T lymphocyte Recruitment to the Lung in Asthma

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Kugathasan Mutalithas
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ABSTRACT

T cells play an important part in the pathogenesis of asthma. In this study greater numbers of T cells were seen in the bronchial epithelium of severe asthmatics compared to normal subjects. There were greater percentages of both IL-4$^+$ and IFN-γ$^+$ T cells in the bronchoalveolar lavage of asthmatics compared to normal subjects. Chemokines and their receptors are thought to act as signals that guide particular subsets of T cells to the lung in asthma. Using flow cytometry I had explored whether the chemokine receptors CCR8 and CRTh2 played any role in recruiting T cells and in particular IL-4$^+$ T cells to the lung in asthma. Both receptors selectively identified IL-4$^+$ and IL-13$^+$ T cells in the blood and bronchoalveolar lavage from asthmatics and normal subjects. Cells expressing CCR8 but not CRTh2 were found at a higher percentage in the blood of severe asthmatics compared to normal controls. The percentage of CCR8$^+$ T in the bronchoalveolar lavage of asthmatics was higher compared to normal subjects and furthermore, there was a greater percentage of CCR8$^+$ T cells in the bronchoalveolar lavage compared to blood within the same asthmatic subject. This difference in the percentage of CCR8$^+$ T cells between blood and BAL was not seen in normal subjects. This suggests that there may be a role for CCR8 in the recruitment of T cells to the lung in asthma. In support of this, higher concentrations of the ligand CCL1, were seen in the bronchoalveolar lavage of asthmatics compared to that from normal subjects. Little experimental evidence was found that supported the contention that CRTh2 played a significant role in T cell recruitment to the lung. As a marker of IL-4$^+$ T cells in asthmatics, CCR8 compared favourably with CRTh2 as they identified a greater percentage of IL-4$^+$ T cells in bronchoalveolar lavage than CRTh2. CCR8 also compared favourably with CCR4 as a marker for IL-4$^+$ T cells due to higher specificity. The iNKT subset of T cells has been claimed to be an important group of T cells in asthma and was reported to be present at high percentages in the lung of moderate to severe asthmatics. In this study we had shown that in asthmatics these cells are present in very low percentages, similar to that in normal subjects and that they probably do not play a significant role in severe asthma.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AHR</td>
<td>Airway hyperreactivity</td>
</tr>
<tr>
<td>APC</td>
<td>Allopyocyanin</td>
</tr>
<tr>
<td>ATS</td>
<td>American thoracic society</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BHR</td>
<td>Bronchial hypersensitivity</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BTS</td>
<td>British Thoracic Society</td>
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<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
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<tr>
<td>CD</td>
<td>Cluster differentiation antigen</td>
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<tr>
<td>CI</td>
<td>Calcium Ionophore</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRTh2</td>
<td>Chemokine receptor homologue expressed on T&lt;sub&gt;H2&lt;/sub&gt; cell</td>
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<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte antigen</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DP</td>
<td>Prostanoid receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second.</td>
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<tr>
<td>FITC</td>
<td>Fluorocein Isothyocyanate</td>
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<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>NKT cell</td>
<td>Natural killer T cell</td>
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<tr>
<td>iNKT</td>
<td>Invariant natural killer T cell</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>OCS</td>
<td>Oral corticosteroid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Pc20</td>
<td>Provocation concentration of methacholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGD$_2$</td>
<td>Prostaglandin D2</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
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<tr>
<td>SSC</td>
<td>Side scatter</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>T$_{H1}$</td>
<td>T helper type 1</td>
</tr>
<tr>
<td>T$_{H2}$</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
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1 Introduction

1.1 Airway inflammation and asthma

Asthma is one of the most common chronic diseases in the world. It is estimated that approximately 300 million people currently suffer from asthma. The incidence of asthma has increased in westernized societies in the past 20 years and there had also been an increase in fatal asthma, especially in children. Asthma now accounts for ~1 in every 250 deaths worldwide [1]. Looking ahead, it is estimated that by 2025 there will be an additional 100 million people with asthma (Global burden of asthma, GINA). In the UK asthma is a major cause of impaired quality of life, primary care usage, consumption of prescription drugs and hospital admissions [2]. Internationally the UK ranks high on the prevalence of asthma. Although the prospect for a cure for asthma still remains remote much has been learnt about the disease over the last 30 years. Asthma was once viewed as a simple case of bronchoconstriction needing treatment with bronchodilators. It is now recognized as a complex inflammatory disorder of the airway with a range of clinical features. In contrast to earlier asthma therapies which were aimed at bronchodilation, current focus is now on designing therapies that will reduce the underlying airway inflammation.

Clinically most asthmatics are identified by their adverse reaction to allergens and other environmental factors with or without diminished lung function. As a disease asthma is characterized by variable airflow obstruction, peribronchial inflammation, and airway hyperresponsiveness. It is now widely accepted that inflammation within the airway is an integral and necessary component of asthma pathophysiology. Early evidence for the role of inflammation in asthma came from autopsy examinations of patients with fatal asthma. Airway histology revealed increased numbers of leukocytes within the mucosa, thickening of the basement membrane, airway epithelial cell injury, increased mucous glands and mucus impaction within the airway lumen. Although originally thought to be
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a feature specific to fatal asthma, with the use of bronchoscopy this was later shown in non fatal asthma across a range of severities [3].

In the 1980’s bronchoscopy provided researchers with a relatively non-invasive tool to sample and analyze lavage and tissue samples from the airway of living subjects with asthma. This together with immuno-histochemical tools provided conclusive proof for the role of inflammation in asthma[3]. A number asthma studies showed increased numbers of eosinophils, mononuclear cells and inflammatory mediators such as cytokines and chemokines in mucosal biopsies lavage samples of asthmatics compared to healthy subjects. Bronchoscopy also allowed comparisons to be made between asthma of different severities, before and after treatment and following airway stimulus (allergen challenge). Clinical correlation was then possible with underlying features of inflammation within the airways. Therapies aimed at reducing inflammation such as glucocorticoids are now the mainstay treatment for asthma emphasizing the importance of inflammation. Improvement in asthma symptoms occurs in parallel with reduction in the degree of inflammation within the airway.

Although a necessary part, the presence of airway inflammation does not always lead to the development of clinical asthma. This is demonstrated by patients with bronchiectasis, and smoking related chronic obstructive pulmonary disease. The asthmatic phenotype appears to develop when airway inflammation leads to airway hyperresponsiveness (AHR) [4]. Why some individuals with airway inflammation develop AHR is still not entirely clear but may possibly be related to modulation of the airway smooth muscle by the inflammatory process. The degree of airway inflammation does not determine the likelihood of developing airway hyperresponsiveness either. Development of the asthma phenotype appears to be multifactorial and depends not only on the presence of inflammation in the airway but also the type of inflammation, environmental risk factors and genetic predisposition.

A lot of effort had been made over the years to identify the specific pathophysiological defect that leads to asthma. 90% of asthmatic children and 50-60% of asthmatic adults
are atopic as defined by the presence of allergen specific IgE [5]. In these individuals an IgE mediated inflammatory response to the allergen could play a role in the initiating and sustaining inflammation. In experimental models of allergic asthma clearly defined roles can be seen for naïve T cells, dendritic cells and B cells during allergen sensitization resulting in the production of allergen specific IgE and sensitized T cells. Primed T cells, preformed IgE and mast cells then take part in an effector phase which eventually leads to bronchoconstriction [6]. However, about 50% of adult asthmatics have no detectable allergen specific IgE. Although IgE to an undetectable allergen has been suggested in these patients, it is not clear whether these processes actually contribute significantly in non experimental asthma whether atopic or non atopic [7].

A number of descriptive studies of asthmatic airways had reported the presence of eosinophils [8-10]. It has become widely accepted that inflammation within the asthmatic airway is characteristically eosinophilic. However, the presence of eosinophils is not specific for asthma as demonstrated by eosinophilic bronchitis, a condition associated with airway eosinophilia, but without all the clinical features of asthma [11]. Not all studies have shown increased eosinophils in the asthmatic airway [12]. With the increasing use of bronchoscopy to characterize asthmatics by their underlying airway pathology, there had been an increasing awareness of the heterogeneity in the inflammatory profile that drives this disease. We now know that a number of different immune cells play key and necessary roles in human asthma pathogenesis including neutrophils [13], lymphocytes [14], mast cells [15] and dendritic cells. In experimental asthma using animal models, each of these immune cells has been shown to play an important part in the eventual development of cardinal features of asthma. Data from experimental asthma over the years suggests that different immune cells contribute differentially to the multiple features that characterize asthma [3].

Similarly there also appears to be heterogeneity in the clinical features shown by asthmatics. Clinicians have long been aware of the distinct clinical phenotypes of asthma for example severe, moderate, mild, allergic (extrinsic), non-allergic (intrinsic), nocturnal, exercise induced and brittle asthma each requiring different treatment strategies. Clinical
categorization can be imprecise as they often include subjective variables. Development of new assessment tools, have given the opportunity to characterize asthma patients further and more precisely, for example with the quantification of sputum eosinophils and other surrogate markers of airway inflammation. This also has clinical relevance in choosing the most effective therapeutic strategy as has been shown by the effective use of monitoring sputum eosinophils in guiding asthma treatment [16]. Cluster analysis of cohorts of carefully characterized (clinical and pathological) asthmatics has identified distinct asthma phenotypes which has given an insight into the correlation of the different clinical patterns to the underlying pathology. Interestingly groups showing poor response to treatment showed discordance between their symptoms and the underlying inflammatory state of their airway [17].

Asthma is clearly heterogeneous in its underlying pathophysiology and clinical manifestation and careful correlation suggests that the varied clinical manifestation including response to treatment can be explained in part by the varying underlying airway pathology. Identifying the critical cell or a critical step that eventually leads to the development of asthma is an attractive concept for drug discovery, however, all evidence to date suggest that the underlying mechanism is more complex and heterogenous. A multi-targeted therapy covering different constituents of the asthma inflammation is likely to be more promising. Different immune cells are likely to be involved at different steps in the cascade that eventually leads to the asthma phenotype. In the following chapters I will focus on just one of these participants in asthma inflammation, the T cell, and explore how this cell may be involved in the pathogenesis of asthma.
1.2 T cells and asthma

T cells constitute ~ 70% of the total pool of lymphocytes. They have long been suspected to have a coordinating role in the inflammatory cascade due to their wide range of function. T cells are a heterogeneous group of cells with multiple subsets, distinct on the basis of maturation, activation requirements, activation status, migratory properties and function. T helper cells (T<sub>H</sub>) are activated by the cognate interaction of the T cell receptor (TCR) with the peptide-MHC II complex on antigen presenting cells. Once activated T<sub>H</sub> cells orchestrate adaptive antigen specific cell mediated and humoral responses. The wide array of cytokines that they are able to produce makes them very versatile immune cells, able to interact and modulate effector functions in other immune cells. The involvement of T<sub>H</sub> cells in the production of IgE by B cells initially triggered the interest in T cells in understanding asthma pathophysiology. In the last 30 years knowledge in T cell biology has expanded considerably and new roles are still being added to this already versatile group of cells. I have summarized below the some of the evidence frequently quoted for the involvement of T cells in asthma.

Human studies

- Overall there is only weak evidence that T cells are seen in increased numbers in the lungs of stable mild to moderate asthmatics compared to non asthmatics. A handful of studies have shown an increase. In a study comparing subjects with atopic asthma, non-atopic asthma with normal subjects more T cells were seen in the asthma groups compared to normal, without prior use of allergen challenge [9]. Increased T cells were also shown in the lung parenchyma of asthmatics using transbronchial biopsies [18]. However, these are outnumbered by the number of good studies that have not shown any significant difference in the number of T cells within the asthmatic lung [19, 20].

- Increased CD<sub>4</sub><sup>+</sup> cells were found in the BAL fluid of asthmatics following allergen challenge compared to normal controls. T cell numbers were shown to
be increased in the lung following allergen challenge compared to sham challenge in atopic asthmatics [21]. In one study allergen specific T cells were shown to increase in the lung when an inhalational challenge by that allergen was given with a reciprocal decrease in their numbers in blood [22].

- A change in the T cell subset composition has been shown in asthmatics following allergen challenge. Mucosal biopsy specimens from asthmatics following allergen challenge showed increased numbers of T cells with surface IL-2R (CD25) [23, 24]. Although once thought to be a marker of activation it is now known that they also identify a subset of regulatory T cells [25]. BAL T cells obtained from asthmatics treated with corticosteroids show less activation markers and cytokine expression assessed using in-situ hybridization [26]. Interestingly T cells obtained from steroid resistant patients failed to show a reduction in their surface activation markers after therapy with corticosteroids, indicating a T cell defect in these patients [27].

- Approximately 50% of all adult asthmatics are atopic and therefore would be expected to have allergen specific IgE. T cells play a key role in allergy mechanisms in part through the induction of IgE synthesis. The initiation of IgE synthesis by inhaled allergens requires the interaction of dendritic cells (antigen presenting cell), T cells and B cells. Activation of B cells occurs in lymph nodes through the interaction of the B cell with the allergen. In order for B cells to switch to the production of the IgE isotype, they will require two further signals provided by T cells. Firstly the presence of IL-13 and IL-4 supplied by the activated T cells within the lymph nodes, and secondly, interaction of surface CD40 on the B cells with its ligand expressed on T cells. T cells therefore contribute to asthma pathology in part through its interaction with B cells and production of IgE. In support of this allergen specific T cell clones have been isolated from blood T cells obtained from subjects with atopic asthma [28]. Similarly allergen specific T cell clones have also been isolated from BAL T cells from asthmatics [29].
• Activation markers (IL-2R, HLA-DR and VLA-1) on T cells were increased in the blood of patients with attacks of acute severe asthma compared to normal controls with levels decreasing following improvement in symptoms [30].

• In humans the use of anti CD4 monoclonal antibodies (Keliximab) had some beneficial effect in chronic severe asthma [31]. Cyclosporin which blocks NF-AT reduces IL-5 production by T cells and improved lung function in chronic corticosteroid dependent asthma [32].

• Challenge with synthetic T-cell epitopes lead to a late asthma reaction suggested to be mediated through allergen specific T\(_{\text{H2}}\) cells [33].

• In recent years regulatory T cells have attracted a lot of attention. Unlike other subsets, this group of T cells is thought to have an attenuating effect on allergic inflammation and asthma. Although only a limited number of studies are available at present and much still remains to be explored, some observations to date do indicate their effect in modulating the disease. Patients with unstable severe asthma were shown to have reduced numbers of IL-10 producing T cells (Tr1) compared to those with less severe disease [34]. Use of glucocorticoids resulted in increased numbers of Treg cells as well as mRNA for IL-10 (produced by Tr1 subset of Treg cells) [35].

