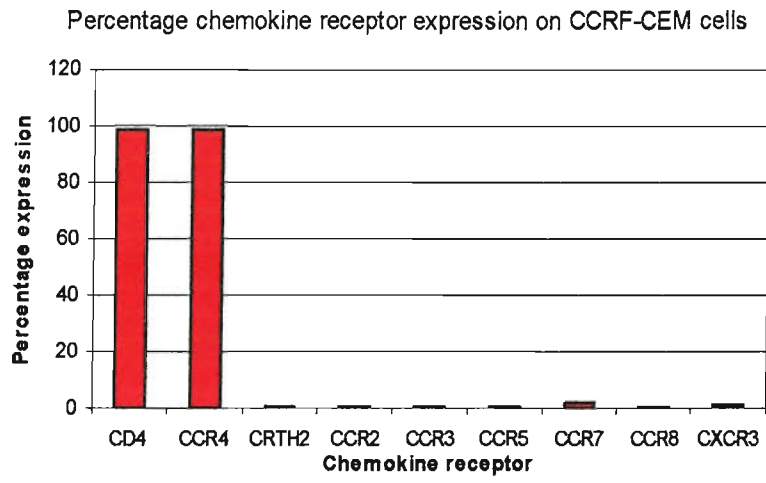
Figure 5.2   Percentage expression of 8 chemokine receptors on the CCRF-CEM cell line.

Figure 5.3 (a-c)

Chemotaxis of CCRF-CEM Cells to Recombinant Human Chemokines
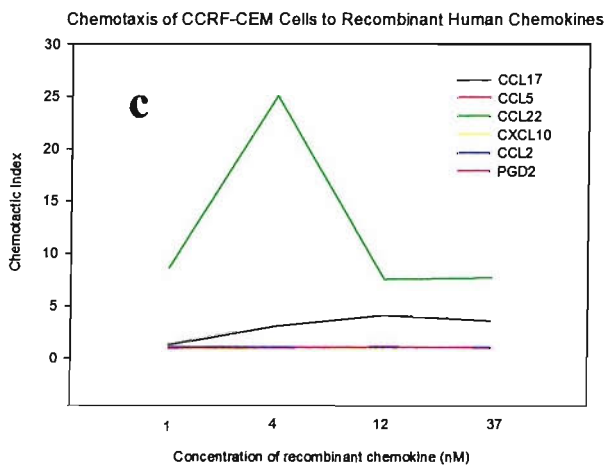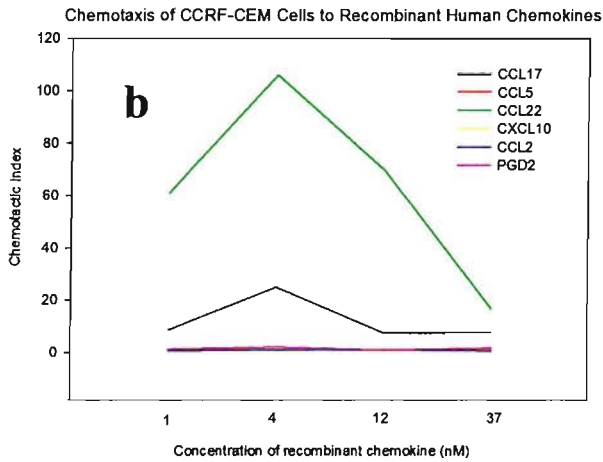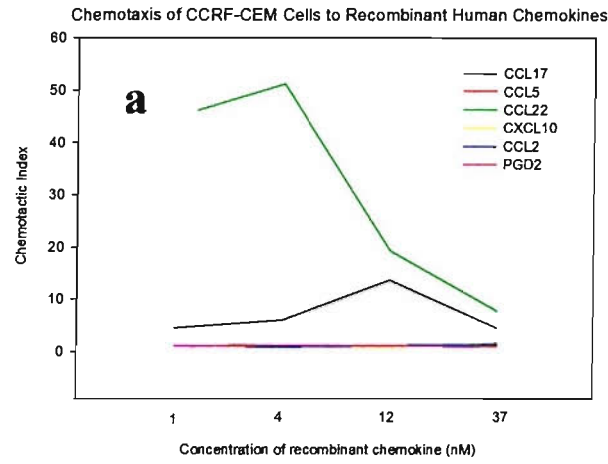
Figure 5.3 (a-c) Chemotaxis of CCRF-CEM cells to recombinant human chemokines: CCL17, CCL5, CCL22, CXCL10, CCL2 and PGD_2 recombinant chemokines. Each panel represents one individual experiment (a-c).

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Figure 5.4

Titration of the GSK CCR4blocking molecule using CEM and CCL22
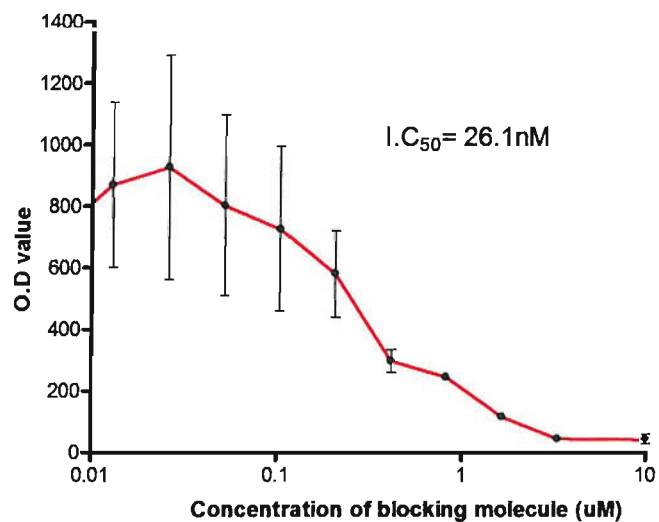
Figure 5.4   Titration of the GSK CCR4 small blocking molecule. Using a constant concentration of 3.7nM CCL22 the IC_{50} for the blocking molecule was 26.1nM. A representative experiment of a total of 3 is shown.

Figure 5.5

Chemotaxis of CEM cells to healthy control biopsy supernatants n=6

Chemotaxis of CEM cells to asthmatic subject biopsy supernatants n=6
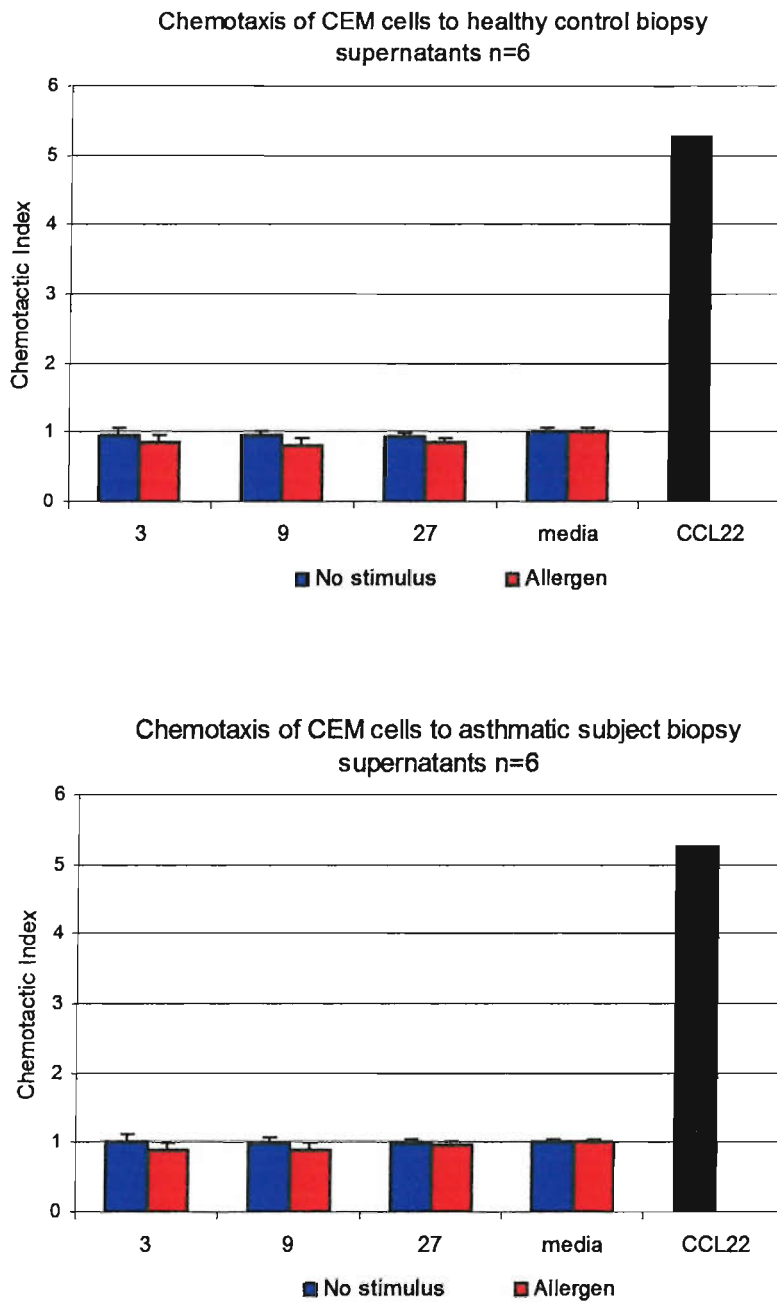
Figure 5.5   The chemotactic responses of CCRF-CEM T cells to biopsy supernatants from 6 healthy controls (upper panel) and 6 mild asthmatic subjects (lower panel) with and without treatment with HDM allergen (n=6). Bars represent SEM.

Figure 5.6

Percentage chemokine receptor expression on PBMC at baseline
in healthy controls and mild asthmatics (n=3)
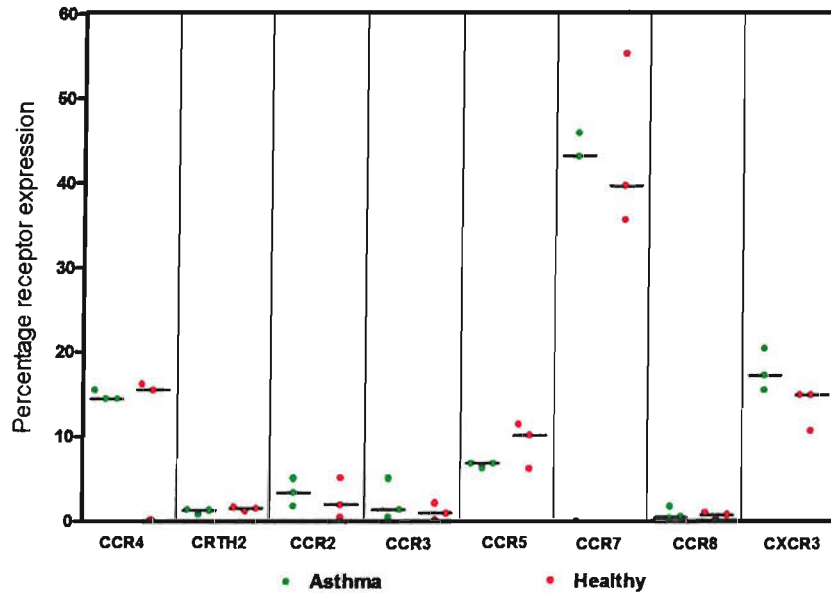
Figure 5.6   Percentage expression of 8 chemokine receptors on PBMC from
healthy control subjects (red symbols) and asthmatic subjects (green symbols) at
baseline. Horizontal lines represent the median of three patients (n=3).

Figure 5.7

Percentage chemokine receptor expression on Th1 cells at
week 1 in healthy controls and mild asthmatics (n=3)

Figure 5.7 Percentage expression of 8 chemokine receptors on Th1 cells from
healthy control subjects (red symbols) and asthmatic subjects (green symbols) at week 1.
Horizontal lines represent median of three patients.

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Figure 5.8

Percentage chemokine receptor expression on Th2 cells at
week 1 in healthy controls and mild asthmatics (n=3)

Figure 5.8   Percentage expression of 8 chemokine receptors on Th2 cells from
healthy control subjects (red symbols) and asthmatic subjects (green symbols) at week 1.
Horizontal lines represent median of three patients.

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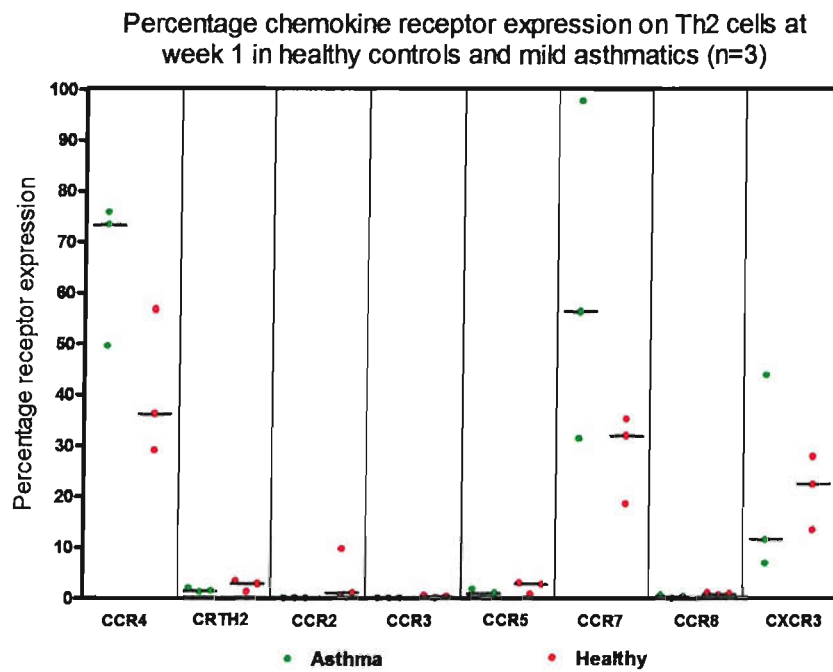
Figure 5.9

Percentage chemokine receptor expression on Th1 cells at week 2 in healthy controls and mild asthmatics (n=3)
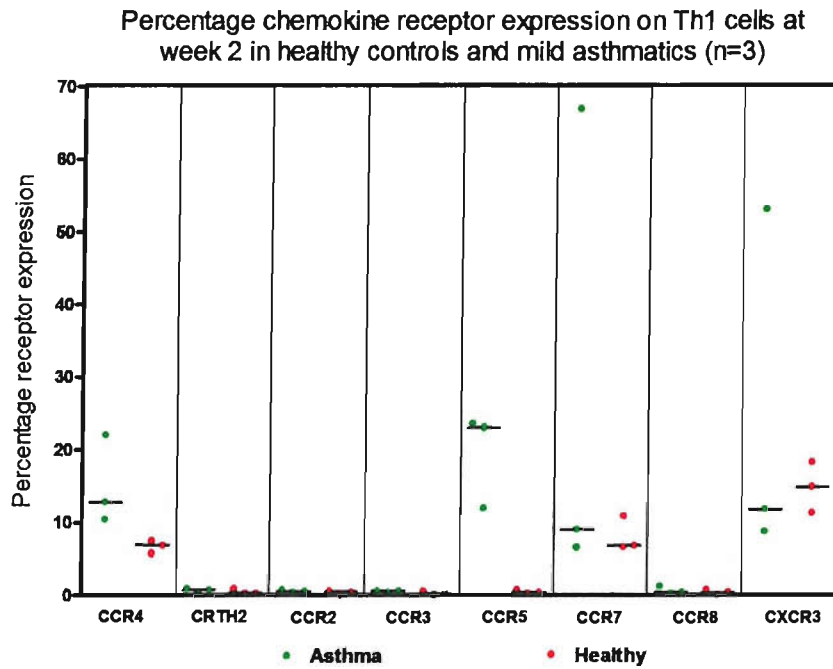
Figure 5.9   Percentage expression of 8 chemokine receptors on Th1 cells from healthy control subjects (red symbols) and asthmatic subjects (green symbols) at week 2. Horizontal lines represent median of three patients.

Figure 5.10

Percentage chemokine receptor expression on Th2 cells at week 2 in healthy controls and mild asthmatics (n=3)
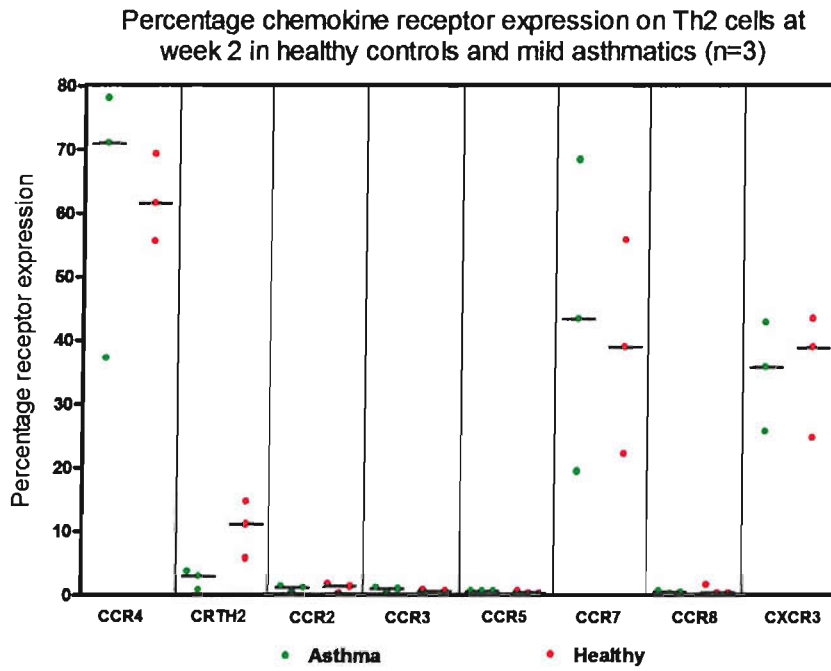
Figure 5.10   Percentage expression of 8 chemokine receptors on Th2 cells from healthy control subjects (red symbols) and asthmatic subjects (green symbols) at week 2. Horizontal lines represent median of three patients.

Figure 5.11

Th1 subject 1

7.21% of total

Th2 subject 1

31.77% of total

Th1 subject 2

8.13% of total

Th2 subject 2

68.11% of total

Chemotaxis of Th1 and Th2 polarised cells from 2 patients to CCL22
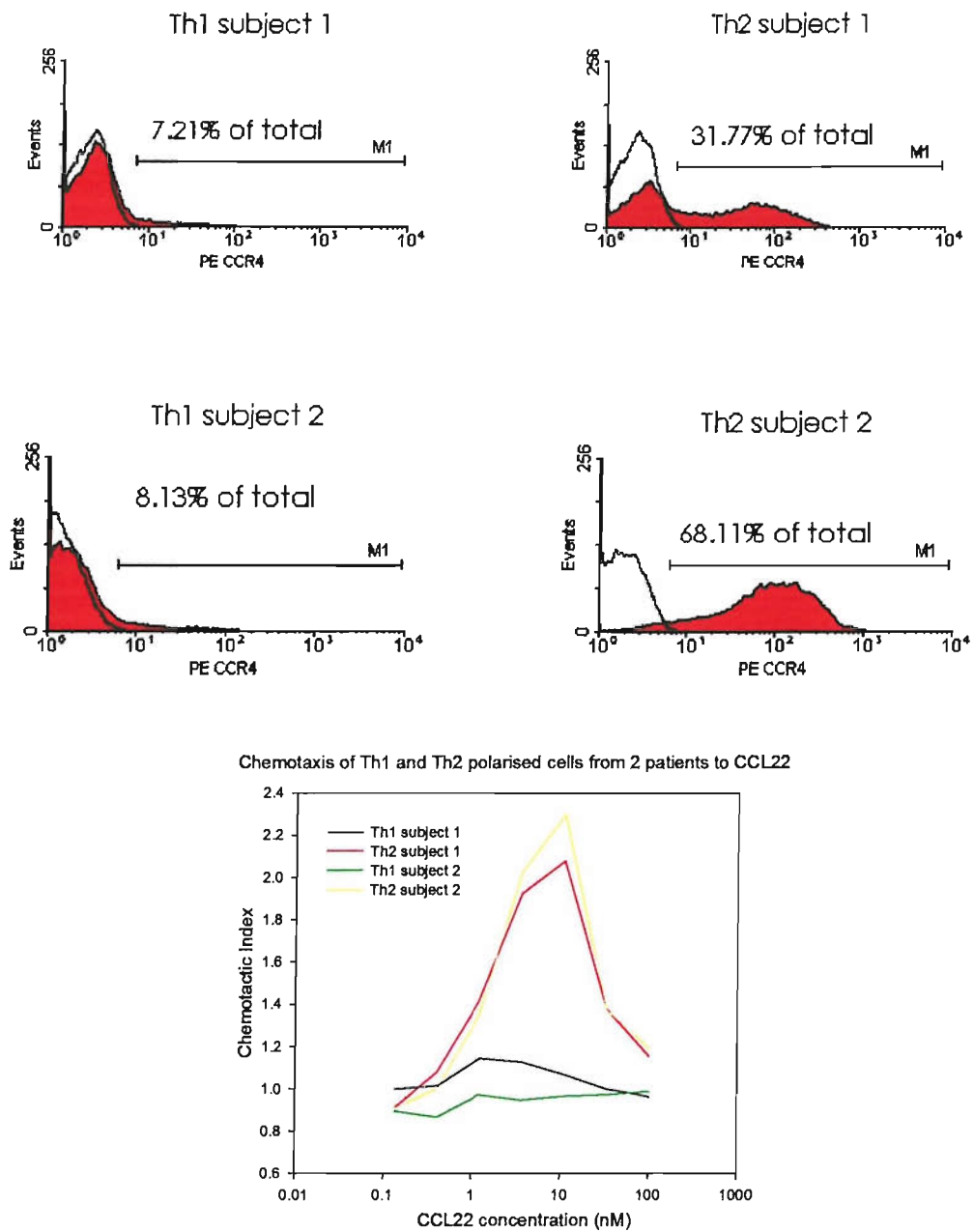
Figure 5.11   The difference in the chemotactic response of Th1 and Th2 polarised PBMC from 2 healthy subjects to CCL22. Maximal migration of Th2 polarised cells was observed at CCL22 concentrations of 11.1nM.

Figure 5.12 (a-c)

Chemotaxis of Th2 polarised cells from a healthy control subject to 6 recombinant chemokines
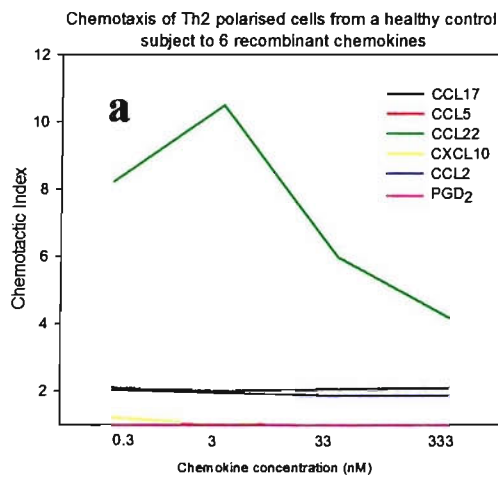
Fig 5.12 (a-c)   Chemotaxis of Th2 polarised PBMC from a healthy control subject to six different recombinant chemokines.

CCR4 expression 55.58%
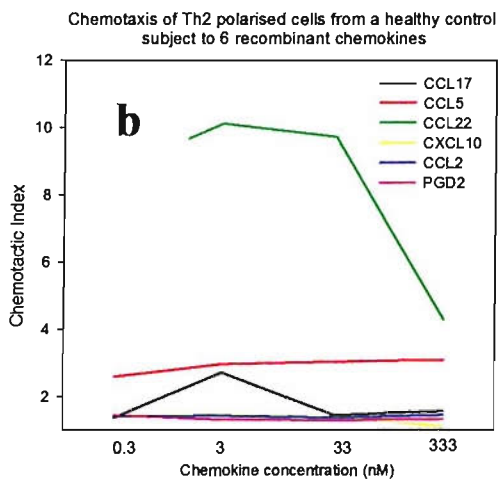
Chemotaxis of Th2 polarised cells from a healthy control subject to 6 recombinant chemokines

Chemotaxis of Th2 polarised PBMC from a healthy control subject to six different recombinant chemokines.

CCR4 expression 61.60%

Chemotaxis of Th2 polarised cells from a healthy control subject to 6 recombinant chemokines

Chemotaxis of Th2 polarised PBMC from a healthy control subject to six different recombinant chemokines.

CCR4 expression 69.61%

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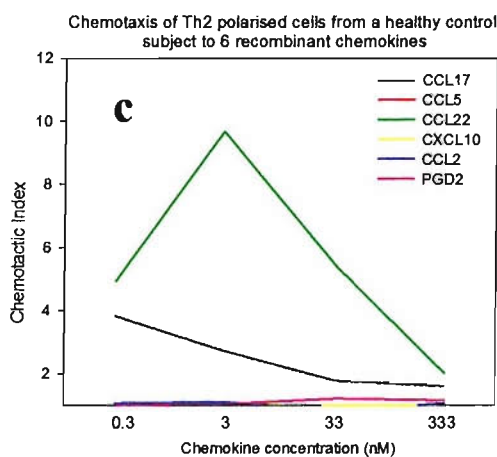
Figure 5.13 (a-c)

Chemotaxis of Th2 polarised cells from a mild asthmatic to 6 recombinant chemokines
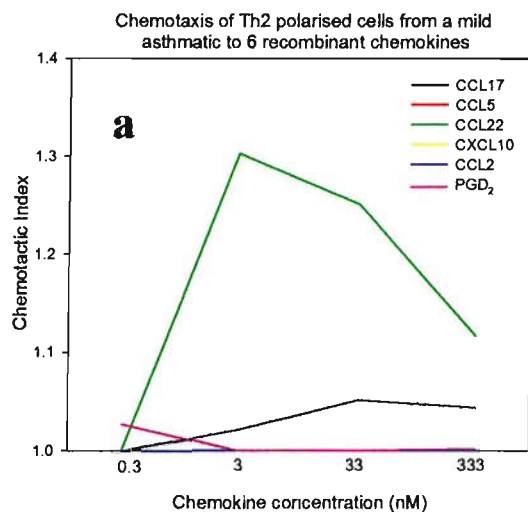
Fig 5.13 (a-c) Chemotaxis of Th2 polarised PBMC from a mild asthmatic subject to six different recombinant chemokines.
CCR4 expression 37.37%
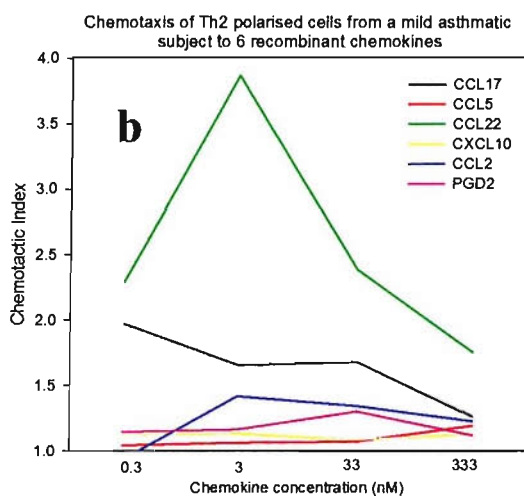
Chemotaxis of Th2 polarised cells from a mild asthmatic subject to 6 recombinant chemokines