Animal Studies

• Mice depleted of CD4+ T cells using a monoclonal antibody were spared from developing airway hyperreactivity (AHR) and airway eosinophil infiltration [36]. Adoptive transfer of allergen specific T\(_{\text{H2}}\) cells induced AHR in mice [37]. T cell targeted therapy has been effective in animal models of asthma. Antagonists of TCR activation reduce airway eosinophilia in rats (PI3-K
inhibitor, P8 MAPK inhibitor and ERK-1/2 inhibitors reduced IL-5 secretion from freshly isolated human T cells).

- In animal models of asthma a subset of T cells, the (invariant) iNKT cells has been suggested to play a role in asthma pathogenesis. These cells were shown able to produce T\(_{H2}\) cytokines. Mice devoid of iNKT were unable to develop airway hyperresponsiveness [38]. Similar involvement in human asthma has also been suggested, but awaits further confirmation. This will be explored in detail in subsequent chapters.

- T cells have also been shown to play a role in airway remodelling, a feature characteristic of chronic asthma. In rats adoptive transfer of antigen specific T cells into naive animals resulted in the greater expansion of airway smooth muscle (ASM) following antigen encounter than those that did not receive the T cells [39].

- Gene deficient mice resulting in absence of mast cells, B cells and IgE can still develop features of asthma [40, 41] whereas mice deficient of CD4\(^+\) T cells were not able to develop asthma [42].

A schematic representation of the possible T cell interactions in asthma pathogenesis is given in Figure 1-1. Despite the many lines of evidence for its involvement in asthma pathophysiology, the presence of T cells in the airway is certainly no *sine quo non* of asthma. A bronchial biopsy study of asthmatics had shown that the presence of T\(_{H2}\) cells in the airway mucosa in asthma and eosinophilic bronchitis in similar numbers. In this study authors had shown that the presence of mast cells embedded in the ASM to be the discriminating feature between the two conditions [11, 43]. Over the last 10 years the major contribution by T cells to asthma pathology was thought to be the production of T\(_{H2}\) cytokines. However, amongst the immune cells known to be involved in the inflammatory cascade occurring within the asthmatic airway, T cells are by no means the
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only cells able to elaborate T_\text{H}2 cytokines (IL-4, IL-5 and IL-13). In fact, there is no evidence that T cells are the major producers of these cytokines in quantity.

Figure 1-1: Possible roles for T cells in asthma pathophysiology.
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Although there is little evidence that T cells are the major contributors of Th2 cytokines in the asthmatic airway, there is evidence that they are probably the major inducible source of Th2 cytokines. In bronchial biopsies Th2 cytokine mRNA were localized to T cells whilst the protein product was mostly localized to mast cells and eosinophils [14, 44, 45]. This would suggest that unlike T cells, non-T cell sources have stored preformed Th2 cytokines within them, which they can readily release upon activation. The contributions by T cells are likely to be related to the timely secretion of these cytokines at critical stages in the evolution of the disease, especially in the early stages of the disease and therefore act as important players in the initiation, regulation and maintenance of the disease. Moreover, there are other T cell subsets that could have a role in asthma pathogenesis, independent of the involvement of Th2 cytokines (NKT cells, Treg). Given their varied function, it is difficult to assign a universal role for T cells in the causation of asthma. This is further complicated by the fact that some T cells may even have a beneficial role in asthma, exemplified by regulatory T cells that have the potential to attenuate inflammation. Contributions made by Th cells as well as some of the other subsets will be explored next.
1.3 The $\text{T}_1/\text{T}_2$ Hypothesis

Much of the research into airway inflammation relating to asthma over the last 10 years has been influenced by the $\text{T}_1$ and $\text{T}_2$ paradigm of inflammation. This stemmed from observations of T cell function in rodents by Mossmann and colleagues in the 1980’s. Two types of helper CD4$^+$ T cells were identified based on their cytokine secretory profile. The T helper 1 ($\text{T}_1$) cells which secreted predominantly IFN-$\gamma$, IL-12 and TNF-$\alpha$, and the T helper 2 ($\text{T}_2$) cells which secreted IL-4, IL-5, IL-9 and IL-13. These two groups of cytokines have distinct functions. The cytokines IFN-$\gamma$, IL-12 and TNF-$\alpha$, are involved in the defense against bacterial and viral pathogens whereas IL-4, IL-5, IL-9 and IL-13, are involved in defence against parasites and allergic inflammatory processes. Furthermore in experimental models these two types of cells have shown reciprocal inhibition such that IL-4 inhibits the development of $\text{T}_1$ cells and IL-12 inhibits $\text{T}_2$ development.

There is strong evidence to support the involvement of $\text{T}_2$ cytokines in asthma pathogenesis in animal models of asthma. In murine models of asthma, IL-4 was closely related to airway hyperresponsiveness, IL-5 with eosinophil recruitment and IL-13 with airway remodeling. These are features closely associated with asthma [46, 47]. Lung specific over expression of IL-5, IL-9 and IL-13 in transgenic mice induced AHR in mice without the need for allergen challenge [48, 49]. Adoptive transfer of $\text{T}_2$ cells into naïve mice induced airway eosinophilia and AHR [50]. $\text{T}_2$ cytokine gene deficient mice were unable to mount an allergic or asthma response [51].

The $\text{T}_1/\text{T}_2$ dichotomy of cytokines first described in mice was later shown in human T cells using T cell clones [52]. Later the involvement of $\text{T}_2$ cells in allergy was demonstrated. In atopic subjects, allergen specific T cell clones were shown to express predominantly $\text{T}_2$ cytokines [28]. In asthma, the first evidence for the involvement of $\text{T}_2$ cells came from the demonstration of an increased number of T cells expressing mRNA (using in-situ hybridization) for IL-4, IL-5 and GM-CSF in the BAL fluid from asthmatics compared to normal subjects. The mRNA was predominantly localized to T cells [14]. In this study mRNA for IFN-$\gamma$ was found in similar quantities in asthma and
normal control subjects. In a subsequent study, the number of cells expressing mRNA for IL-4 and IL-5 were also shown to be increased in bronchial biopsies from asthmatics compared to normal subjects [53]. In addition to mRNA, increased amounts of IL-4 and IL-5 protein was also shown in the BAL fluid of atopic asthmatics [54]. The increased numbers of T\(_H2\) cells was also shown to correlate with disease severity. The number of cells expressing IL-4 and IL-5 mRNA was also shown to correlate with FEV\(_1\) and degrees of airway hyperresponsiveness in asthmatics [55].

The evidence for the T\(_H1\)/T\(_H2\) imbalance with a T\(_H2\) dominant inflammatory profile in the airway of asthmatics led to the ‘T\(_H2\) hypothesis in asthma’ [56]. Central to this hypothesis was the important role played by the T\(_H2\) cell. They were suggested to act as the instigators for the inflammation that characterize the asthmatic airway. Allergen sensitized T\(_H2\) cells can recognize allergen peptides when presented by APC and secrete T\(_H2\) cytokines (IL-4, IL-5, IL-13 and GM-CSF). These cytokines recruit eosinophils and mast cells, activate mast cells, induce mucus hypersecretion and modulate airway smooth muscles leading to AHR.

Given the importance of the T\(_H2\) cell, studies focused on identifying the molecular mechanisms controlling the development of T\(_H1\) and T\(_H2\) cells from naive T cells. Determinants of either a T\(_H1\) or a T\(_H2\) lineage derive from the specific interaction of surface co-stimulatory molecules during allergen sensitization, the surrounding cytokine milieu during T cell development and gene transcription factors. Transcription factors STAT-6 (activated by IL-4), c-\(\text{maf}\) and GATA-3 were restricted to the development of T\(_H2\) cells, whilst T-Bet and STAT-4 was associated with T\(_H1\) cells. GATA-3 which is associated with the expression of IL-5 was shown also to be increased in the bronchial biopsies from human asthmatics compared to healthy controls [57]. In mice negation of GATA-3 resulted in less airway eosinophilia [58]. Mice deficient in STAT-6 were not able to develop airway eosinophilia and AHR [59].

Although the T\(_H2\) hypothesis is now an accepted paradigm for the mechanisms underlying asthma immunopathogenesis, several observations especially over the recent
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years have questioned the importance of the T\textsubscript{H}2 hypothesis in asthma [60]. In animal models mice treated with neutralizing antibodies to IFN-\gamma and then subjected to an allergen challenge did not show AHR whereas those treated with anti IL-4 and anti IL-5 monoclonal antibodies still did [61]. Despite the T\textsubscript{H}1 vs T\textsubscript{H}2 antagonism, adoptive transfer of T\textsubscript{H}1 cells did not attenuate asthma airway inflammation in mice [62].

Virtually all the studies showing an increase in T\textsubscript{H}2 cells in asthma have measured mRNA. When protein is measured there is little evidence for a T\textsubscript{H}2 bias. In humans when peripheral blood mononuclear cells were stimulated in vitro, increased IFN-\gamma+ T cells were seen in asthmatics compared to normal controls [63]. Increased T\textsubscript{H}1 cytokines (IFN-\gamma and TNF-\alpha) were shown in BAL cell cultures isolated from mild atopic asthma subjects compared to normal controls following stimulation with PMA [64]. Using flow cytometry to assess cytokines at the single cell level Krug et al showed that IFN-\gamma+ T cells were increased in the BAL of asthmatics compared to normal subjects. There was no difference in the amount of IL-4 in BAL between asthma and normal subjects in this study [65]. Clinical studies where single T\textsubscript{H}2 cytokines were targeted had been inconclusive. Administration of monoclonal antibodies to IL-5 did lead to reduction in tissue eosinophilia but had not translated to clinical improvement. Recombinant IL-12 (to induce a shift towards T\textsubscript{H}1 profile) reduced sputum and blood eosinophilia, but did not show symptom improvement [66, 67]. Medical therapy known to be very effective in treating the clinical symptoms of asthma, against expectation, induces shift towards a T\textsubscript{H}2 immune profile exaggerating the T\textsubscript{H}2 bias even further [68].

It is now accepted that the concept of an imbalance of T\textsubscript{H}1/T\textsubscript{H}2 with bias towards a T\textsubscript{H}2 predominant immune profile is an oversimplification of the complex immunopathology that drives asthma. The complex interactions occurring between cytokines that allow overlap in functions may make some mediators redundant, and therefore antagonism of a single mediator may not always be effective. Given the emerging evidence, it would seem more likely that several parallel pathological processes occur in the evolution of the disease leading to the eventual clinical phenotype. In addition to the effects of the adaptive immune system (T\textsubscript{H}2 cytokines and the contribution of T cells) these processes
probably also include some components of the innate system (defective signaling by epithelial, dentritic, and airway smooth muscle cells as well as neutrophils and eosinophils) as well as a genetic predisposition in some of the asthmatics to acquire airways disease [69].
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1.4 Invariant natural killer T cells in asthma

TH2 related cytokines are closely related to much of the pathophysiology associated with asthma. Although these cytokines were originally described in the TH2 subset of helper lymphocytes it is evident that that other non T cell sources contribute larger quantities of these cytokines to the inflammatory process than T cells. Animal studies suggest that a subset within the family of T cells may possess the ability to produce larger quantities of cytokine including TH2 cytokines. NKT cells are a distinct subset of T cells first characterized in mice, which express markers of both NK cells (NK 1.1) and T cells (TCR) and appear to be potent immuno-regulatory cells. In mice they form up to 30% of the total lymphocytes in the liver. Their contribution in humans is an area of current research. Their mechanism of activation appears distinct from the conventional CD4+ T cells, and following stimulation they possess the ability to secrete large quantities of both TH1 and TH2 cytokines. Observations from animal studies indicate that these cells may be involved in producing some of the clinical features of asthma, which may be through the elaboration of TH2 cytokines.

Conventional T cells are activated by peptide antigens which are protein based, presented to them via MHC class-II and MHC class I molecules found on the surfaces of antigen presenting cells. In contrast NKT cells are activated by glycolipids either exogenous or endogenous presented to them together with the non MHC encoded molecule CD1d (These cells are therefore sometimes called CD1d restricted T cells). CD1d molecules are found on intestinal and airway epithelial cells [70]. This allows the rapid deployment of NKT cells following antigen exposure both in the lung and intestinal surfaces, and makes these cells part of the innate immune system. Their suggested involvement in allergy provides an attractive mechanism by which the innate and the adaptive immune system could be linked. Some pollen based allergens are glycolipids which makes NKT cells even more relevant. Unlike conventional CD4 T cells which require a sensitization step prior to developing into a TH2 cell, NKT cells do not, and committed TH2 secreting NKT cells can rapidly secret TH2 cytokines instantly upon allergen encounter [71].
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In humans NKT cells can be CD4+, CD8+ or CD4-CD8-. There are three types of NKT cells. Type I NKT cells also known as the classical NKT cells or invariant NKT (iNKT) cells and are the most studied group. The T cell receptor (TCR) of conventional CD4+ T cells is a heterodimer composed of α and β chains each with millions of isoforms. The invariant NKT cell group has a restricted repertoire for both α and β chains. In humans this consists of the Vα24 chain on the variable segment and Jα18 for the joining segment (Vα24-Jα18). This in the great majority of cells from this group pairs with the Vβ11 chain. Type II cells are similar to the type I cells except that they have a less restricted repertoire of TCR chains. These cells have been shown to be associated with ulcerative colitis and suppression of tumour growth. Type III NKT cells have an even less restricted repertoire of TCR than the other two groups and are MHC class I and class II restricted [72].

NKT cells have been shown to have immuno-regulatory roles in autoimmune disease, anti-tumour responses and antimicrobial responses. The possible role of NKT cells in asthma was first suggested by the studies of animal models of asthma. NKT cell deficient mice were shown to be unable to develop AHR following allergen sensitization followed by allergen challenge although there was evidence of a T_{H2} response following the challenge [38, 73]. In these studies subsequent adoptive transfer of NKT cells into these mice restored AHR and this was shown to occur via an IL-4 and IL-13 dependent pathway. Furthermore, this seemingly occurs independent of eosinophils, B cells and CD4+ T cells which may be due to an additional effector role by these cells in this model of asthma. In another study selective activation of NKT cells by glycolipids without the simultaneous activation of CD4 T cells resulted in AHR [74].