Chemotaxis of Th2 polarised PBMC from a mild asthmatic subject to six different recombinant chemokines.
CCR4 expression 70.96%

Chemotaxis of Th2 polarised cells from a mild asthmatic subject to 6 recombinant chemokines

Chemotaxis of Th2 polarised PBMC from a mild asthmatic subject to six different recombinant chemokines.
CCR4 expression 78.04%

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Figure 5.14

Chemotaxis of Th2 polarised cells from healthy controls to healthy control biopsy supernatants
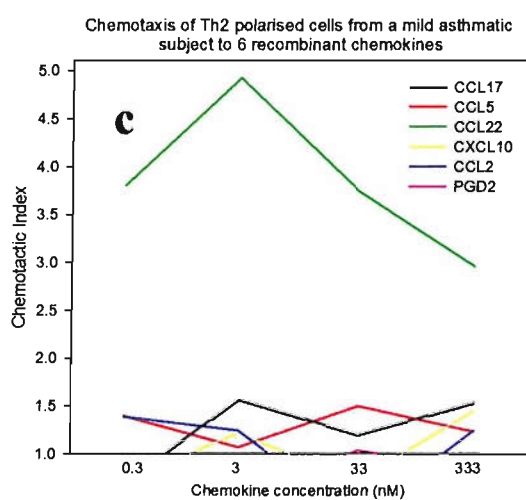
Chemotaxis of Th2 polarised cells from asthmatic subjects to asthmatic subject biopsy supernatants
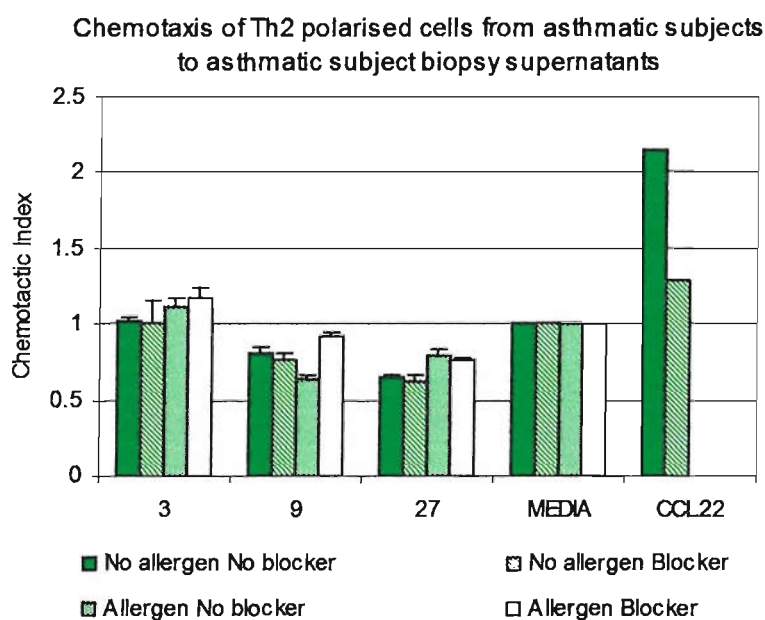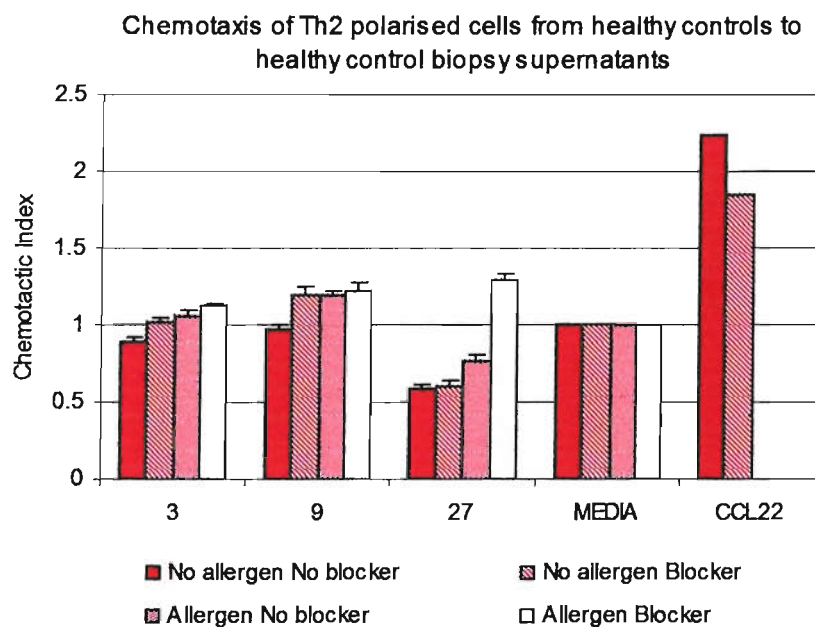
Figure 5.14   The chemotactic responses of healthy control Th2 polarised PBMC to healthy control biopsy supernatants (upper panel) and mild asthmatic Th2 polarised PBMC to asthmatic supernatants (lower panel). Data displays mean of 6 supernatants per subject group. Bars represent 1 SE.

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Chapter 6

Results

The effect of depleting CCR4 positive cells from PBMC
on IL-5 production and cell proliferation

6.1 Introduction

The production of Th2 cytokines by CD4+ T cells is a contributing factor to the cellular influx and subsequent inflammation widely described in asthmatic airways and it is now known that cytokines such as IL-4, IL-5 and IL-13 account for many of the pathophysiological manifestations. Previous research has suggested that CCR4, the chemokine receptor for CCL17 and CCL22, is the major chemokine receptor expressed on Th2 cells and becomes upregulated upon activation (Sallusto *et al.*, 1998, D'Ambrosio *et al.*, 1998, Panina-Bordignon *et al.*, 2001). Despite these observations some scepticism exists as to whether and to what extent this receptor has a part to play in the recruitment of Th2 cells into the airways. Two pieces of work have provided evidence to suggest that CCR4 is a redundant chemokine in the asthmatic response. Using a CCR4-/- animal model, Chvatchko and colleagues have shown that the absence of CCR4 fails to have an effect on *in vitro* Th2 cell differentiation or in a Th2-dependent model of airway inflammation (Chvatchko *et al.*, 2000). Furthermore, the application of a CCR4 blocking antibody by Conroy and colleagues was ineffective and did not inhibit the recruitment of eosinophils into the lungs as assessed in BAL of allergen challenged guinea pigs (Conroy *et al.*, 2003).

The aim of the experiments reported in this chapter was to elucidate further to what extent the CCR4+ cells are responsible for Th2 responses by investigating the levels of production of Th1 and Th2 cytokines, namely, IL-2, IL-4, IL-5, IL-13, IFNγ, the regulatory cytokine IL-10 and the cytokine which has been reported to play an important role in more severe forms of asthma, TNFα (Berry *et al.*, 2006). IL-4, IL-5 and IL-13, account for many of the pathophysiological manifestations of allergy and asthma (Ngoc *et al.*, 2005). IL-4 and IL-13 share a receptor component; they share the use of IL-4 receptor-α (IL-4Rα) for signalling; and both share the ability to induce splicing of an ϵ heavy chain to the immunoglobulin hypervariable region (Fab region), resulting in an immunoglobulin class switch from IgM to IgE production, and B cell proliferation. IL-5 is also pivotal in the allergic asthmatic response, in the recruitment of eosinophils. It is produced by T cells, mast cells, eosinophils and basophils (Yamaguchi *et al.*, 1988) and its main function is on eosinophils, acting to mediate

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their development, differentiation, recruitment, activation and survival (Sanderson, 1988, Clutterbuck and Sanderson, 1988, Wang *et al.*, 1989, Lopez *et al.*, 1988, Sanderson, 1990).

Th1 and Th2 cytokines have a reciprocal antagonistic relationship, whereby IL-10 and IL-4 (derived from Th2 cells) inhibit Th1 responses and IL-12 and IFNγ (generated by Th1 cells) inhibits Th2 responses. By investigating the differences in the production of both Th1 and Th2 cytokines by PBMC and PBMC from which CCR4+ve cells had been removed, a better insight into this reciprocal relationship and the importance of CCR4-expressing cells in response to allergen stimulation could be determined.

It was hypothesised that the depletion of CCR4+ cells from PBMC would deplete Th2 cells, resulting in reduced production, and possible complete abrogation, of one or all of the Th2 cytokines investigated, namely IL-4, IL-5 and IL-13. To test the hypothesis, PBMC of allergic asthmatics were stimulated to detect levels of cytokines and the degree of proliferation in response to stimulation with HDM allergen. These responses were compared to those of PBMC populations from which CCR4+ cells had been removed by magnetic cell sorting (MACS).

Methods

Briefly, PBMC were isolated from peripheral blood of HDM allergic asthmatics and a population of whole PBMC was kept on ice. The remaining PBMC were stained with a phycoerythrine (PE)-labelled antibody to CCR4 (R& D Systems, Abingdon, Oxon, UK) followed by labelling with anti-PE magnetic beads (Miltenyi Biotec, Surrey, UK). The CCR4+ cells were then removed by magnetic separation using the AutoMACS machine (Miltenyi Biotec, Surrey, UK), as described in chapter 2. Cell counts were performed three times to ensure the same densities of cells for both the PBMC and the CCR4-ve populations.

The two populations of cells were treated either with HDM allergen (ALK, Norway) or tetanus toxoid (Diftavax, Aventis Pasteur, Berkshire, UK). Tetanus toxoid was used as a positive control, in order to show that the cells were capable of generating a Th2 response. This is based on the notion that vaccination against tetanus induces a mixed Th2/Th1 response. PHA (Sigma, Dorset, UK) was used as a pan T cell stimulus, working via T cell receptor stimulation.

In order to study the effect of CCR4+ on the proliferative responses, one set of plates for PBMC and another plate for CCR4-ve cells were incubated for 72 hours in quadruplicate before treatment with 1μCi/well of [3H] thymidine for the last 16 hours of culture. The cells were harvested and proliferation detected using a scintillation counter. In parallel experiments, cells were cultured in quadruplicate for 7 days, cell viability was assessed by trypan blue exclusion and supernatants stored to be tested for IL-2, IL-4, IL-5, IL-10, IL-13, IFNγ and TNFα production by multiplex ELISA.

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6.2 Results

6.2.1 The frequency of expression of CCR4 on cells contained within three populations: PBMC, CCR4+ve and CCR4-ve

The effect of CCR4+ cell depletion was studied using PBMC from 4 donors with mild allergic asthma selected during preliminary experiments to identify good responders whose PBMC release high concentrations of IL-5 upon stimulation with HDM allergen. The effectiveness of the CCR4 depletion can be seen in figure 6.1 where in one representative subject 26.93% of the PBMC population of cells were shown to express CCR4 (Fig 6.1a). When the CCR4+ cells were removed and the positively selected fraction was assessed 90.96% were found to be positive for CCR4 (Fig 6.1b). The population of cells that were depleted of CCR4+ cells was shown to have only 5.42% remaining CCR4+ cells that had not been effectively removed by magnetic cell sorting (MACS) (Fig 6.1c). The mean percentage of cells expressing CCR4 was 31.74% in PBMC and only 6.49% after depletion (Fig 6.1d).

6.2.2 The frequency of expression of CD14 on cells contained within three populations: PBMC, CCR4+ve and CCR4-ve

As antigen presentation requires the presence of monocytes, flow cytometric analysis was performed to ensure that the CCR4+ cell depletion had not resulted in depletion of monocytes. These experiments showed that the depletion procedure had no detrimental effects on the percentage of CD14+ve cells. The individual patient values and the mean percentage of CD14+ve cells in the CCR4-ve population remained very close to the percentage of CD14+ve cells detected within the PBMC population. For one representative subject CD14 was shown to be present on 24.23% of cells (Fig 6.2a). The fraction of cells expressing CCR4 that were removed from the whole PBMC population contained only 7.17% CD14+ve cells (Fig 6.2b). The population that had been depleted of CCR4+ve cells had an almost identical percentage of CD14+ve cells as the whole PBMC population, with a value of 24.26% (Fig 6.2c).

The mean percentage of CD14+ cells before depletion was 16.96% and 18.32% after depletion (Fig 6.2d)

6.2.3 The concentration of IL-2 produced by PBMC and CCR4-ve PBMC in response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

There was little IL-2 produced by both cell populations in the non-stimulated and the PHA stimulated conditions, however, with both concentrations of HDM allergen stimulation IL-2 was produced in greater quantities in the CCR4-ve population but this failed to reach significance (Fig 6.3). Little difference in IL-2 production in the tetanus toxoid condition was noted between the whole PBMC and the CCR4-ve population.

6.2.4 The concentration of IL-4 produced by PBMC and CCR4-ve PBMC in response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

IL-4 was detected at low levels, with the exception of the non-stimulated condition. There was less IL-4 produced by the cells in the population from which CCR4+ cells had been removed (Fig 6.4, Table 6.1). This was noted in the PHA, HDM and tetanus toxoid treated conditions. On treatment with 2000 SqU of HDM allergen the differences in the production of IL-4 between the two cell populations reached significance (p=0.019)

6.2.5 The concentration of IL-5 produced by PBMC and CCR4-ve PBMC in response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

There was little spontaneous production of IL-5 (Fig 6.5). However, IL-5 was produced in larger quantities by the PBMC after stimulation with PHA than the CCR4-ve population but this failed to reach significance. For the HDM allergen treated cells, there were much lower amounts of IL-5 produced in the CCR4-ve population and these differences were significant (2000 SqU p=0.021; 5000SqU

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p=0.021). There was also a significant difference in IL-5 production between the PBMC and the CCR4-ve population for the tetanus toxoid treated cells (p=0.021).

6.2.6 The concentration of IL-10 produced by PBMC and CCR4-ve PBMC in response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

There was an apparent difference between PBMC and CCR4-ve cells in the production of IL-10 in the PHA, HDM and tetanus toxoid stimulated conditions (Fig 6.6) but these differences failed to reach significance.

6.2.7 The concentration of IL-13 produced by PBMC and CCR4-ve PBMC in response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

Although the differences in IL-13 production between the two cell populations did not reach significance for the PHA and the tetanus toxoid treated conditions, significance was reached for the two concentrations of HDM allergen (2000 and 5000 SqU) (Fig 6.7, Table, 6.1) showing less IL-13 produced in the population devoid of CCR4+ve cells

6.2.8 The concentration of IFNγ produced by PBMC and CCR4-ve PBMC in response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

There was little IFNγ produced in the non-stimulated condition and no differences between the two cell populations for the tetanus toxoid treated condition. For the PHA and the two concentrations of HDM allergen, there was a trend for greater amounts of IFNγ production in the CCR4-ve population (Fig 6.8).

6.2.9   The concentration of TNFα produced by PBMC and CCR4-ve PBMC in
          response to challenge with PHA, HDM allergen and tetanus toxoid (n=4)

TNFα was produced in greater quantities in the CCR4-ve populations for PHA, both

concentrations of HDM allergen and tetanus toxoid to a lesser extent (Fig 6.9) but

these differences were not significant.

6.2.10   The mean proliferative response of PBMC or cells contained in the CCR4-ve
          fraction in response to challenge with PHA, HDM allergen and tetanus toxoid
          (n=4)

There were no significant differences in the amounts of proliferation between the two

cell populations in either condition (Fig 6.10). There appeared to be less proliferation

of the CCR4-ve population after treatment with PHA, slightly more proliferation in the

CCR4-ve population after treatment with HDM allergen in comparison to the PBMC

population, and more proliferation in the CCR4-ve population after treatment with

tetanus toxoid but these differences were not significant.

6.3 Discussion

The work presented in this chapter aimed to test the hypothesis that CCR4+ cells have a major role in the allergic asthmatic cellular response to HDM allergen by showing that CCR4+ cells were responsible for the production of IL-4, IL-5 and IL-13 and also heightened proliferation in response to challenge with HDM allergen, thus showing that CCR4+ cells were the allergen responsive cells that function to accentuate the allergic asthmatic response in susceptible individuals.

The most profound finding was the significant drop in the production of IL-5, for both concentrations of HDM allergen (2,000 and 5,000 SqU/ml) and after treatment with tetanus toxoid, in the population that was devoid of CCR4+ve cells. There was also a significant drop in IL-4 and IL-13 production in the CCR4-ve population for the cells challenged with 2,000 SqU/ml of HDM allergen. However, surprisingly, there was no effect of depletion of CCR4+ cells on proliferative responses.

IL-4, IL-5 and IL-13 have important roles and profound effects on the allergic asthmatic response. IL-5, produced by T cells, mast cells, eosinophils and basophils (Yamaguchi *et al.*, 1988), serves to mediate eosinophil development, differentiation, recruitment, activation and survival (Sanderson, 1988, Clutterbuck and Sanderson, 1988, Wang *et al.*, 1989, Lopez *et al.*, 1988, Sanderson, 1990). IL-4 is produced predominantly by Th2 cells but can also be produced by mast cells, basophils (Seder, 1991) and eosinophils (Ying *et al.*, 1997). Interleukin-4 is the central differentiation factor for Th2 response development (Nelms *et al.*, 1999) and is an essential cytokine required for class-switching of IgG to IgE antibody production by B cells (Bacharier and Geha, 2000). Additionally, it stimulates the expression of MHC class II molecules, CD80, CD86, CD40, the low-affinity IgE receptor and CD23 by B cells, resulting in an enhanced antigen presenting capacity of the cell (Seder and Paul, 1994). Indeed, activated CD4+ T cells producing IL4 and IL-5 have been detected in BAL and bronchial biopsies of both atopic and non-atopic asthmatics (Ying *et al.*, 1997, Robinson *et al.*, 1992). Interleukin-13, akin to IL-4, upregulates the expression of β_1-integrin and vascular cell adhesion molecule-1 (VCAM-1) and the production of IL-6

and CCL2 from human lung fibroblasts (Doucet *et al.*, 1998). Although IL-13 is well known for its similar functional properties to IL-4, such as its contribution to the production of IgE antibodies and its production by Th2 cells and mast cells, IL-13, unlike IL-4, cannot act on T cells themselves. Although IL-4 drives the Th2 mediated response (Nelms *et al.*, 1999) it does not appear to be necessary for the expression of allergic asthma (Cohn *et al.*, 1998). It is becoming more conclusive that IL-13 has a critical and non-redundant role in the pathophysiology of the lung. Zhu and colleagues aimed to investigate this through generating a mouse which overexpressed IL-13 in the lungs (Zhu *et al.*, 1999). They noted that the mice, in comparison to the transgene negative littermates, developed several characteristics of asthma such as pulmonary eosinophilia, airway epithelial cell hyperplasia, mucus cell metaplasia, subepithelial fibrosis, airway obstruction and non-specific airway hyperresponsiveness to cholinergic stimulation (Zhu *et al.*, 1999). IL-5 is a Th2 cytokine and was identified as an eosinophil differentiation factor in the mid-1980's (Campbell *et al.*, 1987) and it has since been the focus of much attention. It is now known to play an essential role in the orchestration of inflammation in asthma, with eosinophils being one of the first cell types to be recruited into the airways in asthmatic individuals. Although IL-5 was detected in reduced concentrations for all subjects in the current piece of work, the amounts produced between patients were very variable. The amounts of IL-5 mRNA detected in asthmatic subjects have been shown to correlate with the severity of the condition (Humbert *et al.*, 1997) and this may be an indicator of disease severity in the current study. Despite this evidence, Cho and colleagues, found this not to be the case (Cho *et al.*, 2002). Interleukin-5 has been detected in raised concentrations in asthmatics following challenge with allergen (Teran *et al.*, 1999) and this has been confirmed by a number of studies. In their study, Teran and colleagues obtained BAL fluid by bronchoscopy from 12 asthmatic subjects that had evidence of allergy to either HDM, grass pollen, dog, feathers and cat dander and had undergone endobronchial allergen challenge. They discovered that there was a significant increase in the amounts of IL-5, detected by ELISA in segments of the airways that had been challenged with allergen in comparison to the site challenged with saline only (Teran *et al.*, 1996). In the patient group where BAL was performed taken only 4h after

allergen challenge, slight but significant differences were observed. On a separate group of patients where BAL was taken at 24h the amounts of IL-5 detected and the significance were much greater (Teran *et al.*, 1999). In support of this, Pilette and co-workers demonstrated a significantly higher rise in IL-5 concentrations in BAL from atopic asthmatics 24h after endobronchial allergen challenge in comparison to those at baseline levels (Pilette *et al.*, 2004). The results generated in this chapter investigating IL-5 production by peripheral blood cells support these earlier findings. In all patients an increase in the concentrations of IL-5 produced was noted after challenge with PHA; both of the HDM allergens; and tetanus toxoid, in comparison to concentrations found for the unstimulated condition. Bullens and colleagues investigated the allergen induced cytokine profile of 15 birch pollen allergic patients. They tested supernatants after stimulating PBMC with the birch-pollen allergen rBet v 1, for 7 days and in accordance with the results generated in the current study, found that the production of IL-5 had significantly increased (Bullens *et al.*, 2004). The pivotal role of IL-5 in eosinophil recruitment has been demonstrated using knock-out mice and blocking monoclonal antibodies. For instance, a human monoclonal antibody to IL-5 has been developed and upon intravenous administration to allergic mild asthmatic patients, the influx of eosinophils into the airways was prevented and the numbers of eosinophils in the blood greatly reduced (Leckie *et al.*, 2000). Despite this, several studies have shown that the treatment with anti-IL-5 blocking antibodies has little, if any effect on the resultant early and late phase responses and/or the subsequent airway hyperresponsiveness (Leckie *et al.*, 2000, Kips, 2000, Hogan *et al.*, 1998).

Relative to CCR4, Yamamoto and colleagues investigated the expression of chemokine receptors in another Th2 dominated condition, atopic dermatitis (Yamamoto *et al.*, 2000). Their study aimed to identify the cytokine profiles of Th cells expressing different chemokine receptors through intracellular staining and flow cytometric detection after stimulation with PMA and ionomycin, to induce cytokine production. Their results revealed that the cells capable of producing Th2 cytokines, IL-4, IL-5, and IL-13, were restricted to the CCR4-expressing population within memory CD4+ T cells (Yamamoto *et al.*, 2000). A study by Imai and colleagues also

supports the findings of the current study. They found that T cells attracted by CCR4 ligands CCL17 and CCL22, generated cell lines predominantly producing Th2-type cytokines, IL-4 and IL-5 (Imai *et al.*, 1999). They concluded that CCR4 is selectively expressed on Th2-type T cells (Imai *et al.*, 1999).

Based on the literature above, IL-4, IL-5 and IL-13 were chosen as important Th2 cytokines to study. There were statistically significant differences in the production of the Th2 cytokines IL-4 and IL-13 between the two different cell populations upon challenge with 2,000 SqU/ml of HDM allergen. Both cytokines were detected in lower concentrations for all 4 subjects in the CCR4-ve population of cells indicating that the CCR4-expressing cells were responsible for the production of the majority of these two cytokines.

For IL-5, two subjects had a heightened response to 2,000SqU of ALK HDM allergen and the other 2 subjects showed a greater IL-5 production at 5,000SqU, suggesting that the latter 2 subjects are less sensitive to the allergen. In initial experiments where IL-5 production was investigated at day 7, a 10,000 SqU/ml concentration of HDM allergen was tested, and this concentration was shown to have a deleterious effect on the cells, indicating that the HDM allergen at this concentration had a negative effect on the allergic response. This observation was found in all 4 subjects tested. One possible explanation for this may be the fact that a certain concentration of allergen required to produce a Th2 mediated response is required. Indeed, this has been shown by Gardner and colleagues (Gardner *et al.*, 2004). In their study they too used PBMC from HDM allergic donors and challenged the cells with three concentrations of HDM allergen, low, intermediate and high. Using flow cytometry, they discovered that the highest concentrations of this Th2 response-inducing allergen resulted in a Th1-type response of the cells, determined by enhanced IFNγ production and diminished IL-4 production (Gardner *et al.*, 2004). This was not seen for the intermediate and lowest allergen concentration. The lowest allergen concentration seemed to have resulted in the most effective Th2 response (Gardner *et al.*, 2004).

There were no statistically significant differences between the two cell populations in terms of production of IL-2, IL-10, IFNγ and TNFα. Despite a lack of significance, there was an overall trend for the production of these cytokines, with the exception of IL-10, to be detected in increased concentrations in the CCR4-ve population. Taking into consideration that the allergic asthmatic response is of a Th2-mediated nature, in the PBMC population, the production of IFNγ would have been inhibited by IL-4 and in the absence of IL-4 in the CCR4-ve population it is not unreasonable to have expected to see higher concentrations of Th1 cytokines. IL-10 was detected in lower concentrations in the CCR4-ve population, although these differences failed to reach significance. IL-10 is a pleiotropic cytokine that can exert either immunosuppressive or immunostimulatory effects on a variety of cell types. Wong and co-workers investigated the differences in plasma concentrations of a number of cytokines between allergic asthmatic and healthy control subjects and they noted that IL-10 levels were significantly higher in the plasma samples of the allergic asthmatics compared with the healthy controls (Wong et al., 2001). The results from the current piece of work suggest that the majority of IL-10 is produced by the CCR4-expressing cells. Although the differences in IL-10 concentrations between the PBMC and the CCR4-ve populations failed to reach significance, there was a strong trend, in 3 of the four subjects, for IL-10 levels to be lower in the CCR4-ve population and it is possible that the effect of CCR4+ cell depletion would become evident if more experiments were conducted.