Studies in humans have been variable. A small increase in the percentage of NKT cells was seen in the BAL of children with asthma compared to normal controls [75]. In a previous study circulating NKT cells were shown to be decreased in adult patients with asthma of all severities compared to normal controls [76]. In contrast, in a recent study of pulmonary NKT cells, which examined patients with moderate to severe persistent asthma up to 60% of the CD4 T cells in BALF were identified as NKT cells using a
combination of a CD1d tetramer and 6B11 monoclonal antibodies (directed against Vα24) and, furthermore, the pulmonary NKT cells were shown to produce exclusively Th2 cytokines. If correct this data has important implications in understanding the pathophysiology of asthma especially steroid resistant asthma, and the development of future treatment strategies [77]. The findings are in contrast with the previous study of NKT cells in humans where such a profound presence of these cells was not seen (< 1% in asthmatic children). Subsequent study using milder adult asthmatics had not been able to detect NKT cells in the lungs on a similar scale [78]. This will be explored further in the subsequent chapters with results from the present study.
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1.5 Regulatory T cells

The immune system plays a vital role in protecting us from invading pathogens. At the same time it is also important that collateral damage to tissues is kept to the minimum. Regulatory T cells (Treg) are thought to play an important role in maintaining this balance between immunity and tolerance [79]. Although the presence of regulatory T cells had been suggested as early as 30 years ago, definite evidence for their existence came from Sakaguchi et al in the mid eighties [80]. Absence of any markers that could help identify these cells hampered initial work on this T cell subset. Breakthrough came following discovery that the IL-2R α-chain (CD25) could act as a phenotypic marker for Tregs [79]. This had lead to the revival of interest in Tregs. Various subsets of Tregs have now been described that are either CD25+ or CD25-. They have been implicated in autoimmune diseases, allergic diseases and anti-tumour immune responses [81]. Functionally Treg cells are defined by their ability to suppress immune responses. Treg cells have been identified within T cell subsets that are CD4+, CD8+, CD4-CD8- and in those showing the NKT cell phenotype. Of these the CD4+ Tregs have been relatively better characterized. Amongst CD4+ Treg cells, three further subsets have been identified to date. These are the naturally occurring Treg cells, T_{H}3 and Tr1 cells, defined according to their origin and predominant mode of action [82].

The naturally occurring regulatory T cells (nTreg) constitute about 5-10% of the peripheral CD4+ T cells in mice and in humans [83]. They are thought to be derived from a distinct lineage of T cells from the thymus and reside in blood and peripheral lymphoid tissue under normal state. Recent reports however, suggest that there may be an inducible form of Treg cells that can be generated in vitro by the repetitive stimulation CD4 T cells in the presence of IL-10 or TGF-β [84]. Like other CD4 T cells, nTreg cells also require activation via the TCR to exert their function and have also been suggested to have a high affinity to self antigens. Although they seem to show poor proliferative response to stimulation in vitro, upon activation nTreg cells can potently suppress the proliferation and cytokine production of effector CD4+ and CD8+ T cells by inhibiting IL-2 gene transcription. They also seem to be able to suppress B cell function [25].
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The mechanism by which nTreg cells suppress other cells is not entirely clear. nTreg produce only low levels of the inhibitory cytokines IL-10 and TGF-β. Furthermore, suppressive effect of nTreg cells, are not abolished by neutralizing antibodies to the above. In vitro nTreg cells had been shown to exert their suppressive effect by mechanisms dependent on cell-cell contact rather than expression of mediators. Surface CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid induced tumour necrosis factor), LAG-3 (lymphocyte activation gene-3) and galectin-1 appear to be important in this process. In addition to having a negative effect on proliferation of effector cells, nTreg cells were also shown to be able to modulate antigen presenting cells rendering them less effective at stimulating effector T cells, directly kill activated CD4+ and CD8+ T cells in a perforin dependent mechanism and also induce differentiation of naïve T cells into Treg cells [82, 85].

nTreg cells were identified by their surface expression of CD25 and CD4. Although CD25 is a marker of activation, the level of expression on Treg cells appear to be much higher than that on activated T cells. nTreg cells are CD45RBlow and CD62Lhigh. A subpopulation of nTreg cells also express αEβ7 (CD103), a surface marker associated with retention of T cells within inflamed tissue. Recently, FOXP3 a nuclear transcription factor has been shown to be increased in nTreg cells and appears to be significantly more specific to nTreg cells than the surface markers. FOXP3 also seems to be closely related to nTreg function. Retroviral gene transfer of FOXP3 into conventional CD4+ T cells transformed them into nTreg cells. In mice and in human absence of the FOXP3 gene results in a phenotype characterized by severe autoimmunity [86].

Human nTreg cells have been shown to express chemokine receptors CCR4 and CCR8. Treg cells migrated towards CCL1, CCL17 and CCL22 in chemotaxis assays. Dendritic cells were shown to produce CCL17 and CCL22 and the authors had hypothesized that this could play a role in drawing Treg cells towards dendritic cells in vivo. CCR8 was shown to be more specific for CD4+CD25+ T cells than CCR4 and CD4+CD25+ T cells were shown to migrate preferentially towards CCL1 compared to CD4+CD25+ T cells[87].
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In a recent study 20% of CCR8⁺CD4⁺ T cells were shown to express FOXP3 compared to ~3% of CCR8⁻CD4⁺ T cells and accounted for 60% for the total nTreg cells [88].

T₃ and Tr1 are adaptive regulatory T cells. They are inducible and are able to be generated from conventional CD4 T in vivo. They exert inhibitory effects on effector cells via the production of inhibitory cytokines IL-10 and TGF-β. They are generated from naïve T cells following interaction with tolerogenic dendritic cells. These dendritic cells are distinct from immunogenic DC which results in the generation of effector T cells. Tr1 cells produce predominantly IL-10 but also IL-5, IFN-γ and TGF-β to a lesser extent. They produce very low levels of IL-2 and IL-4 compared to conventional CD4 T cells. T₃ cells produce predominantly TGF-β but also variable amounts of IL-4 and IL-10. Both IL-10 and TGF-β modulate antigen presenting cells making them less effective at inducing effector T cells. TGF-β also directly inhibits proliferation of effector cells. Tr1 and T₃ are also CD25⁺ and some also express CTLA-4. However unlike nTreg cells they do not express FOXP3. We have limited knowledge on the chemokine receptor profile of these cells. One study had shown expression of chemokine receptors CCR3, CCR4, CCR5, CCR8 and CXCR3 on Tr1 cells [89].

Treg cells in allergy and asthma

In animal studies, T cells engineered to produce TGF-β and adoptive transfer of IL-10 producing T cells both reduced allergen induced airway inflammation and AHR [90]. Regulatory T cells are likely to be important in human asthma although clear evidence for this is currently awaited. There is some evidence for the association between impaired Treg function and allergic inflammation. It seems to suggest their involvement in the development of asthma and other allergic diseases at a number of levels including the sensitization phase to antigens and tolerance as well as disease persistence and severity [91]. I have listed some human studies which suggest the possible involvement of Treg cells in allergy and asthma.
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- In a study comparing atopic subjects to healthy controls, reduced Treg cell mediated suppression of IL-5 production by CD4 T cells was seen in atopic subjects compared to the healthy controls [92].

- Children who outgrew cow’s milk allergy had increased numbers of CD4^+CD25^+ T cells compared those with persistent symptoms [93].

- Patients with severe unstable asthma were shown to have lower levels of IL-10 producing T cells compared with those with less severe disease [34].

- Glucocorticoids, both administered inhaled or systemic resulted in increased expression of FOXP3 and production of IL-10 mRNA [35].

- Specific allergen immunotherapy (SIT) had been shown to be very effective in reducing allergic inflammation and reducing symptoms [94]. Allergen immunotherapy in bee venom allergic patients resulted in decreased allergen induced T cell proliferation as well as reduced IL-5 and IL-13 production. This effect was antagonized by neutralizing antibodies to IL-10 [95].

There is limited evidence for the involvement of Treg cells in asthma at present. This is because these cells had not been studied in any great detail to date. This is a relatively new area of study for asthma biologists. New markers are being developed to identify these cells and current knowledge on these cells is rapidly progressing. We are likely to learn more of their involvement in asthma pathogenesis in the next few years.
1.6 T cell migration

The T cell mediated immune response is dependent on the direct contact between the T cell and pathogen derived antigens. T cell activation also requires the antigens to be presented by specialized antigen presenting cells (APC), a process that could only occur in lymphoid tissue. Therefore in order to mount an immune response, T cells would firstly require the ability to migrate to sites where encounter with antigen and activation could occur, and secondly upon activation should have the capacity to migrate to the site where the antigen had originated from, where they could then interact with other immune cells and exert their function. To achieve this, lymphocytes, unlike granulocytes are able to re-circulate between blood, lymphoid or peripheral tissue and back into the bloodstream. This ability enables a small number of antigen specific T cells to effectively provide surveillance for their cognate antigens in the various organs throughout the body [96].

The migratory pattern of a T cell is determined by its activation and maturation status. Non-experienced or naïve T cells predominantly circulate between the blood stream and secondary lymphoid tissues (peripheral lymph nodes, Peyer’s patches, spleen) and back to blood via efferent lymphatics. A naïve T cell will continue to circulate until it encounters its cognate antigen, presented to it by an APC, within secondary lymphoid tissue. This encounter will induce proliferation of that T cell clone. Some of these cells will acquire effector functions, exit the lymphoid tissue and then enter peripheral tissue inflammatory sites to exert their effector functions (effector T cells). Most effector cells will die after antigen is cleared. Some may remain long term as memory T cells which enables a rapid response to be mounted on future antigen encounter [96, 97].

Only a small percentage (~2%) of the T cell population is present within the vascular space at any one time. When radio labelled T cells were infused into animals the majority of the cells were traced to lymphoid tissue [98]. The accumulation of T cells in different organs in steady state and disease is not a random process. Subsets within the T cell population show preference to different tissue sites. For example, naïve T cells
preferentially accumulate within secondary lymphoid tissue with minimal entry into non lymphoid tissue. Preferential accumulation of T cells or a particular subset of T cells within an organ or tissue site could occur through one of a number of different mechanisms. Firstly, there could be preferential entry of a subset of T cells into the tissue site. Secondly, in-situ proliferation or a reduction in the rate of apoptosis of a particular T cell subset within the tissue site could lead to preferential accumulation. Finally, reduction in the rate of exit from the tissue site could lead to retention and accumulation of that subset of T cell within the tissue. It seems likely that a combination of all these mechanisms play a role in T cell accumulation within tissues in vivo. The past 20 years has seen a rapid growth in the knowledge of molecular signals that control these processes. Several techniques have contributed to this knowledge including electron and intravital microscopy techniques, in vitro adhesion assays combined with blocking monoclonal antibodies and inhibitors, and gene knockout animals to study the impact of different molecular markers on leukocyte trafficking [99].

In order for a T cell to migrate to a tissue site it must first enter the vascular bed within the tissue. Interactions must occur between the T cell and the endothelium to arrest its movement and a guidance system is then needed to guide the cell to migrate across into the connective tissue. The molecular signals involved in this process could play an important role in the preferential accumulation of some subsets into certain tissue sites [100]. Most of the knowledge of mechanisms involved in the process had been gained using in vitro adhesion assays. Extravasation of T cells appears to occur at specific parts of vasculature where the flow of blood is slow. In non-lymphoid tissue these are the post capillary venules. Within lymphoid tissue T cell egress occurs across specialized vessels called high endothelial venules, which get their name from the characteristic tall cuboidal endothelial cells that line their walls [101].

Extravasation of lymphocytes is thought to occur through four stages (Figure 1-2) [102, 103]. Firstly, the flow of the cells along the vessel must be slowed down. Firm adhesion of lymphocytes to the wall at this stage is not be feasible due to speed at which they travel, therefore multiple weak contacts are made to slow the flow of the cell and allow it
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to roll on the luminal surface of the endothelial wall (tethering and rolling). Predominant molecules involved in this stage of extravasation are selectin molecules and their ligands expressed on the leukocyte and endothelial walls. Selectins are a family of three C-type (calcium dependent) lectins [104]; L-selectin (CD62L) is expressed by leukocytes, E and P-Selectins (CD62E and CD62P) are both expressed by endothelial cells. In humans P-selectin expression is inducible by cytokines such as IL-13 and IL-4. P-selectin is found within granules in endothelial cells (Weibel-Palade bodies) and also within platelets (α-granules) and are able translocate to the surface upon activation. All selectin molecules exhibit high affinity towards oligosaccharides with sialyl-Lewis\(^x\) determinants. L-selectin expressed on leukocytes binds to sulphated sialyl-Lewis\(^x\) like sugars called peripheral node addressin (PNAd) expressed on high endothelial venules. P-selectin expressed on endothelial cells binds to P-selectin glycoprotein ligand 1 (PSGL-1) found on the surface of all leukocytes. PSGL-1 also binds to L and E-selectin although to a lesser extent. E-selectin expressed by inflamed endothelial cells in most organs and by non-inflamed skin micro-vessels also binds to cutaneous lymphocyte antigen (CLA) expressed on some T cells. E-selectin expression is induced by pro-inflammatory cytokines (IL-1, TNF-α) [105].

The slower movement and rotation of the leukocyte on the endothelial wall allows contact to be made between chemokine receptors expressed on the leukocyte surface and their chemokines. This leads to the second stage of leukocyte extravasation (activation). Upon secretion, some chemokines bind to heparin-like glycosaminoglycans found on cell surfaces as well as the extracellular matrix. The interaction of endothelial surface bound chemokines to their receptors found on the adhering leukocytes leads to conformational change within the cell and also activation of surface molecules which then leads to the third stage, where the cell forms stronger adhesions to the endothelial cell and its movement is halted (arrest). The predominant adhesion molecules involved at this stage of extravasation are the integrins [103].

Integrins are a large family of cell adhesion molecules and exists as heterodimers composed of an \(\alpha\) and \(\beta\) subunit. There at least 16 different combinations known. Two \(\beta_2\)
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Integrins (αLβ2, also known as leukocyte function-associate type-1 and αMβ2, also known as Mac-1) and, two α4 integrins (α4β1, also known as very late antigen 4 (VLA-4) and α4β7) are known to be expressed on leukocytes. Ligands for the integrins are found on the endothelial surface belong to the immunoglobulin superfamily and includes intercellular adhesion molecule-1 and 2 (ICAM-1 and ICAM-2), mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) and vascular cell adhesion molecule 1 (VCAM-1). The integrins show little affinity to their ligands in the quiescent state, however, upon cellular activation triggered by surface chemokine-receptor interaction, affinity for their ligands is greatly enhanced leading to the formation of firm adhesions. The integrin ligands ICAM-1 and ICAM-2 are upregulated by cytokines TNF-α, IL-1 and IL-4. Leukocyte surface related LFA-1 binds to both ICAM-1 and ICAM-2. α4β1 binds mostly to VCAM-1. α4β7 found mostly on gut associated lymphocytes binds to the ligand MAdCAM-1, which appears to be restricted to intestinal sites [106].