Evidence exists that suggests a role for both IL-2 and TNFα in the asthmatic response. For instance, McHugh and colleagues investigated the proliferative responses of PBMC and the production of IL-2 by PBMC in subjects with Der. p rhinitis with and without asthma after stimulation with allergen (McHugh et al., 1993). They noted significantly higher concentrations of IL-2 detected in supernatants in the allergic asthmatics compared with non-atopic controls (McHugh et al., 1993), thus suggesting a role for IL-2 in allergic asthma. Inline with this are results from studies investigating the role of TNFα in the asthmatic response. TNFα is a cytokine that acts synergistically and has immunomodulating properties, resulting in the enhancement of

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chemokine production and hence increased recruitment of cell types into the airways. It has also been found in asthmatic airways in substantial concentrations (Bradding *et al.*, 1994). In the current study, the levels of IL-2, IFNγ and TNFα in the PBMC population challenged with 2,000 SqU of HDM allergen were the same as the unstimulated control, but in the CCR4-ve population the concentrations of these three cytokines were raised. Despite existing evidence suggesting that these cytokines are implicated in asthma, the current study indicates that the CCR4+ve cells are not responsible for the production of these cytokines. Non-CCR4-expressing cells in addition to other cell types, such as structural cells of the airways and/or PBMC locally in the airways may be the source of these cytokines.

In order to ensure that any differences in the production of cytokines studied were not as a direct result of reduced antigen processing and presentation ability, flow cytometry was used to investigate the presence of CD14+ve cells which would serve to function as antigen presenting cells, leading to cytokine production and proliferation. The data shown in the current chapter demonstrates that there were negligible differences between the percentage of CD14+ve cells in the PBMC and the CCR4-ve population, implying that the profound differences in cytokine production were as a result of the removal of CCR4+ve cells and not as a result of fewer monocytes/macrophages in either cell population.

In the current study, tetanus toxoid was used as a control non-allergenic recall antigen to confirm that the cells were healthy and capable of IL-5 production. Tetanus toxoid evokes a recall response, characteristic of both a Th1 and Th2-type based on cytokine profile. The results suggest that the majority of cells expressing CCR4 are of the Th2 phenotype, based on their ability to produce IL-5, but they are not solely HDM-allergen specific. One way to determine whether the allergen specific T cells were those responsible for IL-5 production would have been to retain the CCR4+ cells and treat them with the same stimulants as both the PBMC and the CCR4-ve fractions. Irradiated monocytes would have to have been added in order for antigen presentation. Using this method, it would have been possible to find out whether the HDM allergen

specific cells had produced the IL-5. If this were the case, then the amounts of IL-5 produced by the CCR4+ cells would be equal to the IL-5 produced by CCR4-ve fraction subtracted from the PBMC population. Although this would have been a very informative experiment, there were technical considerations: Firstly, the number of CCR4+ cells recovered in the positively selected fraction was very low and the amount of blood needed from each asthmatic patient, in order to provide enough CCR4+ cells, was unethical. Secondly, there was no way of determining whether the anti-PE microbeads would have had any deleterious effect on the behaviour of the cells because they remained bound to the CCR4+ cells.

In terms of proliferation, surprisingly, higher levels of proliferation were seen in the population devoid of CCR4 and this was the opposite of what was hypothesised. It was anticipated that the PBMC population would proliferate more than the CCR4-ve population due to the presence of CCR4+ve cells with their selective ability to respond to antigen. Possible reasons for why the opposite was noted might lie in the contribution of the CCR4+ve cells to dampening down the proliferative response. It has been shown by Kim and colleagues that although in the CCR4+ population, the majority of cells are of the Th2 phenotype and very few are Th1 cells (Kim *et al.*, 2001) many of these CCR4 expressing T cells, in addition, express CXCR3, the receptor for CXCL10. Kim and others further went on to show that most of the CCR4+CXCR3+ cells were of the Th0 phenotype (Kim *et al.*, 2001) which are characterised by the production of a combination of Th1 and Th2-type cytokines. If the majority of CCR4+ve cells that were removed from the PBMC population were either Th2 and/or Th0 cells then all remaining cells within the CCR4-ve population should be Th1 cells. However, it is unknown as to what percentages of the removed CCR4 expressing cells were capable of producing Th1-type cytokines. The fact that these cells proliferate more in response to stimulation with HDM allergen is surprising. Although this work has shown that the CCR4+ve cells within the PBMC population are responsible for the majority of IL-5 production, this does not necessarily mean that these are the cells that proliferate in response to allergen.

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Although the statistical analyses have shown highly significant results, these values may not be a true representation of the data. The number of subjects investigated was low. Approximately 15 HDM allergic mild asthmatics were initially screened for IL-5 production and all but 4 of those failed to produce appreciable amounts of IL-5 whilst others produced such low levels it would have been meaningless to include them in the study. Time constraints and the shortage of mild asthmatic HDM allergic patients also put limits of the content of this chapter. It is possible that if more patients were tested, the statistical significance would increase, based on the fact that each of the 4 included subjects showed greatly reduced IL-4, IL-5 and IL-13 production on the removal of the CCR4+ve population of cells.

In conclusion, it has been shown that CCR4+ve cells are those responsible for the vast majority of IL-4, IL-5 and IL-13 produced in response to HDM allergen. This demonstrates that these cells are those capable of Th2 cytokine production and would play an important role in the allergic asthmatic response to inhaled allergen. The results of the proliferation experiments went against the hypothesis which proposed that there would be more proliferation detected in the PBMC population.

Figure 6.1 (a-c)
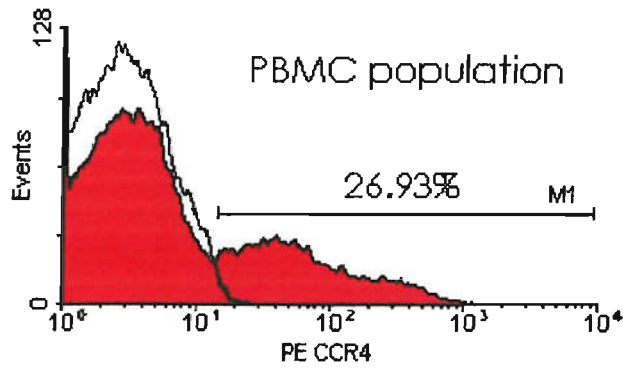
Figure 6.1a   Percentage of CCR4 expressing cells in the PBMC population. One representative HDM allergic mild asthmatic subject with a good IL-5 response. n=4.
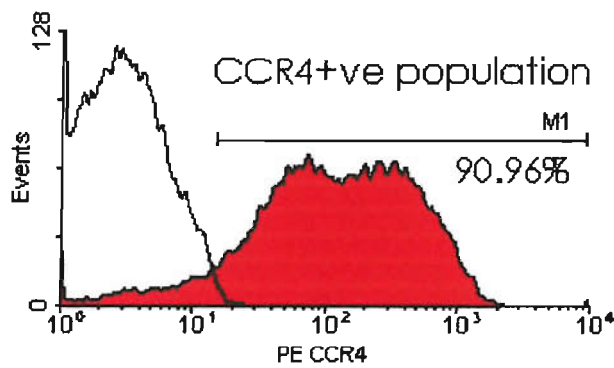
Figure 6.1b   Purity of the CCR4 positively isolated population. One representative HDM allergic mild asthmatic subject with a good IL-5 response n=4.
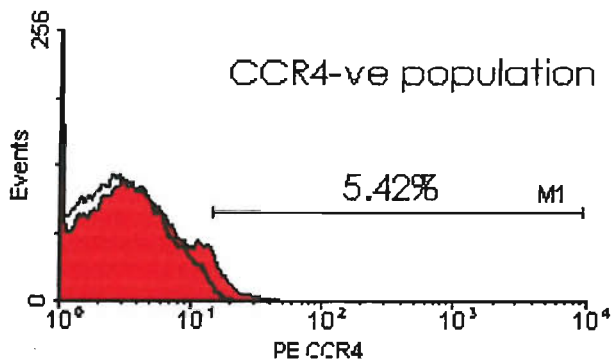
Figure 6.1c   Percentage of contaminating CCR4+ cells within the CCR4-ve population. One representative HDM allergic mild asthmatic subject with a good IL-5 response. n=4.

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Figure 6.1d

Mean percentage of CCR4+ cells in
three PBMC populations
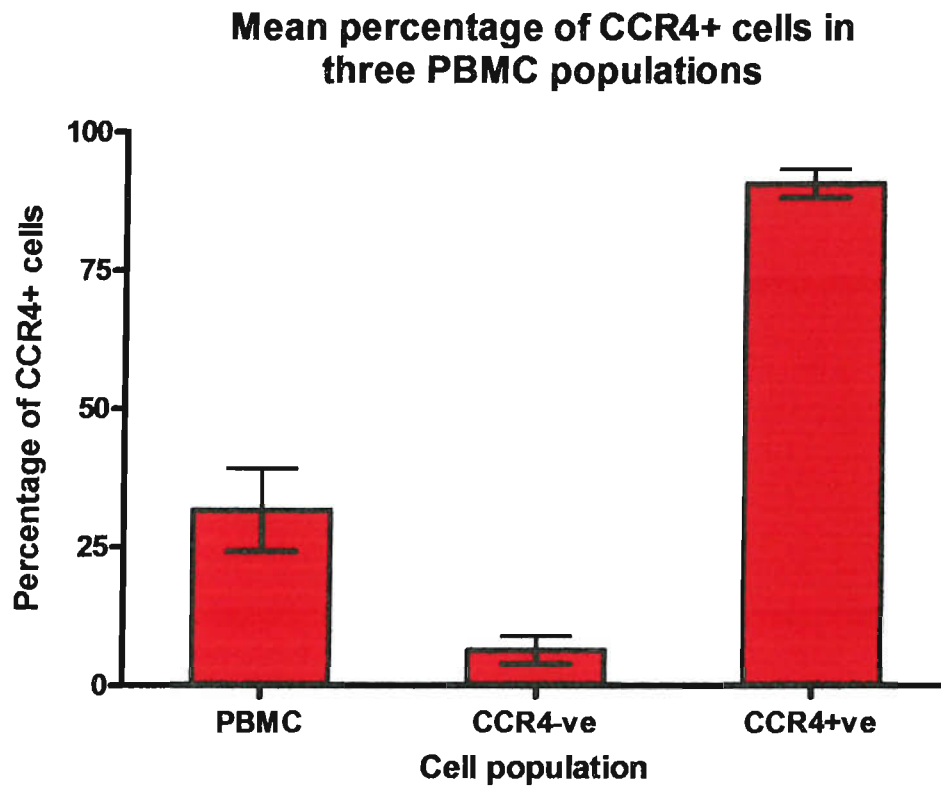
Figure 6.1d   Mean percentage of CCR4+ cells in the three populations of
PBMC; whole PBMC, CCR4- and CCR4+ (n=4).   Black bars indicate +/- 1 SD.

Figure 6.2 (a-c)

PBMC population

24.23%   M1
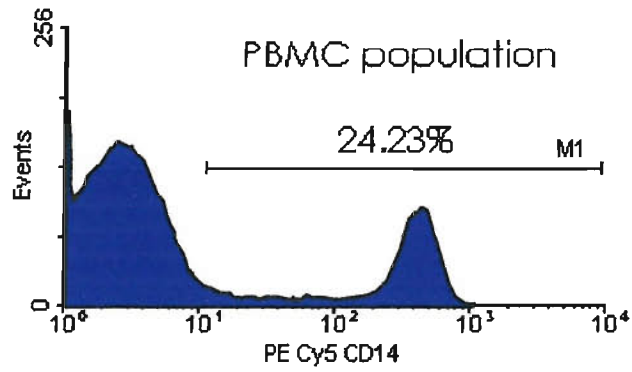
Figure 6.2a   Percentage of CD14+ cells in the PBMC population. One representative HDM allergic mild asthmatic subject with a good IL-5 response. n=4.

CCR4+ve population

7.17%   M1
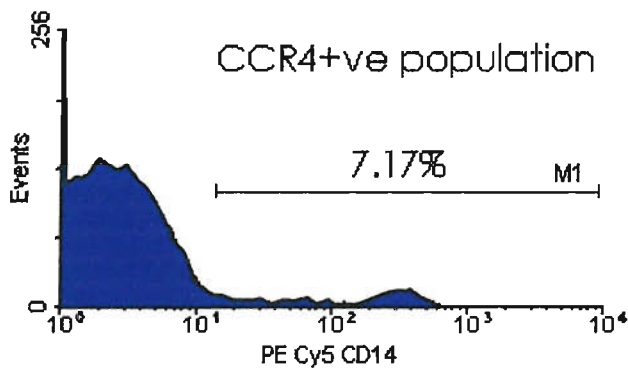
Figure 6.2b   Percentage of CD14+ cells in the CCR4+ population. One representative HDM allergic mild asthmatic subject with a good IL-5 response. n=4.

CCR4-ve population

24.26%   M1
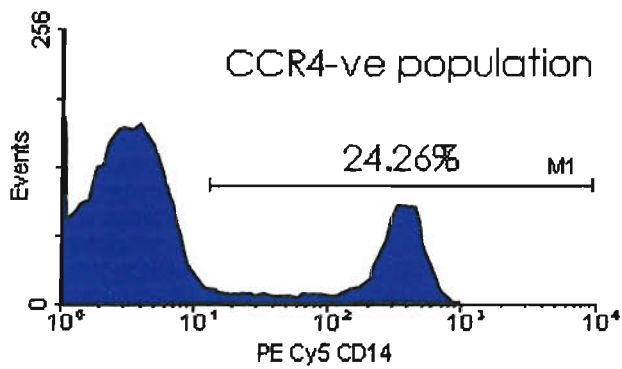
Figure 6.2c   Percentage of CD14+ cells in the CCR4-ve population. One representative HDM allergic mild asthmatic subject with a good IL-5 response. n=4.

Figure 6.2d
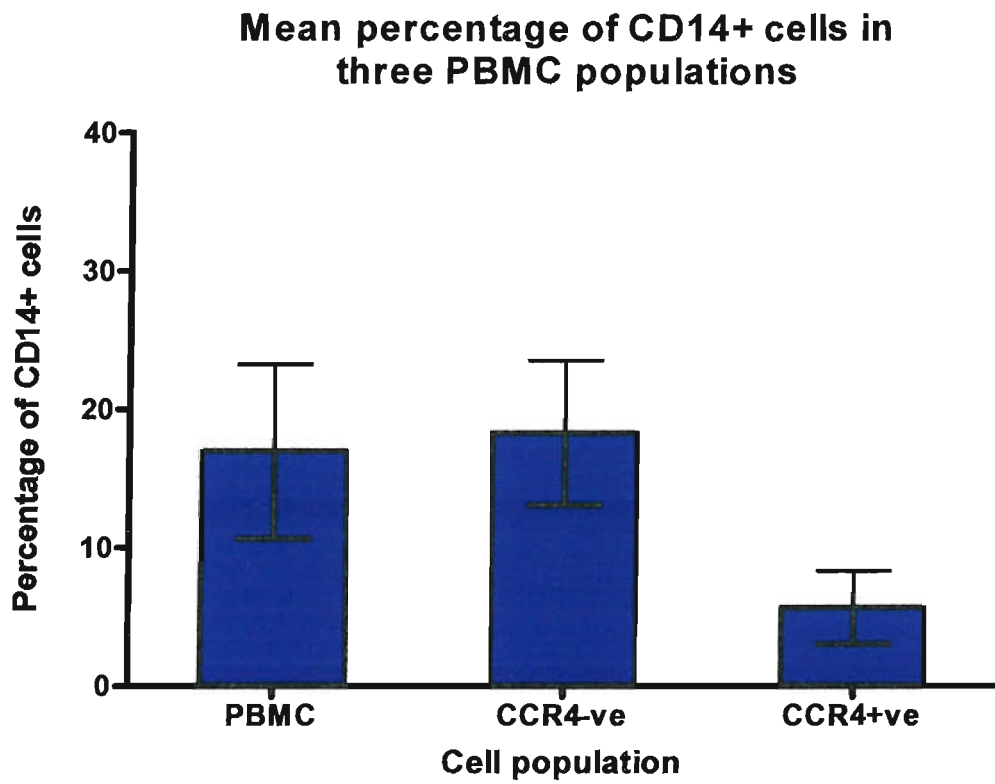
Mean percentage of CD14+ cells in three PBMC populations

Figure 6.2d   Mean percentage of CD14+ cells in the three populations of PBMC; whole PBMC, CCR4- and CCR4+ (n=4). Black bars indicate +/- 1 SD.

Figure 6.3

Production of IL-2 by PBMC or CCR4-ve PBMC after various treatments
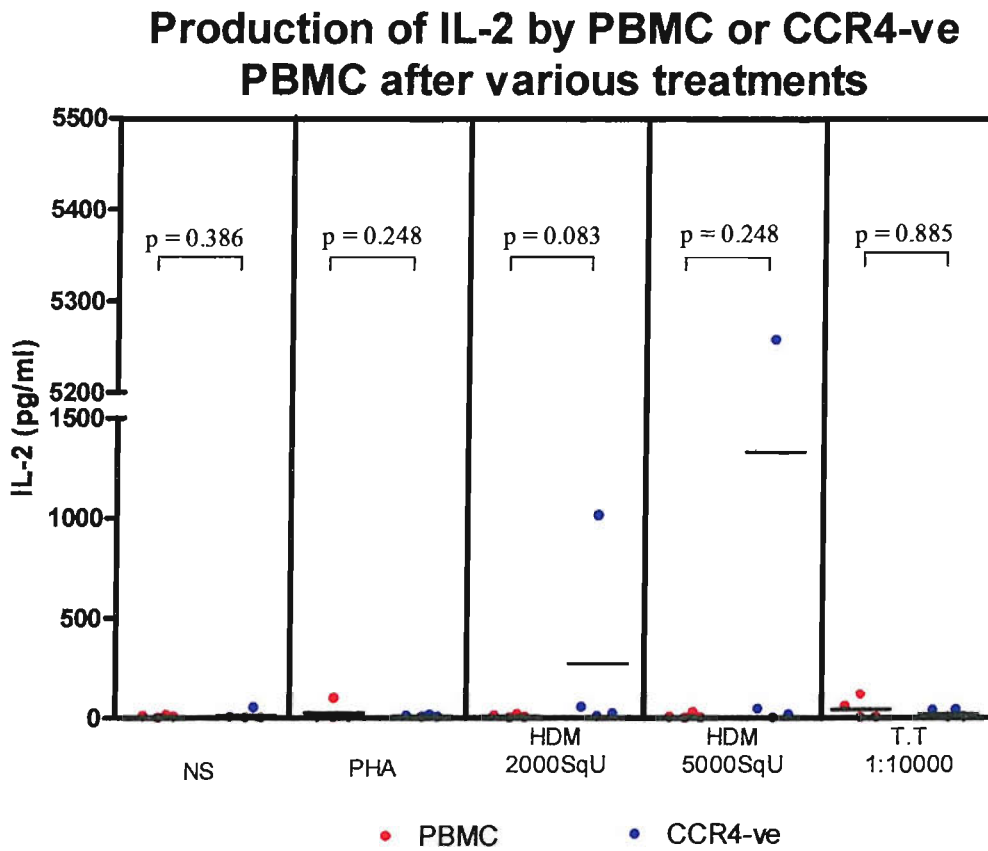
Figure 6.3 Production of IL-2 by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

Figure 6.4
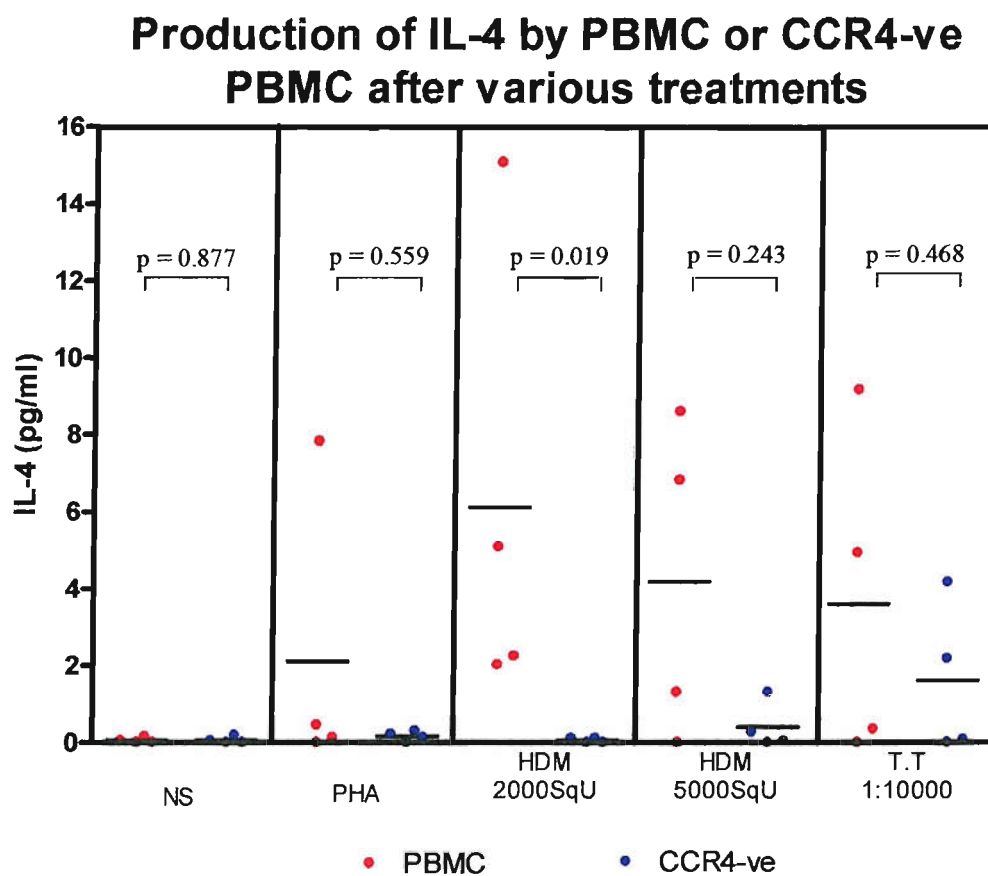
Production of IL-4 by PBMC or CCR4-ve PBMC after various treatments

Figure 6.4   Production of IL-4 by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

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Figure 6.5

Production of IL-5 by PBMC or CCR4-ve PBMC after various treatments
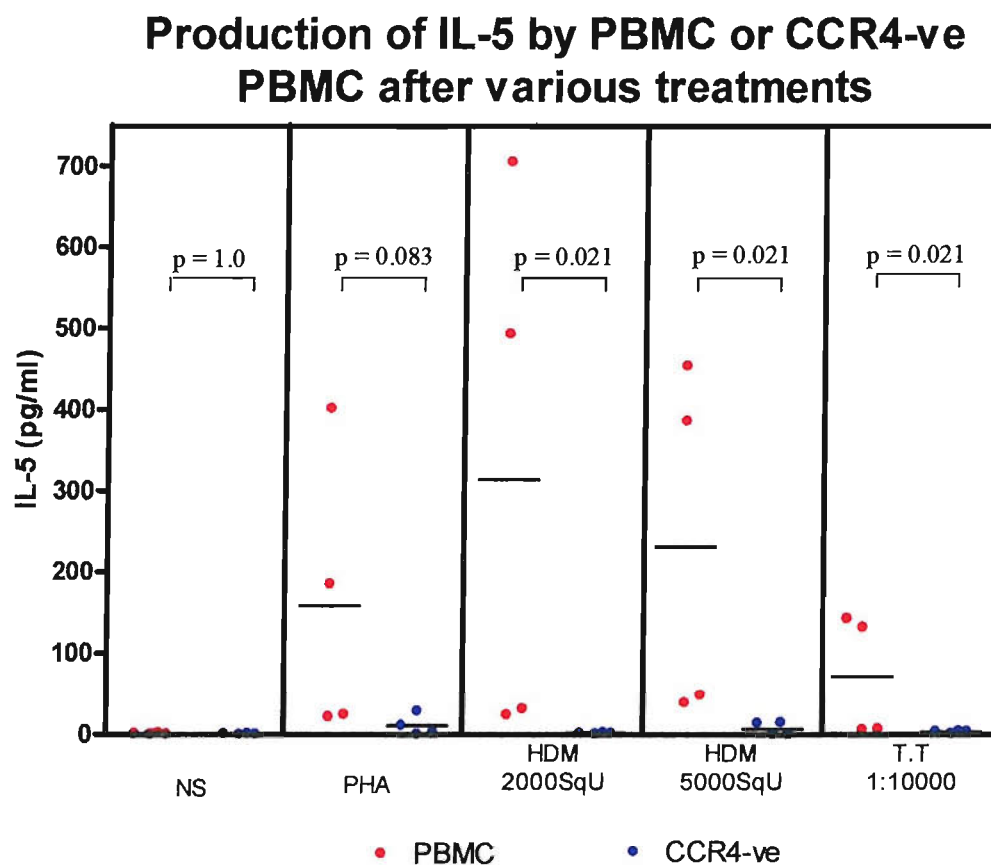
Figure 6.5 Production of IL-5 by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

Figure 6.6

Production of IL-10 by PBMC or CCR4-ve PBMC after various treatments
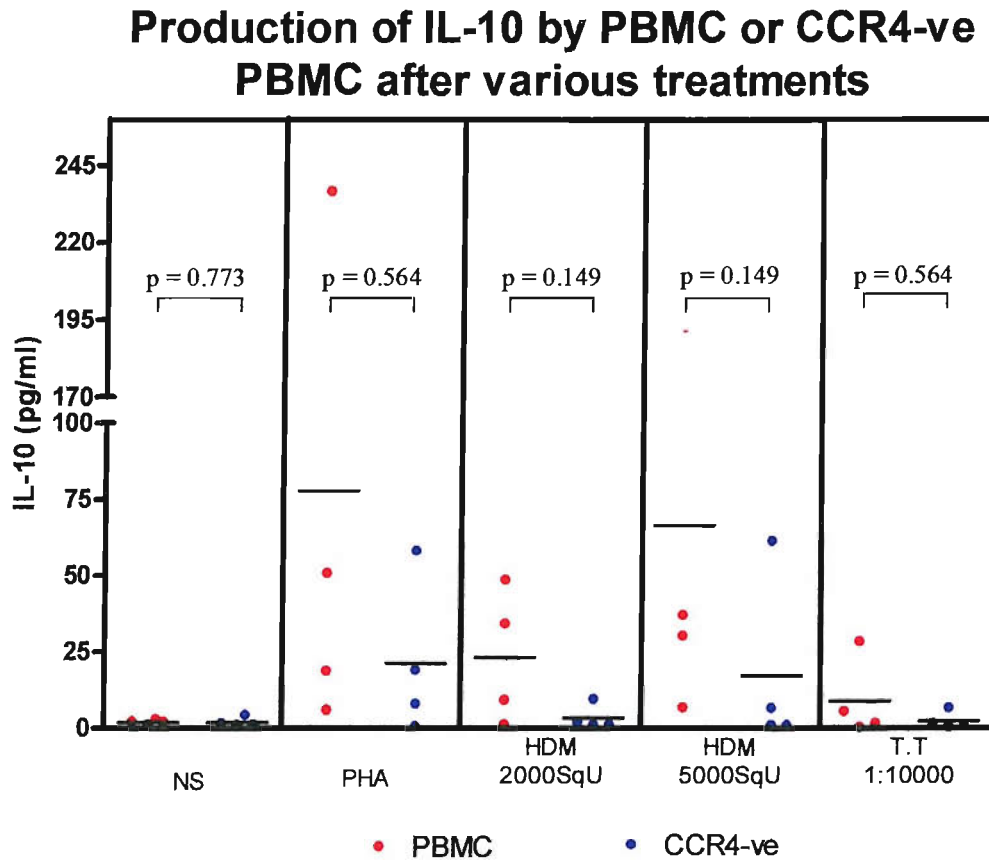
Figure 6.6   Production of IL-10 by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