Lastly leukocyte emigration occurs across the endothelial layer and the perivascular basement membrane into the extravascular tissue. Migration through endothelial cell junctions (paracellular) is the predominant route. New imaging techniques have shown that cells use the transcellular route infrequently. Interactions between the migrating leukocyte surface adhesion molecules and the adhesion molecules between adjacent endothelial cells (ICAM-1 and 2, junctional adhesion molecules, platelet endothelial cell adhesion molecule (PECAM-1) play important roles in facilitating the intra-mural course of the lymphocyte [107]. Once within the tissue space chemokine gradients may guide the onward course of the cell. There may be other adhesion molecules involved in retaining T cells once within the tissue. The activation marker VLA-1 is thought to help retain T cells within tissue. The integrin CD103 (αεβ7 is another molecule expressed predominantly by tissue infiltrating T cells including those within the lung. Very little expression is seen within Lymph node T cells and those within blood [99].
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1.6.1 Tissue specific migration of T cells

Naïve and memory T cells are distinguished by their expression of CD45RA and CD45RO isoforms respectively. Naïve T cells show preferential migration to lymphoid tissue whereas memory T cells circulate to both lymphoid and non lymphoid tissue. Surface adhesion molecules could act as address codes that direct T cell subsets to a specified tissue site. Naïve T cells are L-selectin (CD62L)\textsuperscript{high} and CCR7\textsuperscript{high} compared to memory T cells, and this could play a role in there preferred migratory route lymphoid tissue. CCR7 was thought to be a marker of ability of the cell to enter lymphoid sites. Some groups have suggested that memory T may themselves have two further subsets distinguished by their expression of CCR7 [97]. Firstly a central memory T cell subset which is CCR7\textsuperscript{high} and thought not have any effector function, but provide immune-surveillance for their cognate antigen in both lymphoid and non lymphoid tissue, and secondly an effector memory T cell subset which are CCR7\textsuperscript{low} which have effector functions but are not able to enter lymphoid tissue. Instead, they were suggested to have surface receptors that guide them to peripheral tissue sites. However, subsequent work has shown that such segregation is by no means absolute. A significant percentage of CCR7\textsuperscript{high} T cells have been shown to have effector functions, and both CCR7\textsuperscript{high} and CCR7\textsuperscript{low} T cells have been shown to enter both lymphoid as well as non lymphoid tissue [108].

1.6.2 Migration of T cells into lymphoid tissue

Using electron microscopy the entry of T cells from blood into the lymph tissues was shown to occur across HEV within the lymph node cortex. L-selectin has been shown to play the predominant role in tethering in this context [104]. Mice lacking L-selectin, showed reduced T cell accumulation within peripheral lymph nodes. In peripheral lymph nodes L-selectins bind to PNAd on high endothelial venules. In gut associated lymphoid tissue however, L-selectin binds to MAdCAM-1 and the interaction between α4β7-MAdCAM-1 also plays a significant part in rolling. Surface CCR7 on T cells interacting
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with CCL19 and CCL21 expressed by high endothelial venules has been shown to be important in the activation stage. Gene knockout mice absent of these chemokines showed very few T cells within peripheral lymph node. LFA-1 and VLA-4 binding to ICAM-1 and VCAM-1 respectively are thought to be involved in the arrest step. Within the lymph node, chemokine CCL19 guide T cells to the T cell zone via its receptor CCR7, whilst CXCL13 would guide B cells toward the B cell zone via its receptor CXCR5 [96, 109].

1.6.3 Migration of T cells to non lymphoid tissue

A mechanism for tissue selective migration (homing) of T cells would improve their chance of re-encountering their cognate antigen. T cells showing tropism to non-lymphoid tissue are CD62L\textsuperscript{low}. The two well studied pathways of non lymphoid homing T cells are those migrating to the skin and the intestine [96]. Skin homing lymphocytes are characterized by their surface expression of cutaneous lymphocyte antigen (CLA), CCR4, CCR8 and CCR10. CLA and its interaction with E-selectin on skin micro-vessels is involved in the initial tethering and rolling. Activation and arrest is triggered by the interaction of CCR4 and CCR10 with their ligands CCL17 and CCL27 found on skin endothelium. CCL17 then induces integrin dependent adhesion to ICAM-1. CCL1 and CCR8 have been shown to play roles in recruiting T cells to the skin in atopic dermatitis [110].

T cells homing to the small intestine were shown to express \( \alpha_4 \beta_7 \) and CCR9. \( \alpha_4 \beta_7 \) interacts with MAdCAM-1 which facilitates tethering and rolling. MAdCAM-1 expression is restricted to intestinal tissue. CCR9 interacting with its ligand CCL25 leads to integrin mediated adhesion and transmigration of these T cells. Interestingly T cell migration to the large intestine seems slightly different. Here both VLA-4 and \( \alpha_4 \beta_7 \) are involved in the integrin mediated adhesion, and CCL25-CCR9 does not appear to play a part in integrin activation [99].
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1.6.4 T cell recruitment to the lung

A significant proportion of radio-labelled T cells infused into animals were shown to accumulate within the lungs (~15%) [98]. Lung T cells are almost exclusively antigen experienced memory T cells (CD45RO+) and also express markers indicating an activated phenotype (CD69+, VLA-1+) [111]. The majority of lymphocytes in the lung were localized to the parenchyma [111]. T cells are also distributed to the other compartments within the lung including the airway and the intraepithelial compartments. Unlike other tissue sites, lung T cells are not maintained through in-situ proliferation. In rats, using fluorescent labelled lymphocytes, it was shown that lymphocytes in the lung were recruited predominantly from the bloodstream [112]. Similarly, in humans accumulation of lung T cells is maintained by the continual recruitment from the systemic memory T cell pool [113].

T cell migration from blood into the lung differs from that in other tissue sites in a number of ways [111, 114]. Unlike other tissue the lung is supplied by two arterial systems, the pulmonary and the bronchial arteries, which supply most of the lung parenchyma and the airways respectively. It should be noted that rodents which are often used as models to study lung disease lack a separate bronchial circulation. The pulmonary arterial system consists of a large expanse of capillaries, with smaller sized vessels compared to the systemic circulation, and also is under considerably lower pressure than the bronchial system. Molecules involved in tethering and rolling are therefore not always required for the slowing of leukocytes within the pulmonary circulation and leukocyte egress could occur across capillaries rather than post capillary venules. Furthermore, in the pulmonary circulation chemotactic signals for the extravasating leukocytes come from cells lining the alveoli, as opposed to bronchial epithelial cells which are the dominant source in the bronchial circulation. The molecular signals that regulate T cell extravasation are therefore different for the two circulations, and it has been hypothesized that the pulmonary system may have relatively less stringent requirements [115]. At the present moment in time the exact contributions and relevance
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to human lung T cell recruitment of these two circulations under steady state and in
disease is not entirely clear.

The bronchial circulation is composed of relatively larger caliber vessels and T cell
egress is likely to have an initial capture step and subsequent integrin mediated firm
adhesion as in other tissue sites. Inhibition of PSGL-1 resulted in decreased T cell
recruitment to the lung suggesting a prominent role for P-selectin and PSGL-1 in the
initial phase of lymphocyte extravasation into lung. P-selectin expression is increased by
the cytokines IL-4 and IL-13. LFA-1 inhibition also decreased lung T cell accumulation
suggesting a role for the integrin LFA-1 and its ligands ICAM-1 or 2 in arrest and
extravasation into lung [116]. Increased expression of ICAM-1 and VCAM-1 on
endothelium has been shown following inflammatory stimuli and their expression is
increased after allergen challenge [117]. Unlike skin and gut specific T cells, T cells
collected from the lung did not show any unique combination of chemokine receptors
and/or adhesion molecules. As would be expected lung T cells were CD62L\textsuperscript{low}. Unlike
gut homing T cells, lung T cells were α\textsubscript{4}β\textsubscript{7}\textsuperscript{low} [118].

Dispersed lung T cells from normal subjects and ex-smokers were shown to have higher
level of expression of the chemokine receptor CCR5 and to a lesser extent CXCR3,
CXCR4 and CCR4. In bronchoalveolar lavage samples T cells showed high levels of
expression of CCR5, CXCR3 and CXCR6 [118, 119]. The ligands for CXCR3, CXCL9,
10 and 11 are all expressed by bronchial epithelial cells. Blockade of CXCR3 resulted in
reduced T cells within lungs in an animal model [120]. Ligand for CCR5, CCL3 and
CCL5 (RANTES) are produced by lung resident cells. CXCL16, the ligand for CXCR6
has been shown in bronchoalveolar lavage fluid [119]. The above chemokine receptors
with their ligands may all have a role in integrin activation during T cell migration into
the lung. CCR5 and CXCR3 however are expressed by most antigen sensitized T cells
and are not specific to the lung. Similarly CXCR4 is a putative marker found on all T
cells. CCR4 expression is also found on skin homing T cells. To date a lung specific
surface ‘address code’ had not been identified.
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The important role of T cells in asthma has been covered earlier. Allergen sensitized T cells play a pivotal role in orchestrating the allergic inflammation. In an asthma study using an allergen challenge model, the number of allergen sensitized T cells decreased in the blood following inhalational challenge with that allergen, with a reciprocal increase in their numbers in the BAL fluid [22, 111]. The bronchial vasculature in asthmatic lung showed increased expression of E-selectin and integrin ligands ICAM-1 and VCAM-1 compared to non asthmatic lungs [121]. VCAM-1 has also been shown to play a significant part in the recruitment of eosinophils to the lung. Chemokines and receptors that could be involved in T cell migration in asthma will be explored next.

Figure 1-2: Multistep model of Leukocyte migration
INTRODUCTION

Figure 1-3: Surface molecules involved in tethering, rolling, activation and adhesion

* Possible involvement in T cell migration to Lung
1.7 Chemokines

Chemokines are a group of small chemotactic cytokines. These range in size from 8-14kDa, and their predominant function is to act as signals for immune cell trafficking and activation. The early chemokines were discovered in association with their involvement in inflammatory responses. Thus the first chemokines to be discovered were platelet factor 4 (CXCL4) and IP-10 (1977). Their important role in providing chemotactic signals that aid cellular movement was first described with IL-8 (CXCL8) on neutrophils 10 years later. The availability of large databases of expressed sequence tags in bioinformatics (computer assisted sequence analysis) has greatly aided the identification of the rest of this family of small molecular signals in a relatively short period of time [122]. During the early stages after their initial description several different groups often reported the same molecule under different names. This lead to significant confusion, and eventually a consensus agreement was made on the classification and a universal nomenclature was adopted to describe these molecules [123].

The family of chemokines now includes 48 chemokines, not all of them expressed in humans (Table 1-1 to Table 1-4). A rudimentary chemokine system has been seen in life forms as primitive as the |Drosophila [124]. Gene deficient mice with absence of some chemokine receptors resulted in major developmental abnormalities and were not compatible with life, indicating the vital role played by these molecules. The extent of the involvement of these molecules in human physiology and pathology is continually being extended. Their involvement has been shown in a variety of human inflammatory diseases including asthma, rheumatoid arthritis and inflammatory bowel disease, HIV pathogenesis, fibrosis, angiogenesis, haematopoiesis and during embryogenesis. In addition to their small size and function all chemokines also share a particular common structural feature. They all have 4 cysteine residues at their NH\textsubscript{2}-terminus. They have been divided into four subfamilies on the basis of the position of two of the conserved cysteine residues located near the N-terminus. The four structural motifs with X denoting a non-cysteine residue, are: CXC (Alpha), CC (beta), C (gamma) and CX3C (delta).
INTRODUCTION

The biological effects of chemokines are mediated by the interaction of these molecules with specific receptors which belong to the family of G-protein-coupled 7 transmembrane receptors. This is similar to other non chemokine, chemoattractant receptors such as C5a which also signal through G-protein-coupled 7 transmembrane receptors. Activation of these receptors leads to the initiation of several intracellular signaling cascades including the Rho protein family, which is in involved in cellular motility. G-protein coupled receptors are amenable to pharmacological manipulation and have been convenient targets in the design of therapeutic drugs by the pharmaceuticals industry. Thus chemokines and their receptors have great potential to provide future therapeutic strategies.

There are 19 chemokine receptors identified to date. Depending on the chemokine class they bind, the receptors have been named CXCR (bind to CXC chemokine), CCR (bind to CC chemokine), XCR (binds to the C chemokine lymphotactin) and CX3CR (binds to CX3C chemokines). It would be easier to design experimental models to study the effect of each the chemokine-CCR pair if every chemokine had its own faithful receptor. However, as is apparent in Table 1-1, there are more chemokines than receptors, which means one chemokine receptor binds to more than one chemokine (receptor promiscuity) and a receptor can be activated to induce a function by more than one chemokine (ligand redundancy). This makes the study of chemokines and their effector pathways more complicated than initially anticipated. Furthermore, animal models are frequently employed to study the function of chemokines, however, the chemokine system in animals differs that of humans, which adds to the difficulty in dissecting the many functions of these molecules.

Chemokines are thought to provide the directional cues for cellular movement, especially of immune cells during inflammation. They play a significant role in the extravasation of leukocytes at the target inflammation site by aiding adherence of cells to the vessel wall. Following egress of cells in to the tissue a chemokine concentration gradients aid directional movement of the cell towards the target. Chemokines are produced at increased quantities in response to inflammation in various organs including the lungs.
INTRODUCTION

They have avidity for heparan sulphate proteoglycans, a tissue matrix component, which allows locally, produced chemokines to be retained at the tissue site. At the other end chemokines that diffuse into the vessels are removed by the non-functional receptor DARC (Duffy antigen receptor for chemokines) expressed on red blood cells which acts as a chemokine sink.

There are various stimuli that can induce the production of chemokines including pro-inflammatory cytokines such as IL-1, TNF-α as well as bacterial toxins, lipopolysaccharides and viral infections. The type of inflammatory infiltrate that characterizes a specific disease may be in part controlled by the type of chemokines secreted at the disease site. For example increased concentration of the monocyte attractant chemokines (MCP-1) was found in atherosclerotic lesions which show monocyte accumulation [125]. Increased concentrations of CCL2 (MCP-1) and CXCL8 (IL-8) are found in the synovium in rheumatoid arthritis and the intestinal mucosa in ulcerative colitis which leads to the accumulation of monocytes and neutrophils at these disease sites [126]. The different immune cells have distinctive CCR or combination of CCRs which allows their identification and specific recruitment, for example CCR3 expression is relatively selective for eosinophils. As discussed earlier the immune cells may also be organ specific showing preferential accumulation within that particular organ (α4β7high, CCR9high T cells and the gut). The eventual accumulation of a particular immune cell subgroup may therefore be the result of a combination of several factors. Multiple chemokines and other mediators are produced in response to pro-inflammatory stimuli. Identification of a particular subgroup of immune cell may be through a unique combination multiple adhesion and chemokine receptors. The different chemokines and adhesion receptors may be involved at different stages of the migratory path of the cell to its eventual destination [127].
### Table 1-1: The human chemokine/Chemokine receptor family and their functions (CC Chemokines). Adapted from *Zlotnik and Yoshie* [123].