Figure 6.7

Production of IL-13 by PBMC or CCR4-ve PBMC after various treatments
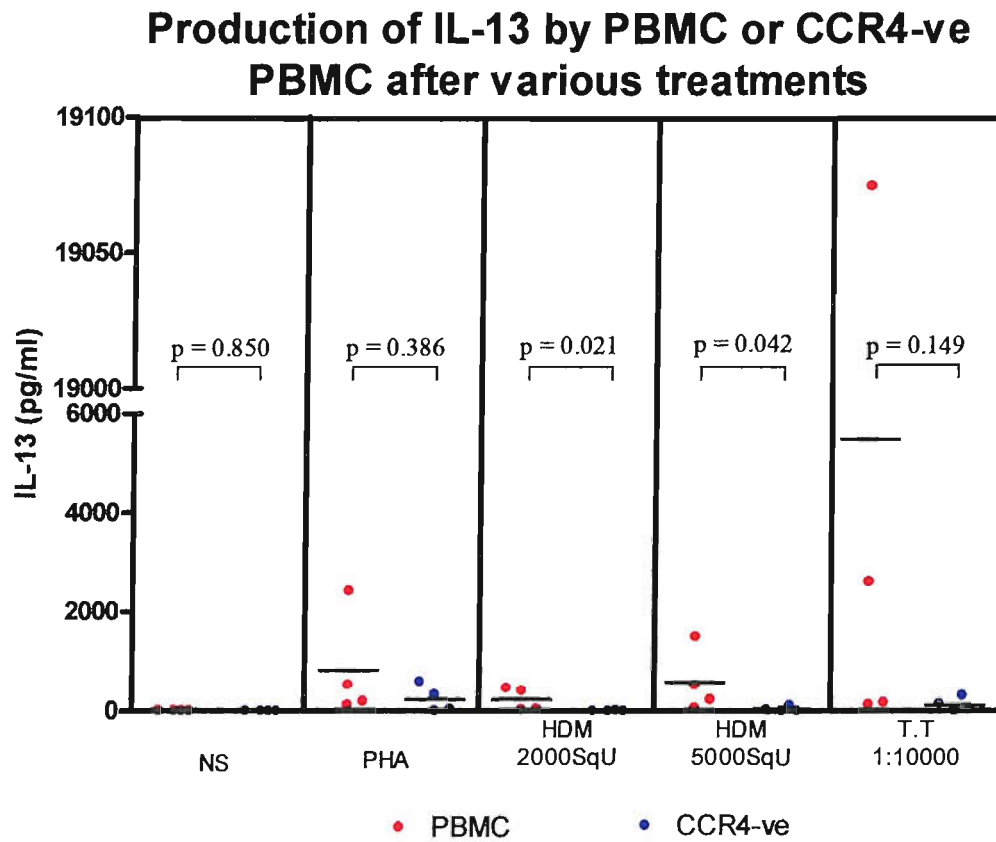
Figure 6.7 Production of IL-13 by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

Figure 6.8
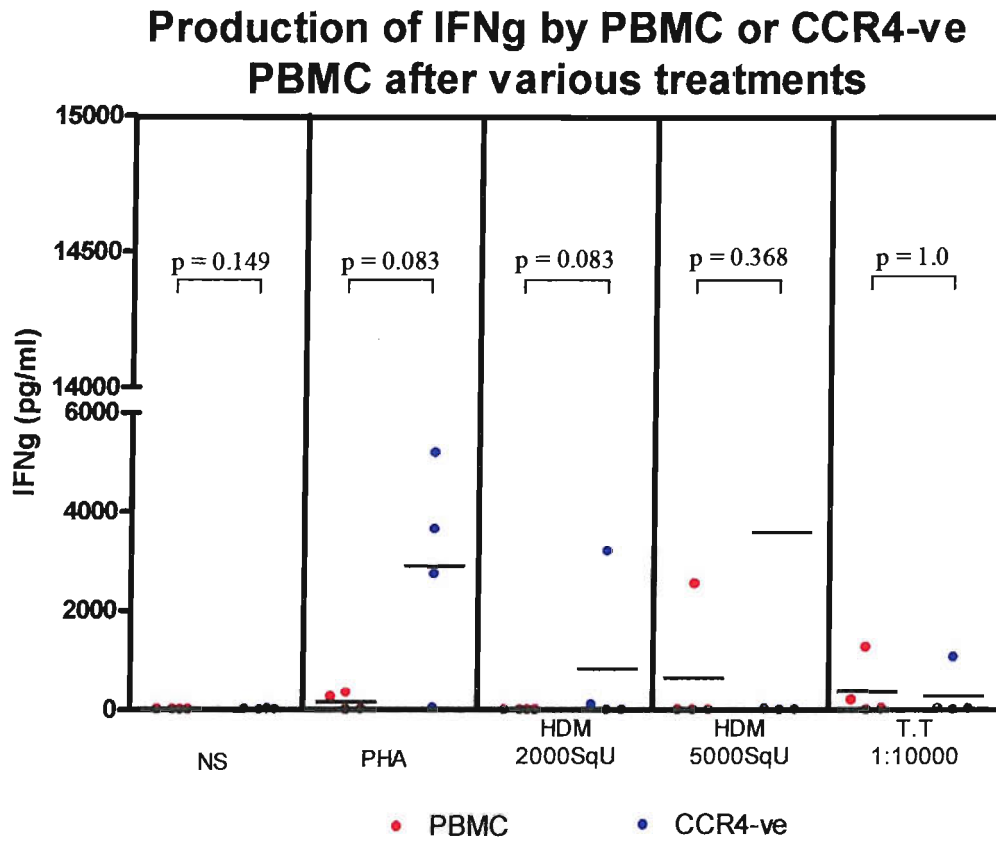
Production of IFNg by PBMC or CCR4-ve PBMC after various treatments

Figure 6.8 Production of IFNγ by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

Figure 6.9

Production of TNFa by PBMC or CCR4-ve PBMC after various treatments
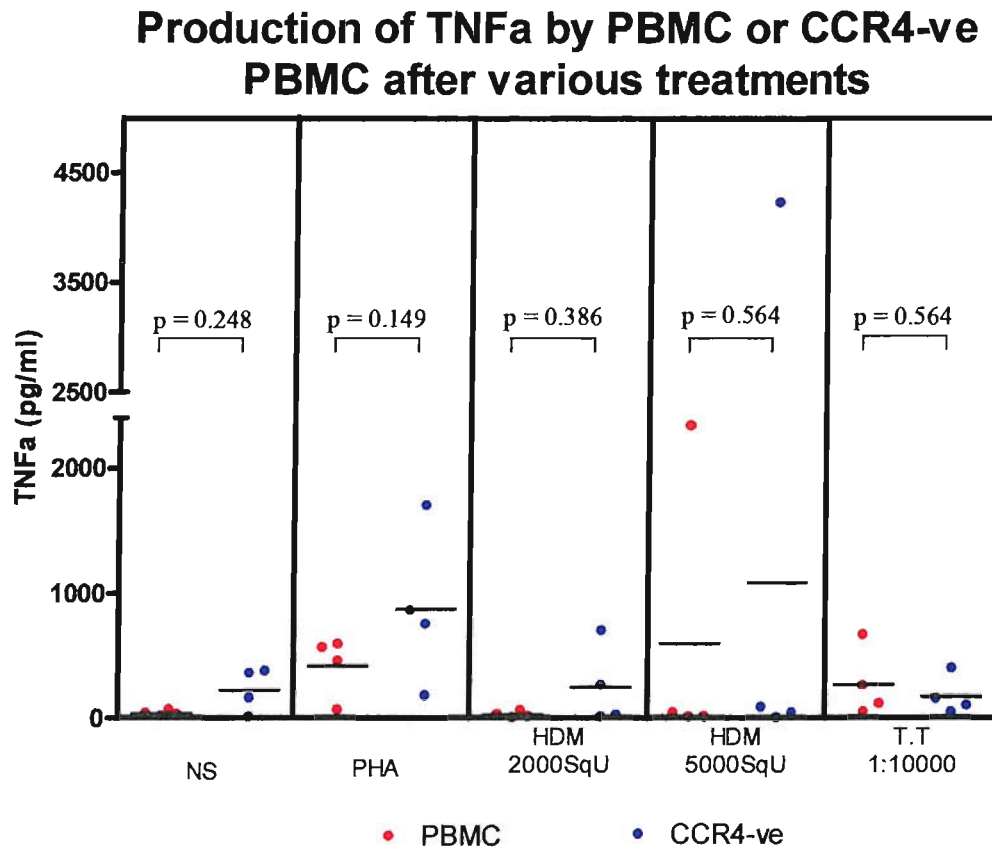
Figure 6.4   Production of IL-4 by PBMC (red symbols) or CCR4-ve cells (blue symbols) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values of 4 allergic mild asthmatics.

Table 6.1

| Table of statistics					
	NS	PHA	HDM 2000 SqU	HDM 5000 SqU	T.T 1:11000
Proliferation	1.000	0.248	0.564	0.564	0.248
IL-2	0.386	0.248	0.083	0.248	0.885
IL-4	0.877	0.559	0.019	0.243	0.468
IL-5	1.000	0.083	0.021	0.021	0.021
IL-10	0.773	0.564	0.149	0.149	0.564
IL-13	0.850	0.386	0.021	0.042	0.149
IFNg	0.149	0.083	0.083	0.386	1.000
TNFa	0.248	0.149	0.386	0.564	0.564

Table 6.1   Table of statistical significance for the drop in cytokine production upon removal of CCR4+ cells from PBMC after treatment with PHA, HDM allergen and tetanus toxoid.

Figure 6.10

Proliferation of PBMC or CCR4- PBMC after various treatments
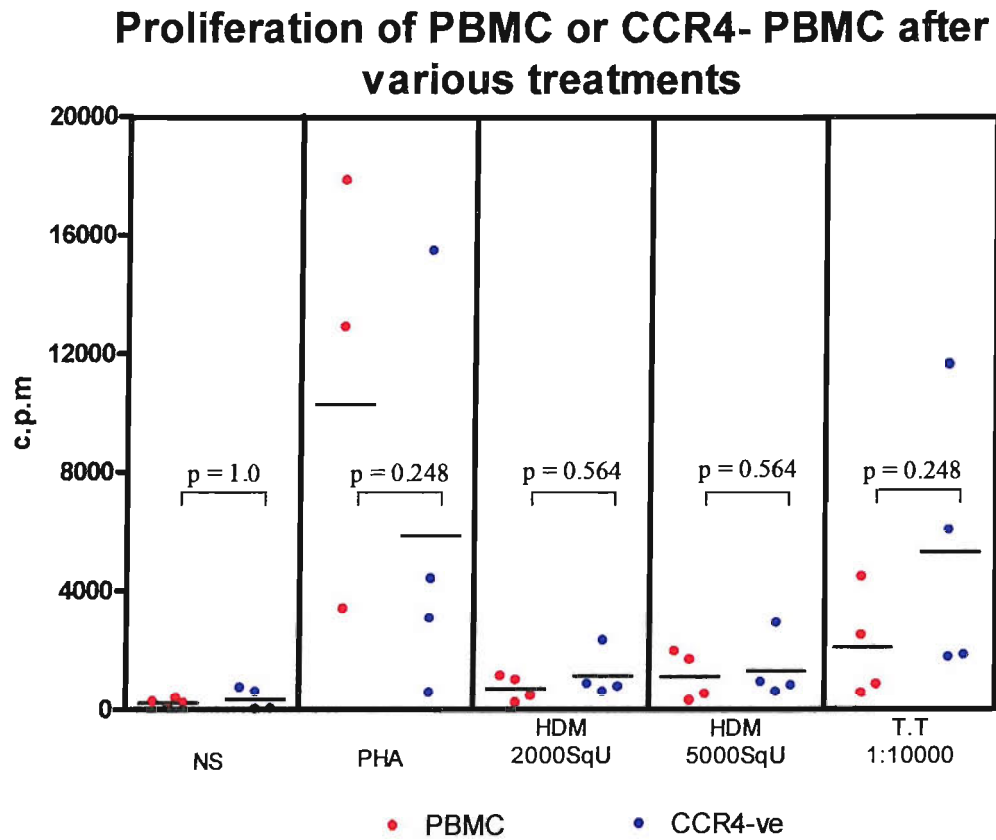
Figure 6.10 Proliferation of PBMC (red symbols) or CCR4-ve cells (blue symbols) detected by thymidine uptake (c.p.m) in response to treatment with: PHA; 2 concentrations of ALK HDM allergen (SqU); one concentration of tetanus toxoid (TT); and an untreated control (NS). Horizontal lines indicate median values for 4 allergic mild asthmatics.