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Synonyms</th>
<th>Chromosome</th>
<th>Expressed by</th>
<th>Binds to receptor/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL1</td>
<td>I-309</td>
<td>17</td>
<td>T cells</td>
<td>CCR8; chemoattracts Monocytes, NK and T cells</td>
</tr>
<tr>
<td>CCL2</td>
<td>MCP-1</td>
<td>17</td>
<td>Osteoblasts, osteoclasts, mast cells</td>
<td>CCR2, CCR5, LTC4 release from mast cells</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α</td>
<td>17</td>
<td>macrophages</td>
<td>CCR1, CCR5; Activates neutrophils; It’s induced by IL-1, IL-6 and TNF-α</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>17</td>
<td>macrophages</td>
<td>Activates neutrophils. It’s induced by IL-1, IL-6 and TNF-α</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>17</td>
<td>T cells</td>
<td>CCR1, CCR3, CCR5; Recruitment of T cells, eosinophils, basophils to inflammatory sites, induces proliferation of NK cells.</td>
</tr>
<tr>
<td>CCL6</td>
<td>Found only in Rodents</td>
<td>11</td>
<td>macrophages</td>
<td>Interacts with CCR1</td>
</tr>
<tr>
<td>CCL7</td>
<td>MCP-3</td>
<td>17</td>
<td>macrophages</td>
<td>CCR1, CCR2, CCR3</td>
</tr>
<tr>
<td>CCL8</td>
<td>MCP-2</td>
<td>17</td>
<td>Neutrophils, macrophages, airway smooth muscle</td>
<td>CCR1, CCR2 and CCR5; chemoattract mast cells, eosinophils, basophils, monocytes and basophils</td>
</tr>
<tr>
<td>CCL9</td>
<td>MIP-1γ (CCL 10)</td>
<td>11 (mice)</td>
<td>Macrophages, myeloid cells, found in peyers patches.</td>
<td>CCR1 (Osteoclasts)</td>
</tr>
<tr>
<td>CCL11</td>
<td>Eotaxin-1</td>
<td>17</td>
<td>Eosinophils, airway epithelium, macrophages</td>
<td>CCR2, CCR3; chemoattracts Eosinophils.</td>
</tr>
<tr>
<td>CCL12</td>
<td>MCP-5</td>
<td>18</td>
<td>Found in thymus, lymphoid tissue and allergic reactions</td>
<td>CCR2; Chemoattracts Eosinophils, monocytes and lymphocytes.</td>
</tr>
<tr>
<td>CCL13</td>
<td>MCP-4</td>
<td>17</td>
<td>Mononuclear cells, airway epithelium</td>
<td>CCR2, CCR3 and CCR5; allergic reaction. It’s induced by IL-1, TNF-α</td>
</tr>
<tr>
<td>CCL14</td>
<td>HCC-1</td>
<td>17</td>
<td>Found in spleen, bone marrow, liver, muscle and gut</td>
<td>Monocytes activation</td>
</tr>
<tr>
<td>CCL15</td>
<td>Leukotactin, MIP-5</td>
<td>17</td>
<td>Small intestine, colon</td>
<td>CCR1, CCR3</td>
</tr>
<tr>
<td>CCL16</td>
<td>Monotactin-1</td>
<td>17</td>
<td>Liver, thymus, spleen; induced by IL-10, IFN-γ, Bacterial LPS</td>
<td>CCR1, CCR2, CCR5; chemoattracts monocytes, lymphocytes.</td>
</tr>
<tr>
<td>CCL17</td>
<td>TARC</td>
<td>17</td>
<td>Thymus</td>
<td>CCR4; Chemoattracts T cells.</td>
</tr>
<tr>
<td>CCL18</td>
<td>PARC</td>
<td>16</td>
<td>Lung, lymph nodes</td>
<td>Chemoattracts activated T cells.</td>
</tr>
<tr>
<td>CCL19</td>
<td>MIP-3β</td>
<td>9</td>
<td>Thymus, Lymph nodes</td>
<td>CCR7</td>
</tr>
<tr>
<td>CCL20</td>
<td>LARC</td>
<td>2</td>
<td>Seen in MALT, induced by LPS, TNF-α, IFN-γ, lymph nodes, liver.</td>
<td>CCR6; chemoattract dendritic cells and lymphocytes</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>---</td>
<td>----------------------------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>CCL21</td>
<td>6CKine, SLC</td>
<td>9</td>
<td>Seen in lymphoid tissue</td>
<td>CCR7</td>
</tr>
<tr>
<td>CCL22</td>
<td>MDC</td>
<td>16</td>
<td>Dendritic cells, macrophages.</td>
<td>CCR4; chemoattract T cells, monocytes, dendritic cells, NK cells</td>
</tr>
<tr>
<td>CCL23</td>
<td>MIP-3</td>
<td>17</td>
<td>Seen in lung, liver, bone marrow</td>
<td>CCR1</td>
</tr>
<tr>
<td>CCL24</td>
<td>MPIF-2, Eotaxin-2</td>
<td>7</td>
<td>Eosinophils, macrophages</td>
<td>CCR3; chemoattract Eosinophils, T cells.</td>
</tr>
<tr>
<td>CCL25</td>
<td>TECK</td>
<td>19</td>
<td>Thymic dendritic cells</td>
<td>CCR9; development of T cells, thymocytes, macrophages, dendritic cells.</td>
</tr>
<tr>
<td>CCL26</td>
<td>Eotaxin-3, MIP-4α</td>
<td>7</td>
<td>Seen in heart, lung, ovaries, endothelial cells</td>
<td>CCR3; chemoattract Eos, basophils</td>
</tr>
<tr>
<td>CCL27</td>
<td>IL-11Rα locus chemokine (ILC)</td>
<td>9</td>
<td>Seen in gonads, skin, thymus</td>
<td>CCR10; homing of memory T cells to skin.</td>
</tr>
<tr>
<td>CCL28</td>
<td>Mucosa associated epithelial chemokine (MEC)</td>
<td>5</td>
<td>Epithelial cells in gut, lung, breasts</td>
<td>CCR3, CCR10; Homing of T/B cells.</td>
</tr>
</tbody>
</table>
Table 1-2: The human chemokine/Chemokine receptor family and their functions (CXC Chemokines). Adapted from Zlotnik and Yoshie [123].

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Synonyms</th>
<th>Chromosome</th>
<th>Expressed by</th>
<th>Binds to receptor/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>GRO1 oncogene, NAP-3</td>
<td>4</td>
<td>Macrophages, neutrophils, epithelial cells</td>
<td>CXCR2; angiogenesis, inflammation around healing</td>
</tr>
<tr>
<td>CXCL2</td>
<td>MIP-2α</td>
<td>4</td>
<td>Monocytes, macrophages</td>
<td>CXCR2; polymorphs, haemopoietic stem cells</td>
</tr>
<tr>
<td>CXCL3</td>
<td>MIP-2β</td>
<td>4</td>
<td>CXCR2; chemoattract monocytes.</td>
<td></td>
</tr>
<tr>
<td>CXCL4</td>
<td>Platelet factor 4 (PF4)</td>
<td>4</td>
<td>Alpha granules of platelets</td>
<td>CXCR3; wound repair; chemoattract neutrophils, fibroblasts, monocytes.</td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td>4</td>
<td>Eosinophils stimulated by IL-1, TNF-α</td>
<td>CXCR2; connective tissue remodelling</td>
</tr>
<tr>
<td>CXCL6</td>
<td>Granulocyte chemotactic protein GCP-2</td>
<td>4</td>
<td>CXCR1, CXCR2; chemoattract neutrophils.</td>
<td></td>
</tr>
<tr>
<td>CXCL7</td>
<td>Pro-platelet basic protein</td>
<td>4</td>
<td>platelets</td>
<td>CXCR1 and CXCR2; Synthesis of ECM</td>
</tr>
<tr>
<td>CXCL8</td>
<td>IL-8</td>
<td>4</td>
<td>Macrophages, epithelial, endothelial cells</td>
<td>CXCR1, CXCR2</td>
</tr>
<tr>
<td>CXCL9</td>
<td>MIG</td>
<td>4</td>
<td>Induced by IFN-γ</td>
<td>CXCR1, CXCR2; chemoattract T cells. Reduces AHR, encourages TH1 skewing.</td>
</tr>
<tr>
<td>CXCL10</td>
<td>IP-10</td>
<td>5</td>
<td>Monocytes,</td>
<td>CXCR3; Acts on mast cells. Increases AHR.</td>
</tr>
<tr>
<td>CXCL11</td>
<td>I-TAC</td>
<td>4</td>
<td>Leukocytes</td>
<td>CXCR3; chemoattract activated T cells.</td>
</tr>
<tr>
<td>CXCL12</td>
<td>SDF-1</td>
<td>10</td>
<td>Directs migration of haemopoietic stem cells in embryogenesis.</td>
<td>CXCR4, CXCR7</td>
</tr>
<tr>
<td>CXCL13</td>
<td>B lymphocyte chemoattractant (BLC)</td>
<td>4</td>
<td>Dendritic cells</td>
<td>CXCR5; B cells</td>
</tr>
<tr>
<td>CXCL14</td>
<td>Breast and kidney expressed chemokine (BRAK)</td>
<td>5</td>
<td>Constitutively expressed at high levels in normal tissue</td>
<td>Unknown</td>
</tr>
<tr>
<td>CXCL15</td>
<td>Lungkine</td>
<td>Found only in rodents</td>
<td></td>
<td>Recruits neutrophils in lung inflammation</td>
</tr>
<tr>
<td>CXCL16</td>
<td>Surface bound and soluble forms</td>
<td>17</td>
<td>Dendritic cells, T cells</td>
<td>CXCR6; T cells, NK cells</td>
</tr>
<tr>
<td>CXCL17</td>
<td>VEGF co-regulated chemokine 1 (VCC-1)</td>
<td>19</td>
<td>Constitutively expressed in lung</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1-3: The human chemokine/Chemokine receptor family and their functions (CX3C Chemokines). Adapted from Zlotnik and Yoshie [123].

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Synonyms</th>
<th>Chromosome</th>
<th>Expressed by</th>
<th>Binds to receptor/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX3CL1</td>
<td>Fractalkine</td>
<td>16</td>
<td>Endothelial cells</td>
<td>CX3CR1; Membrane bound receptor; T cells, monocytes. Increased expression on asthmatic peripheral T cells.</td>
</tr>
</tbody>
</table>

### Table 1-4: The human chemokine/Chemokine receptor family and their functions (C Chemokines). Adapted from Zlotnik and Yoshie [123].

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Synonyms</th>
<th>Chromosome</th>
<th>Expressed by</th>
<th>Binds to receptor/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>XCL1</td>
<td>Lymphotactin</td>
<td>1</td>
<td>Spleen, Thymus, CD8+T cells</td>
<td>XCR1; Chemoattracts T cells</td>
</tr>
<tr>
<td>XCL2</td>
<td></td>
<td>1</td>
<td></td>
<td>XCR1; Chemoattracts activated T cells</td>
</tr>
</tbody>
</table>
INTRODUCTION

1.8 Chemokines and chemokine receptors in asthma

The asthmatic airway is characterized by eosinophilic infiltration, mucus hypersecretion and airway hyperresponsiveness. Many more immune cells also take part in the cascade of events that leads up to this characteristic phenotype. Most of the immune cells are recruited to the lung from peripheral blood. Chemokines are the principal leukocyte navigators. It is therefore not surprising that chemokines had become an area of interest to those studying inflammatory processes within asthmatic airway. Chemokine receptor bearing immune cells are guided towards their target site within lung by the presence of their respective chemokines. The source of the chemokines within the lung are the resident airway epithelial cells, macrophages and other recruited immune cells [128]. Evidence that chemokines contribute to the development and maintenance of inflammation leading to asthma comes from both animal models of asthma and human studies. Most of the early evidence for the role of chemokines in asthma had come from studies investigating the accumulation of eosinophils in and around the airway.

Evidence from animal models:

- Increased concentrations of CCL5 (RANTES), CCL3 (MIP-1a), CCL11 (eotaxin-1), CCL12, CCL24 (eotaxin-2) and CCL26 (eotaxin-3) were shown in the airways of allergen challenged animals [129, 130]. These chemokines have the ability to chemoattract eosinophils.

- CCL1 (eotaxin-1) is the major ligand for CCR3 expressed mostly by eosinophils [131] and to a lesser extent by T_{H}2 lymphocytes. Neutralisation of CCL1 resulted in less eosinophil accumulation within the airway and reduced BHR after allergen exposure. Neutralisation of CCL5 resulted in less accumulation of lymphocytes and eosinophil within the airway [130, 132].

- Chemokines have been shown to work in a co-ordinated manner with different chemokines contributing at different time points of the inflammatory cascade.
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CCL2 (MCP-1) is released during the early phase of allergic inflammation and interacts with mast cells to degranulate and produce the early asthma response. CCL5 and CCL3 help in eosinophil recruitment in the early phase and CCL11 in the chronic phase of asthma [133].

- Co-ordinated production of multiple chemokines by resident cells helps establish chemokine gradients. Several chemokines were found to be able to chemoattract eosinophils including CCL3, CCL5, CCL7, CCL11, CCL13 and CCL22. Of these CCL3, CCL7 and CCL22 are expressed mostly by macrophages and help draw eosinophils out of the blood vessel. CCL5, CCL11 and CCL13 are produced by airway epithelial cells, which then guide eosinophils from the interstitium into the airway [134].

- Neutralisation of CCL17 (TARC) and CCL22 (MDC), involved in recruiting CCR4 expressing cells (T\(_{H2}\) cells), were shown to abrogate lung eosinophilia and AHR [135, 136].

- Monoclonal antibodies to CXCR4 (AMD3100) resulted in reduction in both airway inflammation and AHR [137].

Gene knock out studies have produced conflicting messages. CCR2 deletion, against expectation showed augmented T\(_{H2}\) cytokine responses and increased AHR [138]. CCR3 (receptor for CCL1) deficient mice showed reduced airway eosinophilia but against expectation showed increased AHR. This was later explained by the increased accumulation of mast cells into the airway. Although neutralisation of CCL17 and CCL22 resulted in reduced AHR, reduced expression of their receptor CCR4 afforded no protection against AHR in one study [139] but did in another [140]. Gene deletion studies are difficult to interpret as strain differences can lead to different responses and compensatory mechanisms can develop. Furthermore, mouse models of asthma differ from human asthma in a number of ways most importantly the lack of chronicity. Although useful it provides a useful tool for exploring the acute processes of asthma the
INTRODUCTION

important long term aspect of human asthma such as airway remodelling is not reproduced in animal models, in which these small molecular mediators may participate [141].

*Human studies:*

- Increased CCL5 (RANTES), CCL7 (MCP-3), CCL11 (eotaxin-1) and CCL13 (MCP-4) have been shown in the airway of asthmatics [142, 143].

- Sputum eosinophils are associated with disease severity in asthma. Sputum CCL11 (eotaxin-1) levels and its receptor CCR3 correlated with degree of AHR in asthmatics [144].

- CCL11, CCL24 and CCL26 are increased at different time points after allergen challenge [145]. These are ligands for CCR3 which is expressed by eosinophils.

- Increased levels of CCL2 (MCP-1), CCL3 and CCL5 in BAL fluid was associated with increased asthma severity [146].

- Mast cells are closely involved with the asthmatic airway [11]. CXCR3 is expressed by mast cells and together with CXCL10 is involved in recruiting mast cells to airway smooth muscle in asthma [147].

- CCL17 and CCL22 were increased in the airway epithelium of allergen challenged asthmatics [148]. Their receptor CCR4+ T cells were also increased in allergen challenged asthmatics [148, 149]. CCR8 expressing T cells were also shown to be increased in one of these studies.

- Amongst asthmatics, mutation within the CCL11 gene is associated with reduced levels of circulating CCL11 and eosinophils, as well as better lung function [150].
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It is very likely that chemokines do contribute significantly to the pathogenic process in asthma. The mechanisms are likely to be complex due to the redundancy of function with some of the chemokines and their production that occurs in waves at different stages of the same process [132]. In addition they have roles in normal physiology as well as pathology and this makes the design of experimental models to study their effector roles very difficult. Further work is obviously needed to elucidate more of the effector roles of these molecules. As a therapeutic venture, antagonism of specific CCR to block a specific pathway would avoid the need for broad anti-inflammatory drugs such as steroids. Despite the ambiguous messages from the gene deletion studies in animals, clinical trials using chemokine receptor antagonists for CXCR4, CCR3 and CCR4 are already underway [151, 152].
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1.9 Chemokines, T cells and Asthma

T cells play a variety of roles in the processes that leads to the asthma phenotype. Although a lung homing specific T cell surface marker or chemokine receptor had not been found, lung T cells do have a migration associated receptor phenotype distinct from T cells homing to other organs. T cells from the lung have been shown to be CLA$^{-}$ and $\alpha^4\beta^7_{\text{low}}$ and $\alpha^4\beta^1_{\text{high}}$ [153]. Lung T cells were also CCR4$^{\text{medium}}$ CCR5$^{\text{high}}$ CXCR3$^{\text{high}}$ and CXCR6$^{\text{high}}$ [118, 119, 154]. It was initially thought that an asthma specific surface marker may exist that would be involved in drawing pathogenic T cells relevant to asthma to the airway. However, comparative studies of asthma and normal subjects, using flow cytometry, have shown no significant difference in the expression of chemokine receptors on T cells between the two groups [118, 154]. Chemokine receptors may also be differentially expressed on the different subsets of T cells. For example naïve T cells express CCR7 and CXCR4 which allow them to home to lymph nodes that express their respective ligands CCL21 and CXCL12.

Studies of in vitro polarised T cells have shown that there may be a dichotomy of chemokine receptor expression on T cells based on their cytokine profile. The T$_{\text{H}}$1 subset of lymphocytes was shown to preferentially express CCR5 and CXCR3 whilst the T$_{\text{H}}$2 subset of lymphocytes preferred expression of CCR3, CCR4, CXCR4 and CCR8 [155-157]. Correspondingly, chemokines that bind to the T$_{\text{H}}$1 and T$_{\text{H}}$2 associated receptors may be thought of as T$_{\text{H}}$1 and T$_{\text{H}}$2 associated chemokines. Thus CCL3 and CXCL10 (IP-10), ligands for CCR5 and CXCR3 respectively are T$_{\text{H}}$1 associated chemokines. CCL11, CCL17/CCL22 and CCL1 ligands for CCR3, CCR4 and CCR8 are T$_{\text{H}}$2 associated chemokines. T$_{\text{H}}$2 cytokines themselves were shown to be able to induce the production of T$_{\text{H}}$2 chemokines [158]. This provides a convenient mechanism for the establishment of T$_{\text{H}}$2 bias within an inflammatory site. If within a tissue, T$_{\text{H}}$2 chemokines in concert with their receptors could recruit T$_{\text{H}}$2 cells; these in turn could lead to the production of further T$_{\text{H}}$2 cytokines which will induce more T$_{\text{H}}$2 chemokines and recruit even more T$_{\text{H}}$2 cells. This amplification circuit would eventually result in a T$_{\text{H}}$2 predominant milieu.
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In animal models of asthma, T\(_h\)2 cell recruitment was shown to be dependent on chemokines. CCL11 and CCL22 were involved in recruiting T\(_h\)2 cells to the lung in mice [159]. Neutralisation of MCP-1 and MCP-5 resulted in reduced T cells in the lung [132]. In another study T\(_h\)2 cells pretreated with Pertussis toxin, which blocks G\(\alpha\) protein coupled receptors, were unable to traffic to the lung and, animals infused with these cells were not able to develop allergic inflammation [160]. In humans despite the association of asthma with a T\(_h\)2 predominant response, the message from studies of chemokines had not been straightforward. Some studies have not shown a difference in the frequencies of CCR4 or CCR3\(^+\) T cells in either blood or BAL samples between stable mild asthma and normal subjects [161]. In others, increased CCR4\(^+\) and CCR8\(^+\) T cells were found in the bronchial epithelium of asthmatics following allergen challenge [148]. This will be explored further in subsequent chapters.

The potential role of regulatory T cells in human asthma inflammation has been discussed earlier. Their association with the chemokine receptors CCR4 and CCR8 has been shown in both humans and animals [87, 88]. In contrast to the T\(_h\)2 subset, regulatory T cells are involved in attenuating inflammation. It seems likely that chemokines and their receptors may also be involved in recruiting regulatory T cells to the lung and therefore play a role in curtailing the asthma inflammatory process.
1.10 CCR8/CCL1 and Asthma

Animal and some human studies have indicated that CCR8 and its ligand CCL1 may be involved in Th2 related inflammatory processes and in particular, asthma. Despite being one of the earliest described chemokines, CCL1 (I-309) has not been well studied in the context of human asthma (Table 1-5). Its mouse homologue TCA-3 had been relatively more studied in animal models of asthma. CCL1 was first isolated through subtractive hybridization from an activated T cell cDNA library. CCL1 is secreted by monocytes [162], lymphocytes, mast cells [163, 164] and endothelial cells [165]. Under physiological concentrations CCL1 exists as an 8 kDa monomer. The gene encoding CCL1 maps to chromosome 17 along with most of the other CC chemokines.

Table 1-5: Discovery and progress made with CCL1/CCR8 over last 20yrs

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989</td>
<td>CCL1 (I-309) first described from PBMC [166]</td>
</tr>
<tr>
<td>1990</td>
<td>I-309 gene sequenced [167]</td>
</tr>
<tr>
<td>1991</td>
<td>I-309 shown to chemoattract monocytes. [162]</td>
</tr>
<tr>
<td>1997</td>
<td>CCR8 identified as receptor for I-309. [168]</td>
</tr>
<tr>
<td>1998</td>
<td>CCR8 shown to associated with Th2 cells [155, 169]</td>
</tr>
<tr>
<td>2001</td>
<td>Absence of CCR8 resulted in reduced Th2 cell response and eosinophil recruitment in animal model of asthma. [170]</td>
</tr>
<tr>
<td>2003</td>
<td>Instillation of anti CCL1 into airway of mice resulted in ↓ eosinophil recruitment. [171]</td>
</tr>
<tr>
<td>2005</td>
<td>CCR8 shown to be involved in atopic dermatitis [110]</td>
</tr>
<tr>
<td>2007</td>
<td>Report of CCR8 antagonists. (Oxazolidinones) [172]</td>
</tr>
</tbody>
</table>

CCL1 binds to the receptor CCR8. Unlike most of the other chemokine-CCR associations, CCR8-CCL1 show mutually exclusive binding to each other. Although there had been suggestion that CCL17 and CCL4 may also signal through CCR8 [173], this was later disproved [174]. So far, CCR8 expression has been demonstrated in lymphocytes, monocytes, macrophages, NK (mRNA) cells, vascular smooth muscle and eosinophils (in
INTRODUCTION

mice) [168, 175-178]. Studies in humans suggest involvement of the CCL1-CCR8 in a number of processes and gives clues to their effector roles. CCL1 produced by endothelial cells aid recruitment of monocytes to atherosclerotic plaques suggesting a role in plaque development [165]. CCR8 activation and endothelial cell proliferation has been shown in in vitro systems suggesting their potential role in angiogenesis [179]. In lymphoma cells CCR8 offered protection against apoptosis suggesting a role in carcinogenesis [180]. CCR8+ Dendritic cells were shown to accumulate in the skin demonstrating a role in cellular trafficking [110]. Along with CCL1, viral chemokines can also bind to CCR8 [181]. CCR8 also acts as a co-receptor for HIV and, CCL1 has been shown to inhibit this process [182].

The CCL1-CCR8 axis is also involved in polarisation of immune responses favoring a T\_H2 response. Amongst lymphocytes CCR8 is expressed preferentially on T\_H2 cells in mice [169] and in humans [155]. The first report of the association of CCR8 in asthma came from a gene knock out study. In an ovalbumin challenged allergic inflammation model, mice that were CCR8\(^{-/}\) showed reduced peribronchial eosinophil and lymphocyte recruitment. When Cockroach antigen was used eosinophil recruitment was impaired but T\_H2 cells remained unaffected. In both models the T\_H1 response to mycobacterium tuberculosis antigen remained unaffected [170]. However, two subsequent studies using an ovalbumin challenge model of asthma using a similar protocol had not been able to detect any difference in eosinophil or lymphocyte recruitment to the lung between CCR8\(^{-/-}\) and wild type mice [183, 184]. One of these studies also showed no effect of CCR8\(^{-/-}\) on peritoneal allergic inflammation induced by ragweed challenge. The difference was explained due to the slight difference in the genetic background of the parents used to generate the gene deficient animals. In a mouse model of asthma instillation of neutralizing anti CCL1 antibodies into the airway resulted in reduced airway eosinophil recruitment but did not have any effect on T\_H2 cell recruitment [171]. A detrimental effect of CCR8 in the context of asthma was also shown in a recent mouse model of fungal related asthma. CCR8\(^{+/}\) mice had impaired ability to clear fungal elements from the airway compared to CCR8\(^{-}\) mice, and authors had suggested the use of CCR8 antagonists in fungal related asthma [185].
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The first description of CCL1-CCR8 in human asthma came from a bronchial biopsy study following allergen challenge. In this study 6 atopic asthmatics who underwent allergen challenge were compared to 6 atopic asthmatics who underwent sham challenges, and 3 healthy controls. Allergen challenge resulted in a significant increase in the T cell population within the airway of the asthmatics and of these T cells, ~24% expressed CCR8. CCR8 was undetectable in the airway of healthy controls. CCR4 expressing T cells accounted for ~90% of the T cells in the airway of the asthmatic group and of these T cells 28% expressed both CCR4 and CCR8. In this study all T cells within the biopsies of allergen challenged asthmatics were shown to be IL-4+ using immunofluorescence. This compared with 34% that were IL-4+ in the healthy controls. Although this demonstrates a possible major role for both CCR4 and CCR8 in recruiting Th2 cells to the airway in asthma, a number of observations from this study had been inconsistent with other published studies and the findings had been questioned. Furthermore, in this study, a goat polyclonal antibody was used to identify the CCR8+ T cells. The specificity of this antibody had not been confirmed.

There are other evidence from human studies for the involvement of CCL1-CCR8 in allergic and asthma. A recent study has demonstrated greater concentrations of CCL1 in the airways of atopic asthmatics compared to normal controls [186]. This finding suggests a possible role for CCL1 in recruiting CCR8+ T cells to the lung in atopic asthmatics. Atopic dermatitis is a Th2 associated disease. Increased CCL1 was seen in the skin of patients with atopic dermatitis, which correlated with increased frequency of CCR8 expressing T cells within the skin in these patients. This could suggest a possible role for CCL1 in recruiting CCR8+ T cells to the skin in patients with atopic dermatitis. [110].

The association of CCR8 expression with human Th2 cells was recently demonstrated in peripheral blood from normal subjects [88]. Following anti-CD3/anti-CD28 mAb-mediated activation, approximately 40% of peripheral blood CCR8+CD4+ T cells expressed IL-4 or IL-13 while 13% expressed IFN-γ. In this study CCR8 was also shown
to be associated with the recently characterized subset of T cells, the regulatory T cells. CCR8 was shown to identify a subgroup within CD25^{high} T cells that were also expressing FOXP3 [88].

Recently, ICOS Corporation have developed a highly selective monoclonal antibody to CCR8 with sufficient quality of staining to allow the study of this receptor with flow cytometry and immunohistochemistry. In the following chapters I will describe our efforts at confirming the quality of this antibody and its use to explore CCR8 in asthma.
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1.11 CRTh2 and asthma

Release of preformed mediators from resident lung cells is thought to contribute to the typical early asthma response following allergen encounter. Mast cells are resident lung cells within the asthmatic airway that play an intricate role in its pathophysiology [187]. The arachidonic acid metabolite prostaglandin D$_2$ (PGD$_2$) is a lipid mediator known to be rapidly released by IgE-activated mast cells in significant quantities upon allergen exposure [188]. Dendritic cells are also known sources of PGD$_2$ [189]. In asthmatics large quantities of PGD$_2$ are detected in bronchoalveolar lavage fluid within minutes of allergen encounter. Similar local response to allergen instillation has also been shown in the nasal mucosa and skin in allergic rhinitis and atopic dermatitis respectively [190].

Several lines of evidence now suggests that PGD$_2$ may not only exert local effects within the asthmatic airway as part of the early asthma response, but, may also play a role in recruiting and activating leucocytes that could contribute to the late asthma response [191].

The pro-inflammatory effects of PGD$_2$ includes induction of local vasodilatation which also contributes to oedema, recruitment of eosinophils and T$_{h2}$ cells, induction of T$_{h2}$ cytokine synthesis and in asthma causes bronchoconstriction [192, 193]. PGD$_2$ is known to exert its biological effects via three G-protein coupled chemokine receptors (GPCR); D prostanoid 1 (DP1), TP (thromboxane A2 receptor that also binds to the PGD$_2$ metabolite PGF$_2$), and the recently characterized receptor CRTh2 (chemokine receptor homologous molecule expressed on T helper 2 cells) [191]. The DP1 receptor is the most studied of the three and is expressed by vascular and airway smooth muscle. The DP1 receptor has been shown to contribute to the increased nasal blood flow in allergic rhinitis, however this does not seem to have any significant effect on lung function. The TP receptor appears to be primarily responsible for the bronchoconstrictor response of the airway to PGD$_2$. Despite their local effects, in studies using selective receptor agonists, neither DP1 nor TP receptor had any effect on inflammatory cell activation or recruitment.
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The recruitment and activation of eosinophils and Th2 cells by PGD2 appears to be mediated primarily via CRTh2 [194]. In vitro, activation of CRTh2 (also known as the DP2 receptor) was shown to induce chemotaxis of eosinophils [195], Th2 cells and basophils [196-198]. Furthermore, a selective CRTh2 agonist was shown to exacerbate pathology in a mouse model of asthma [199] and Ramatroban, a moderate CRTh2 and strong TP antagonist was shown to attenuate eosinophil accumulation into the mouse airway [200]. The TP receptor is not expressed by eosinophils, therefore the effect of Ramatroban must have been mediated via the CRTh2 receptor. Interestingly, PGD2 was shown to induce IL-4, IL-5 and IL-13 production by Th2 cells in humans via a CRTh2-dependent mechanism [201]. This effect was also inhibited by Ramatroban.