Chapter 7

Discussion and Conclusions

7.1   Discussion

The main overall objective of this thesis was to improve the understanding of the mechanisms of T cell chemotaxis in atopic asthma, with a focus on the chemokine receptor CCR4 and its ligands. Firstly, any differences in the frequency of circulating T cells which are CCR4+ or CRTH2 + in asthma were determined. Additionally the expression of a range of activation and memory markers on CCR4+ T cells was quantified in peripheral blood and comparisons were made between asthmatic and healthy control subjects. The expression of CCR4 in sections of bronchial biopsy tissue was also investigated in order to find whether there were any differences in the expression of CCR4 in the airways between healthy and asthmatic subjects. Another aim was to extend past observations of chemotactic activity produced in the bronchial mucosa when stimulated with allergen; to that extent the bronchial explant model was employed in order to quantify the production of a selection of cytokines and chemokines that are believed to be important in asthma pathogenesis. The dependency of the chemotactic activity on CCR4 and its ligands was assessed using a cell line that expresses CCR4 and by using T cells that had been polarised towards a Th2 phenotype, thereby augmenting the expression of CCR4. Finally, the role of CCR4 was further studied by examining whether cells that express CCR4 are of central importance to the release of Th2 cytokines which play a key role in asthma and other atopic diseases.

Extensive flow cytometic analysis was conducted on a sufficiently large number of atopic asthmatics and control non-atopic subjects and the results showed conclusively that there are no differences between mild atopic asthmatics and control subjects in respect of the numbers of either CCR4+ or CRTH2+ T cells and with regards to the expression of memory and activation markers on either total CD4+ or CD4+CCR4+ T cells (Chapter 3). When assessing the relevance of these findings, there are a number of important factors to be taken into account. Firstly, the patients studied had mild asthma and CCR4+ cells as well as other activated cells in the blood may be more frequent in the blood of patients with more severe disease. Second, although the asthmatics did not have higher percentages of cells expressing any of the activation

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and memory markers studied in the blood this cannot be extrapolated to the airways. Indeed a similar lack of difference in blood has been found to be the case for CD45RO+ cells (Saltini *et al.*, 1990) while raised RO+ cells have been found in the airways of asthmatics (Marathias *et al.*, 1991), probably resulting from continuous stimulation of T cells within the airways or selective accumulation of these cells by mechanisms as yet unknown. In patients with more severe disease, increased percentages could have been found in the blood possibly due to a spill over from the airways into the peripheral circulation which is less likely to be seen in those patients with milder forms of asthma. The presence of CCR4+CD45RO+ cells has been studied by Kurashima and colleagues (Kurashima *et al.*, 2006) who sought to investigate the relationship between Th2 cells and asthma severity, taking into account that a predominance of type 2 helper T cells (Th2) in bronchoalveolar spaces and peripheral blood is a well-accepted feature of bronchial asthma. To investigate this they assessed the distribution of peripheral blood CXCR3+ and CCR4+ lymphocytes using flow cytometry in asthmatic and healthy subjects and discovered that the percentages of CCR4+/CD45RO+ CD4 T cells were elevated in asthmatic patients compared with controls but they found no significant difference in CCR4+/CD45RO+ cells between the mild to severe asthma patients (Kurashima *et al.*, 2006). Using immunohistochemical staining, the current study showed that there were no CCR4 positive cells in bronchial biopsy tissue sections of 6 asthmatics and 6 healthy subjects. Nasal polyp from one subject of a total of 9 tested, stained positive for CCR4 and this was used as a positive control throughout. This finding is contrary to that of Panina-Bordignon and colleagues who found extensive T cell staining for CCR4 in OCT-stained sections of bronchial tissue, either unchallenged or allergen challenged (Panina-Bordignon et al., 2001). Their choice of poor quality antibody to CCR4 and lack of staining for CCR4 in bronchial biopsy tissue sections in the present study casts doubt for a functional role of CCR4 in asthma.

The assessment of cytokine and chemokine production by bronchial biopsy tissue from healthy and asthmatic subjects (Chapter 4) has so far found significant differences for a number of cytokines, namely IL-2, IL-4, IL-5 and IL-13. The findings for IL-4, IL-5

and IL-13 are in keeping with reports in the literature. These cytokines have a prominent role in the allergic asthmatic response, primarily with roles associated with eosinophil function (Weller, 1991) and isotype switching of B cells and subsequent IgE production (Minty et al., 1993). This observation also validates the methodological approach taken in the current study. Although it is impossible to determine to what extent the differences in cytokine and chemokine production between the atopic asthmatics and the healthy control subjects are due to asthma or atopy and had atopic non-asthmatics also been included in the study, more definitive conclusions could be made.

Assessment of the biopsy supernatants was extended by investigating the chemotactic responses of both a CCR4-expressing T cell line and PBMC that had been artificially polarised towards the Th2 phenotype, with the aim of showing that these CCR4 bearing cells migrate towards the cytokine and chemokine containing biopsy supernatants generated using the explant model (Chapter 5). The ultimate goal was to show that CCR4 was responsible for the migration of both the CEM cell line, which constitutively expresses CCR4, and the Th2-type generated cells, by ablating this migration with a CCR4 blocking molecule. In the validation experiments, the CEM cell line migrated to recombinant CCL22 in a typical bell-shaped manner and migration to CCL22 was abolished at the highest tested concentration of the blocking molecule. Following this, the expression of CCR4 and a panel of other chemokine receptors on PBMC that had been polarised into a Th2 phenotype were investigated. In this instance, the success of polarisation was characterised by a higher percentage of CCR4 expression on the Th2 polarised cells than that of untreated PBMC and PBMC polarised into Th1 type cells. These polarised cells were then used in chemotaxis assays and it was found that Th2 polarised but not Th1 polarised cells responded with prominent chemotactic activity when exposed to CCL22. Previous studies, (Imai et al., 1999, Lloyd et al., 2000) have documented an increased expression of CCR4 on in vitro polarised Th2 cells and their enhanced ability to migrate to the CCR4 ligands, CCL17 and CCL22. For instance, Imai and colleagues noted that when CD4+ T cells from adult peripheral blood were polarised into a Th2 phenotype in vitro, the cells

selectively expressed CCR4 and vigorously migrated toward CCL17 and CCL22 (Imai *et al.*, 1999). Similar findings have been documented by Lloyd and co-workers who noted that human cells that had been polarised into Th2 cells *in vitro* selectively express CCR4 and respond preferentially to CCL22 (Lloyd *et al.*, 2000). Using *in vitro* generated Th2 cells from healthy and asthmatic subjects the ability of these cells to migrate to the biopsy supernatants from healthy and asthmatic subjects respectively was investigated, with a final goal of being able to block cell migration using the CCR4 blocking molecule. Contrary to what was anticipated, there was no migratory activity of the cells to the biopsy supernatants and therefore, no migration to block with the blocking molecule. Having reviewed the data, it was concluded that the cell line and the polarised Th2 type cells have responded to recombinant chemokines but not to native chemokines. The polarisation stages of the fresh PBMC involved what could be viewed as a harsh treatment and lengthy periods of culture which could have rendered the cells less sensitive to the ligands. The concentration of recombinant CCL22 required to induce migration of the Th2 cells was 3nM which equates to 24µg/ml and far exceeded the concentration of CCL22 found in the biopsy supernatants of both the healthy and asthmatic subjects. The highest value obtained for CCL22 from the analysis of the biopsy supernatants, before taking biopsy weight into account was 888pg/ml which is 27-thousand times less than that of recombinant CCL22 required to induce cell migration. It was unlikely, therefore, that any migration would have been seen. Although it this does not rule out migration mediated by the actions of synergy and the action of chemokines on receptors not known to be expressed on the cells, the 27-thousand times lower concentrations in the biopsy supernatants makes even synergistically acting chemokines still unlikely to induce migration of these polarised cells. Whilst it is conceivable that native chemokines produced by diseased tissue are more potent than recombinant chemokines the lack of migration to the biopsy supernatants is unquestionably due to the concentrations not being high enough to induce cell migration. If the cells had migrated to the biopsy supernatants, some form of synergy would have been involved which could have been investigated further by using the CCR4 blocking molecule. A third explanation, albeit less likely, is the effect of labelling cells with calcein and this is something which may

also have affected the cells, however, in validation experiments, calcein appeared to have no detrimental effects on the cells. Finally, the CEM cells and Th2 polarised cells might express more CCR4 receptors on the surface in comparison to Th2 cells migrating into the airways and this could also be a contributing factor, limiting the migration of the cell line and the artificially polarised cells to biopsy supernatants.

Indeed, recent studies, conducted by Pandurangan Vijayanand, within the inflammatory cell biology group in which the current work was performed, has shown that fresh, untreated, negatively selected CD4+CD45RO+ cells from the same patients from which the supernatants were obtained, migrate towards the respective supernatants, in a dose-response manner. The migration that was observed with CD4+CD45RO+ cells was diminished, but not completely abolished, when the blocking molecule was added. The migratory activity of these cell types was elucidated by cell counting and not by detecting fluorescence emitted from calcein stained cells. This suggests; a) that cells from the individual from which the explant supernatants were obtained may respond more optimally; b) that memory cells migrate more readily, and c) that fresh, non-cultured cells are more sensitive to low concentrations of chemokines such as the concentrations found in the biopsy supernatants.

A number of studies have shown that CCR4 is selectively and highly expressed on Th2 cells and is a selective marker for Th2 lymphocytes which is markedly upregulated upon T cell receptor activation (Sallusto et al., 1998, Panina-Bordignon et al., 2001, D'Ambrosio et al., 1998). To this end, a further aim was to assess whether the CCR4+ cells population in HDM allergic asthmatics contains the allergen specific T cells (Chapter 6). By removing CCR4+ cells from HDM allergic individuals, IL-5 production in all the treatment conditions, i.e. not just with HDM allergen, was significantly reduced but not completely ablated. This work has suggested that the IL-5 producing cells have been removed and not just the HDM allergen specific cells, implying that without any prior *ex vivo* manipulation (i.e. polarisation, as has been the case with previous studies) the CCR4+ cell population contains Th2 cytokine

producing cells. The results from this piece of work support the literature, stating CCR4 is preferentially, but not selectively expressed on Th2 / Tc2 cells, as indicated in the current study, by the production of the Th2-type cytokine IL-5 and IL-4 and IL-13 to a lesser extent.

The main limiting factors governing the results of this thesis are the relatively small subject numbers and focus only mild atopic asthmatics and lack of inclusion of atopic non-asthmatics as a separate control group. This project involved the use of bronchoscopy which, in view of the safety and tolerability issues limits the numbers of subjects that can be studied. The complexities involved in reaching a definitive outcome when investigating certain mediators and cell surface markers, or indeed any contributing factor in asthma, include that of redundancy of cytokines and other mediators. For example, a poor IL-5 response in a given asthmatic may be compensated for by an increase in the production of another pro-eosinophil factor such as GM-CSF or IL-3, or even a combination of many cell-derived factors and these may well differ from person to person. Although this research focused on human studies, it is worthwhile mentioning that much of the research investigating the mechanisms and pathology of asthma are performed using animal models of respiratory inflammation and although this gives valuable insight into the mechanisms involved in murine asthma, it cannot be directly extrapolated to humans: For instance, although immunologically similar, the Th1/Th2 arm of the immune system is much more clear-cut in the mouse and additionally, much of the ongoing research investigating asthma is performed on inbred strains of mice with the same genetic background. The incentive behind this kind of work is due to the limitations and ethical restrictions applying to human subjects.

7.2   Conclusions

This work has led to the following conclusions

1. T cells that express CCR4 contain the majority of cells that produce Th2 cytokines;

2. The Th2 cytokine producing CCR4+ cells can be induced to generate these cytokines by both an allergen-specific and a non-specific stimulus;

3. Bronchial tissue stimulated *ex vivo* with allergen as part of the explant model released significant quantities of Th2 cytokines and chemokines, including CCL17 and CCL22, the ligands for CCR4;

4. CCR4+ cells generated *in vitro* using standard protocols to augment the expression of CCR4 and respond to recombinant CCL22; however, these cells do not respond to the low levels of native chemokines produced *ex vivo* by the explanted bronchial mucosal tissue;

5. The reasons for the discrepancy in response to recombinant and native chemokines remains to be elucidated but could include the following;

   a. The need for higher concentrations of chemokines than achieved in the explants;

   b. Requirement for additional chemokine receptors, not just CCR4, which are not present on T cells after the completion of the polarisation protocol;

   c. The need to use enriched CD45RO+ memory cells which, as suggested in the work that followed, migrate better to explant supernatants;

   d. Even though previous research in the Inflammatory Cell Biology group has shown good responses of unfractionated T cells from unrelated donors, it is possible that the use of cells from the same donor might have resulted in more consistent responses.

7.3 Future research

1. Is the expression of CCR4 on circulating T cells different in more severe asthma?

    a. Is this a feature of asthma and atopic inflammation or is this a feature of all more severe inflammatory diseases?

    b. Does the expression of CCR4 change (increase or decrease) at times of asthma exacerbation?

    c. Does any increased expression of CCR4 during exacerbations depend on the cause of exacerbation, i.e. viral versus allergen-induced?

2. Is there any increase in CCR4 expression in the airways of mild asthmatics?

3. Is there any increase in CCR4 expression in the airways of more severe asthmatics?

4. Are the CCR4+ cells also a source of Th2 cytokines in more severe asthmatics?

5. Is the use of unstimulated peripheral blood T cells from the same donors that provided the explant tissue a more sensitive and, therefore, more physiologically relevant means of testing the chemotactic activity?

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