Results from studies using knockout mice have been confusing; IgE production and allergic skin inflammation was diminished in CRTh2 deficient mice in one study [202], however, in another study eosinophil accumulation and IL-5 production was shown to be enhanced in CRTh2 deficient mice [203]. This discrepancy may in part be explained by the fact that mouse Th2 cells do not preferentially express CRTh2 like in humans. A short term human asthma study has shown that treatment with Ramatroban resulted in reduced airway hyper-responsiveness [204].

Several lines of evidence now suggest that CRTh2 may be involved in the recruitment of leucocytes to the asthmatic airway in particular Th2 lymphocytes. Interestingly, functional polymorphisms within the CRTh2 gene have been associated with severe asthma in certain populations [205]. The CRTh2 variants may result in an exaggerated responses to PGD2 leading to more active recruitment of Th2 cells and increased disease severity. Cytokines from Th2 cells are recognised to closely relate to all aspects of asthma pathophysiology [206]. CRTh2 has been shown to be involved in the induction of cytokines by Th2 cells. Although both histamine and PGD2 are produced by mast cells, it is PGD2 that is produced in the early phase of the asthma response to allergen. The selective recruitment of Th2 cells to the airway by mast cell derived PGD2 provides a convenient mechanism by which the innate immune system links with the adaptive immune response in propagating the inflammatory response.
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Given the possible involvement of CRTh2 in the asthmatic inflammation and its potential to lead to future pharmacological intervention, further evidence is needed for its direct involvement in human asthma. There have been no studies to date demonstrating the direct role of CRTh2 in the recruitment of T\textsubscript{H}2 cells in human asthma. Using a commercially available mAb to CRTh2 we have studied the role of CRTh2 in T cell recruitment to the lung in asthma.
INTRODUCTION

1.12 Aims and Hypothesis

1.12.1 Aims of the study

Expression CCR8 and CRTh2 on blood T cells in asthma and normal subjects

The aim of this part of the study was to study the expression of CCR8 and CRTh2 on blood T cells using flow cytometry. A recently characterized novel monoclonal antibody to CCR8 was to be used to identify CCR8 expressing T cells. Firstly, sensitivity and specificity of this monoclonal antibody was to be confirmed using Th1 and Th2 polarised cells and chemotaxis blocking experiments. A commercially available CRTh2 mAb was to be used to identify CRTh2 positive T cells in blood in asthmatic and normal subjects. Using intracellular cytokine analysis for IFN-γ, IL-4 and IL-13 the association of the expression of CCR8 and CRTh2 on Th1 and Th2 cells was to be studied.

Expression of CCR8 and CRTh2 in lung in asthma and normal subjects

The aim of this part of the study was to investigate the expression of CCR8 and CRTh2 on T cells extracted from bronchoalveolar lavage fluid from asthma and normal subjects. Intracellular IFN-γ, IL-4 and IL-13 analysis will be used to determine the association of CCR8 and CRTh2 expression on BAL Th1 and Th2 cells. Comparison of the expression of these chemokine receptors on BAL T cells with that of blood T cells obtained from the same subject would allow assessment of whether these receptors have any role in recruiting T cells to the lung. In addition to flow cytometry the aim was to analyze the frequency of CCR8 and CRTh2 positive T cells in the bronchial epithelium of asthma subjects using immunohistochemistry and compare that with normal control subjects.

Assay of the chemokine CCL1 and PGD2 in asthma and normal subjects

The aim of this part of the study was to quantify the ligands of CRTh2 and CCR8, PGD2 and CCL1, in the BAL fluid from asthmatics, and normal subjects.
INTRODUCTION

Co-expression of CCR8, CRTh2, CCR3 and CCR4

The aim of this part of the study was to investigate the co-expression pattern of CCR8 and CRTh2 with the other known T\(_H\)2 associated chemokine receptors CCR3 and CCR4 in asthma in an attempt to characterize the T\(_H\)2 cell using chemokine receptors.

iNKT cells in asthma

The aim of this part of the study was to assess the extent of involvement of iNKT cells in the pathogenesis of asthma and in particular severe asthma. Using flow cytometry the percentage of iNKT cells will be estimated in blood and BAL from a group of severe asthmatics. This data will be pooled with data on iNKT cells in the BAL of milder asthmatics and non asthma controls obtained by researchers at the MRC centre for infection and inflammation in Birmingham.

1.12.2 Hypothesis

CCR8 and CRTh2 are preferentially expressed on T\(_H\)2 cells and there are increased numbers of T cells expressing these receptors in the blood and airways in asthma.
2 MATERIALS AND METHODS

2.1 Clinical Methods

2.1.1 Subject Recruitment and clinical characterization

The studies described were conducted on asthma subjects of all severities and normal subjects who acted as controls. This was an explorative study and a power calculation had not been possible as the level and variation of the expression of CCR8 and CRTh2 was not known. A total of 38 subjects with asthma and 23 normal controls took part in the various studies. All patients were recruited from Leicester. Asthma subjects were recruited from those attending the out-patient department at Glenfield Hospital in Leicester. Some of the subjects with severe asthma also participated in an ongoing bronchoscopic study of severe asthma conducted at the Institute for Lung Health in Leicester. Normal subjects were recruited from staff and students from University of Leicester. Asthmatics were defined as those with a suggestive clinical history with confirmed airway hyperresponsiveness to methacholine or confirmed reversible airflow obstruction. Normal subjects had no history of respiratory disease. Spirometry measurements, Pc20 measurement, blood eosinophil count, serum total IgE and atopic status were used to characterize patients. Medical history including, ongoing asthma treatment were noted. The study was approved by the Leicestershire and Rutland ethics committee and informed consent was obtained from all participants.

2.1.2 Methacholine challenge test

Airway responsiveness was measured and quantified using methacholine challenge test. Bronchoconstriction to increasing concentrations of inhaled methacholine was assessed. Baseline FEV1 and FVC was first measured at least three times or until reproducible within 5%. The test was not performed in those with FEV1<1.0L. A Wright’s nebuliser (Roxon Medi-Tech Ltd) was used to provide aerosolized methacholine via a mouthpiece.
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to the subject (at a flow rate of 0.13ml/min). The first aerosol given was 0.9% saline which was the diluent and acted as the control. First methacholine concentration was 0.03mg/ml and, subsequent doses were given in doubling concentrations (0.06, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16mg/ml). A volume of 3ml was used and the aerosol was given over 2 minutes. Subjects were instructed to breathe through the mouth for this period. At the end of inhalation subjects were removed from the nebuliser and FEV$_1$ was measured 30 and 90 seconds after inhalation. Perception of breathlessness was measured using Borg scale. Incremental concentrations of methacholine were given until FEV$_1$ dropped by 20% of the baseline value or FEV$_1$ drops to 1.5L or the highest concentration has been given. The concentration of methacholine required to elicit a 20% decrease in FEV$_1$ (Pc20 FEV$_1$) was used as a measure of their airway responsiveness. Those with subnormal Pc20 (<16 mg/ml) were given 2 puffs of Salbutamol at the end of the test.

2.1.3 Skin prick test

Atopic status was assessed by measurement of total IgE concentration and skin prick tests to common aeroallergens (A wheal diameter > 3mm compared to negative control was defined as positive). Saline was used as the negative and histamine as the positive control. Common aeroallergens tested included cat fur, dog fur, tree, grass pollen, house dust mite, cladosporium and Aspergillus fumigatus

2.1.4 Asthma severity classification

Severity of asthma was classified according to the British Thoracic Society (BTS) criteria. Those in step 1 (steroid naïve asthmatics) were classed as mild. Those in steps 2 and 3 of the BTS treatment groups were classed as moderate and those in steps 4 and 5 were classed as severe asthma. (The patients within the severe asthma group also satisfied the American Thoracic Society criteria for refractory asthma with a mean daily inhaled corticosteroid dose of 1035µg (153) Fluticasone equivalent and ongoing symptoms). Some of the patients with severe asthma were also treated with maintenance oral corticosteroids.
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2.1.5 Bronchoscopy

Those who took part in the bronchoscopy part of the study had a plain chest radiograph prior to the test. A separate written consent was obtained from all participants for this part of the study. Fibreoptic bronchoscopy was performed according to standard methods (Olympus, Japan). Local anaesthesia (2% lignocaine) was first instilled via the scope on to the cords and into the airways (10-12ml in total). Some subjects also received some intravenous sedation prior to the test (midazoloam 2-5mg). Asthmatic subjects were pre-medicated with 2.5mg of nebulised Salbutamol 20 minutes prior to the procedure. BAL was performed by sequential instillation and aspirations of 3 aliquots of 60ml of warmed sterile 0.9% saline. BAL fluid was placed and transported in ice prior to analysis. Biopsies were taken from the second and third sub-segmental carina and were first fixed in paraformaldehyde and later embedded in paraffin. All subjects had continuous oximetry, pulse and regular blood pressure monitoring throughout the procedure and for 60 minutes post procedure. None of the subjects studied had needed prolonged stay in hospital following the test.

2.1.6 Venepuncture

All subjects described in this work had provided a sample of blood. 10-15ml volume of blood was extracted from one of the arm veins into a heparinised syringe and was promptly transported to the lab for processing. In those who had a bronchoscopy blood was taken prior to the procedure.

2.1.7 Eosinophil count and Serum IgE

Blood count and serum IgE estimation was performed at the haematology lab and the immunology lab of the University Hospitals of Leicester respectively.
2.2 Laboratory Methods

2.2.1 Isolation of PBMC from peripheral blood

Blood was drawn into a heparinised syringe. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using density gradient centrifugation (Histopaque 1077; Sigma Dorset, UK). Using a pastuer pipette blood was first carefully layered onto 10-15ml of cold Histopaque held in a plastic universal container. This was then centrifuged at 1400 RPM for 25 minutes at 4°C without brakes. The buffy layer was then carefully removed and then washed in cold PBS (Invitrogen, UK) added with 5% BSA (Sigma). Cells were then resuspended in medium (RPMI+10% FCS + 1% antibiotics) and counted using Kimura stain.

2.2.2 Isolation of mononuclear cells from BAL fluid

Warmed 0.9% saline was used to perform the BAL. Samples were first filtered through a 48µm nylon gauze to remove debris. Cells were pelleted by centrifuging at 1400 RPM for 10 minutes and the supernatants were collected and stored at -80°C for later chemokine and mediator estimation. A red cell lysis was performed if significant blood contamination had occurred during the lavage extraction process. Red cell lysis was performed by resuspending the cellular pellet in ice cold distilled water for 20 seconds prior to quenching with a sizeable volume of culture medium. A differential cell count was performed on the BAL cells by preparing cytospins (200 000 cells/ml) and staining them using Romanowsky stain. Cell viability was estimated using a trypan blue count and percentages were > 60-70%.
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2.2.3 Preparation of dispersed lung cells

Resected lung specimens from those undergoing thoracic surgery was obtained after prior consent. Indications for surgery in these patients were for early stage lung carcinoma and lung volume reduction surgery and all patients had underlying COPD. The specimen was chopped and a filtrate produced by passing pieces through a filter. The filtrate was allowed to stand in a large Petri dish overnight to allow macrophages to adhere to the floor of the container. The filtrate above was gently removed without disturbing the cells adhered to the plate floor. This was first pelleted and re-suspended in media and then carefully layered onto Histopaque and centrifuged @ 1400 RPM for 30mins (4°C and no brake). The buffy interface was carefully removed, washed in media and then counted with Kimura and trypan blue (assess viability).

2.2.4 Flow cytometry

Surface expression of chemokine receptors on PBMC and BAL T cells

PBMC or BAL cells suspended in PBS (Invitrogen, UK) and 0.5% BSA (Sigma) were first stained for surface chemokine receptors followed by antibodies specific for CD3, CD4, CD8 and CD45RO. Cells at a concentration of 1X10^6/ml were incubated with the anti CCR mAb at the appropriate concentration on ice for 20 minutes. Co-expression of chemokine receptors in blood was studied by labelling two or more chemokine receptors together in various combinations. Anti CCR8, anti CCR3 and anti CCR4 were unlabelled and required detection with a secondary fluorescence labelled mAb. CCL1-F is a fluorescently labelled ligand for CCR8 and all CD markers used were directly labelled. In experiments that included CCL1-F, cells were first incubated with the labelled ligand (due to the different incubation conditions), washed, and then incubated with the anti chemokine receptor antibodies. (Table 2-1).
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Table 2-1: Antibody sequence and combinations used to detect surface CCR.

<table>
<thead>
<tr>
<th>STEP 1</th>
<th>STEP 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CRTh2-biotin + CCR8 (mouse IgG\textsubsup{2A}{}) )</td>
<td>(CRTh2-biotin + CCR3 (Rat IgG\textsubsup{2A}{}) )</td>
</tr>
<tr>
<td>CCL1-F</td>
<td>(CRTh2-biotin + CCR4 (mouse IgG\textsubsup{2B}{}) )</td>
</tr>
<tr>
<td>CCL1-F</td>
<td></td>
</tr>
<tr>
<td>CCR3 (Rat IgG\textsubsup{2A}{}) followed by anti rat Ig-FITC.</td>
<td>CCR4 (mouse IgG\textsubsup{2B}{}) followed by anti mouse Ig-PE</td>
</tr>
</tbody>
</table>

After incubation with the primary antibodies, cells were then washed with PBS+0.5%BSA and then incubated with the appropriate secondary labelled antibodies for 20 minutes (labelled streptavidin, or anti-mouse/anti-rat labelled antibodies). Cells were washed with PBS+0.5%BSA once and then stained with labelled anti CD monoclonal antibodies at the appropriate concentration. Samples were then analyzed on FACS Canto using FACS Diva software (Becton Dickinson Immunocytometry System). For the surface CCR expression studies at least 10000 live cells were analyzed for each sample. The percentage expression of the chemokine receptor was estimated amongst the CD3 gated population as shown in Figure 2-1. Gates and quadrant markers were set based upon background staining with matched isotype control Abs.

Staining using CCL1-F

CCL1-F was used to identify CCR8 in some of the experiments involving co-staining with multiple antibodies (chemokine receptor co-expression). This Alexa Fluor 647 labelled CCL1 was a kind gift provided by Millennium Pharmaceuticals. The staining protocol used was different to that of the anti CCR mAbs. Cells were suspended in MACS buffer (Ca\textsuperscript{2+}/Mg\textsuperscript{2+} Dulbecco’s phosphate buffered saline +0.5% BSA and 5mM EDTA) at a concentration of 1 X 10\textsuperscript{6}/ml and incubated with 10\textmu l of CCL1-F at 37°C for 30 minutes. Cells were then washed with cold MACS buffer once prior to staining with CD mAbs. The Alexa Fluor 647 signal was detected in the FL3 channel during acquisition on the flow cytometer.
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Surface CCR8 expression following activation of cells

Other groups studying chemokine receptors have shown down regulation of the surface receptors following stimulation of the cells due to receptor internalization [207]. Intracellular cytokines were only detectable following stimulation with Phorbol-Myristate-Acetate (PMA) and the calcium ionophore A2317 (Both obtained from Sigma-Aldrich) (Figure 2-2). As the aim was to study CCR expression in association with intracellular cytokines, it was important to ascertain whether CCR8 or CRTh2 surface receptor down regulation occurred following stimulation. Although there was a small decrease in expression of both receptors following stimulation this was not significantly different (Figure 2-4). Nevertheless in this study cells were stained for surface CCR8 and CRTh2 first prior to stimulation.

Cytokine single cell analysis of CCR8 expressing CD3 T Cells

Intracellular staining was performed using a method adopted from Prussin et al [208]. Following surface CCR8 labeling, cells were suspended in 2mL of RPMI 1640 and 10% FCS in a 12 well culture plate at a concentration of 2 X 10⁶/ml (PBMC) or 1 X 10⁶/ml (BAL Cells). Cells were stimulated with 25ng/mL of PMA and 500ng/ml of calcium ionophore in the presence of 10ug/ml brefeldin (Sigma) for 6 hours at 37°C. Previous groups studying intracellular cytokine staining had shown detection of cytokines after 4 hours of stimulation with PMA and calcium ionophore. In preliminary experiments the highest percentage of cytokine expressing cells (both IFN-γ and IL-4) were seen after 23 hours of incubation with the stimulants. However there were also a significant number of dead cells seen after prolonged culture with brefeldin. An incubation time of 6 hours was therefore used. An example of a time course experiment is shown in Figure 2-3. Two concentrations of PMA (5ng/ml and 25ng/ml) were also assessed during the time course experiment. The 25ng/ml concentration resulted in higher percentage of IFN-γ⁺ and IL-4⁺ T cells at 6 hours.
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Following activation cells were washed in PBS+0.5% BSA and fixed by incubating cells with 100ul of 4% paraformaldehyde for 15mins on ice. Cells were then permeabilised by incubating with 100ul of 0.1% solution of saponin in PBS+ 0.5% BSA for 15 minutes. Cells were then incubated with appropriately labeled anti IL-4, anti IL-13 and anti IFN-\(\gamma\) mAbs for 30mins on ice. Cells were then washed once with 0.1% saponin + PBS + 0.5% BSA to remove intracellular unbound antibodies and then resuspended in PBS + 0.5% BSA prior to analysis by flow cytometry. For the study of intracellular cytokines 30000-40000 live cells were analyzed from each sample. The percentages of cytokine\(^+\) T cells were generally low, especially IL-4\(^+\) T cells. Isotype control was included for each subject. Samples with non specific staining (isotype control staining of >0.1%) were discarded.

2.2.5 Chemokine quantification

CCL1 Quantification.

CCL1 in BAL fluid was assayed using commercially available ELISA kit (R&D Systems Inc. USA) with a detection limit of 15pg/ml. We had not been able to detect any CCL1 in unconcentrated BAL samples from asthma or normal subjects. BAL samples were therefore 20 fold concentrated using Amicon Ultra-15 centrifugal filters (for nominal molecular weight limit 5,000) (Millipore, UK). BAL samples were centrifuged within the centrifugation tubes at 400g for 30 minutes at 25\(^\circ\)c. Filtrate volumes recovered were 0.25-1.0ml. Samples were assayed by ELISA in duplicates and the product protocol was followed. CCL1 values less than 15pg/ml were considered undetectable.

PGD\(_2\) Quantification

Detectable quantities of PGD\(_2\) were found in BAL samples from asthma and normal subjects without the need for concentration. PGD\(_2\) was estimated in BAL samples using a commercially available PGD\(_2\) ELISA kit (Prostaglandin –D2 MOX EIA kit from Cayman chemicals). The detection limit for this product was 6pg/ml.
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2.2.6 T cell polarisation

T cell polarisation was performed using a method adapted from cousins et al [209]. In vitro polarised IFN-γ⁺ and IL-4⁺ T cells were expanded from PBMC isolated from 8 asthma subjects. CD3 cells were stimulated to proliferate using anti-CD3/CD28 beads (Dynabeads® CD3/CD28 T cell expander; Invitrogen, UK) in the presence of recombinant human IL-2. Beads were first washed in PBS + 0.1% BSA prior to use. The starting concentration of cells was 1X10⁶ cells/ml. 1ml of this cell suspension was placed per well in a culture plate. Beads were added to the cells at a ratio of 3 beads per mononuclear cell.

To direct in vitro polarisation towards IFN-γ producing T cells rIL-12 (2.5ng/ml; R&D Systems) and anti IL-4 (0.1µg/ml PreproTech EC Ltd, UK) were added to the culture well. To direct in vitro polarisation towards IL-4 producing T cells rhIL-4 (12.5ng/ml purchased from R&D Systems) and anti IFN-γ antibodies (0.1µg/ml PeproTech EC Ltd, UK) were added to culture well. All cells were fed with rhIL-2 (10-100U/ml). Cells were cultured for 2 weeks under these conditions with replenishment of culture media and rhIL-2 to maintain the cells at a concentration of 0.5 X 10⁶/ml.

Cells were harvested at the end of the 2 week period. Beads were first removed from the cell suspension by transferring the cell suspension into a tube and then placing the tube in a magnet (Dynabeads®, UK) and allowing the beads to adhere onto the wall thus separating them from the cell suspension. The cellular suspension was then carefully removed using a pipette. Cells were washed in PBS+0.5% BSA, and counted. The extent of polarisation was first confirmed by simulation with PMA and calcium ionophore and estimation of intracellular cytokines within the in vitro polarised IFN-γ⁺ and IL-4⁺ T cells (Figure 2-5). Polarised cells were then used for estimation of surface expression of CCR8, CRTh2, CCR3 and CCR4 as well as CCL1 directed chemotaxis assays.
2.2.7 Chemotaxis assay

Chemotaxis assay was performed using in vitro polarised IL-4+ T cells with upregulated expression of CCR8 (19-27% vs ~5% in fresh PBMC). Migration towards recombinant human CCL1 (R&D Systems) was assessed at a concentration of 100ng/mL diluted in RPMI 1640+10% FCS. CCL1 was placed in the lower wells of a Transwell® chemotaxis plate (Costar, USA). Polarised cells were first washed with media and a 100µl cell suspension at a concentration of 10 x 10^6/ml cells was added to the 3µm pore size polycarbonate culture insert. SDF-1 (CXCL12) was used as the positive control and a lower well without rhCCL1 was used as the negative control. The migratory response of the cells pre-incubated with CCR8 mAb or the isotype IgG2A was assessed. The plate was then incubated at 37°C for 2h in a humidified 5% CO₂ incubator. After incubation, migrated cells in the lower wells were recovered and counted using a microscope (x20) after staining with trypan blue. Cells migrating are expressed as a percentage of cells initially added into the culture insert. Assays were performed in duplicate.
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Figure 2-1: Gating of mononuclear cells to identify CCR8/CRTh2 expressing T Cells. All plots shown below were from the same experiment (patient name shaded to protect identity). The purple shaded area within the top left plot represents gate P1.

**P1:** Gated for lymphocytes  
**P2:** Gated for P1 + CD3+  
**P3:** CCR8+CD3+ cells  
**P4:** CRTh2+CD3 Cells

Isotype controls  
**P3:** (Isotype control for anti CCR8) on CD3+ cells  
**P4:** (Isotype control for anti CRTh2) on CD3+ Cells
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Figure 2-2: Intracellular IFN-γ and IL-4 in T cells incubated with PMA + CI for 6 hours vs those incubated with media (n=13). Figures above the graphs represent mean percentages of IFN-γ and IL-4 T cells.

Figure 2-3: Optimal concentration and duration of incubation with PMA (5ng/ml and 25ng/ml) and Calcium Ionophore (500ng/ml). Similar result was obtained for IL-4 (Data not shown).
Figure 2-4: Effect of stimulation of PBMC derived T cells with PMA and calcium ionophore on surface (A) CCR8 and (B) CRTh2. Cells were incubated with PMA/calcium ionophore for 6 hours. Control cells were incubated with media only. (n=11). No significant difference in CCR8⁺ or CRTh2⁺ T cell population was seen following stimulation.
Table 2-2: Antibodies and isotype controls used in flow cytometry/T cell polarisation/chemotaxis

<table>
<thead>
<tr>
<th>Antibody specific for</th>
<th>Antibody</th>
<th>Clone</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR8</td>
<td>Mouse IgG2a</td>
<td>433H</td>
<td>ICOS corporation</td>
</tr>
<tr>
<td>CRTh2-Biotin</td>
<td>Rat IgG2a</td>
<td>BM16</td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>CCR3</td>
<td>Rat IgG2A</td>
<td>61828</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>CCR4</td>
<td>Mouse IgG2A</td>
<td>205410</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>CCL1-F (hCCL1-Alexfluor 647)</td>
<td>N/A</td>
<td>N/A</td>
<td>Milleneum Pharmaceuticals</td>
</tr>
<tr>
<td>IFN-γ RPE</td>
<td>Mouse IgG1</td>
<td>B27</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>IL-4 RPE</td>
<td>Mouse IgG1</td>
<td>8d4-8</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>IL-13 RPE</td>
<td>Mouse IgG1</td>
<td>32007</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Invariant NKT</td>
<td>Mouse IgG1</td>
<td>6B11</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Va24-PE</td>
<td>Mouse IgG1</td>
<td>C15</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ11</td>
<td>Mouse IgG2a</td>
<td>C21</td>
<td>Immunotech</td>
</tr>
<tr>
<td>CD3-PE Cy5.5</td>
<td>Mouse IgG2a</td>
<td>S4.1</td>
<td>Caltag</td>
</tr>
<tr>
<td>CD3-PerCP</td>
<td>Mouse IgG1</td>
<td>SK7</td>
<td>BD</td>
</tr>
<tr>
<td>CD3-FITC</td>
<td>Mouse IgG1</td>
<td>UCHT1</td>
<td>BD</td>
</tr>
<tr>
<td>CD4-FITC</td>
<td>Mouse IgG1</td>
<td>MT310</td>
<td>BD</td>
</tr>
<tr>
<td>CD4-PE</td>
<td>Mouse IgG1</td>
<td>MT310</td>
<td>BD</td>
</tr>
<tr>
<td>CD8-PerCP</td>
<td>Mouse IgG1</td>
<td>SK1</td>
<td>BD</td>
</tr>
<tr>
<td>CD45RO-APC</td>
<td>Mouse IgG2a</td>
<td>UCHL1</td>
<td>Caltag</td>
</tr>
<tr>
<td>I-309/CCL1 (neutralisation)</td>
<td>Mouse IgG1</td>
<td>35305</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Mouse IgG2a</td>
<td>25723</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>IL-4</td>
<td>Mouse IgG1</td>
<td>3010</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Immunoglobulins/FITC</td>
<td>Rabbit F(ab’)_2 polyclonal</td>
<td>DAKO</td>
<td></td>
</tr>
<tr>
<td>Streptavidin-APC</td>
<td>N/A</td>
<td>N/A</td>
<td>Caltag</td>
</tr>
<tr>
<td>Streptavidin-PE</td>
<td>N/A</td>
<td>N/A</td>
<td>DAKO</td>
</tr>
</tbody>
</table>

**Isotype Controls**

<table>
<thead>
<tr>
<th>IgG2a</th>
<th>Mouse IgG2a</th>
<th>X0933</th>
<th>Dako</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IgG2a-Biotin</td>
<td>Rat IgG2a</td>
<td>R2a15</td>
<td>Caltag</td>
</tr>
<tr>
<td>IgG2a-APC</td>
<td>Mouse IgG2a</td>
<td>X5563</td>
<td>Caltag</td>
</tr>
<tr>
<td>IgG1 RPE</td>
<td>Mouse IgG1</td>
<td>MOPC-21</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>IgG PerCP</td>
<td>Mouse IgG1</td>
<td>X40</td>
<td>BD</td>
</tr>
<tr>
<td>IgG2b</td>
<td>Rat IgG2b</td>
<td>DAK-GO9</td>
<td>DAKO</td>
</tr>
</tbody>
</table>
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2.3 Immunohistochemistry

2.3.1 Biopsies

It was important to determine whether the CCR8 monoclonal antibody could be used with GMA embedded sections. This would be ideal as it would have enabled sections thin enough to allow different antibodies on sequential sections allowing the accurate identification of the cell bearing the receptor (co-localization). However during preliminary experiments the anti CCR8 monoclonal antibody had not shown any staining with GMA embedded agar blocks or mucosal biopsies. Sequential sectioning was therefore not performed in the biopsy studies.

2.3.2 Preparation of agar blocks

Preliminary experiments included the use of agar blocks to ascertain antibody binding to GMA or paraffin embedded tissues. Polarised T_{H2} cells, known to have upregulated expression of CCR8 were used to prepare agar blocks. Cells at a concentration of 0.75 X 10^6/ml were first pelleted in a 1.5ml eppendorf tube and the supernatant discarded. Agar (Sigma-Aldrich) was melted in a warm water bath (35°C) and a drop was added to the resuspended cell pellet and allowed to set at room temperature. The blocks were then removed and embedded in GMA or paraffin and processed as per biopsy specimens for CCR8 staining.

2.3.3 Fixation, processing and embedding in paraffin

Mucosal biopsies or agar blocks were first fixed in 4% paraformaldehyde overnight at room temperature. These were then processed through a series of alcohol washes at 36°C 70% for 30mins, 90% for 30mins, three washes with absolute alcohol 30mins each, three washes with cleaning solvent 30mins each and immersed in hot wax twice 60mins each. Following processing the biopsies were embedded in separate paraffin blocks.
2.3.4 Preparation of slides

Leica microtome (RM2155) was used to obtain slices from the paraffin blocks at 4µm thick. Blocks were maintained at 40°C for a few hours prior to sectioning to prevent fragmentation. Cut sections were first allowed to float on water heated in a water bath to 35°C. Sections were then floated onto poly-L-lysine coated microscope slides and then dried in an oven maintained at 55°C for 24 hours. Slides were then stored at 4°C until stained.

2.3.5 De-paraffinisation and rehydration

Prior to staining sections with antibodies, sections needed to be first de-paraffinised. This was done sequential immersions in xylene (5 minutes each) and graded alcohols (2 minutes each) as follow: xylene – xylene - 100% ethanol - 100% ethanol – industrial methylated spirit (IMS) twice and finally deionized water (2 minutes). Slides were then immediately processed for antigen retrieval without allowing the sections to dry.

2.3.6 Antigen Retrieval

Formaldehyde fixation of tissues impairs the antigenicity of many antigens or epitopes. This can be reversed by a number of methods including enzymatic digestion or heat-induced epitope retrieval (HIER). Preliminary experiments showed enzymatic digestion to be ineffective with our anti CCR8 antibody. Heat induced antigen retrieval is performed with sections immersed in a buffered solution and was achieved by several methods including heating to high temperatures in a microwave oven or a pressure cooker. The heating process although whilst aiding antigen retrieval also causes destruction to the tissue and could hinder subsequent analysis. In preliminary experiments, following several attempts using a variety of heating methods and antigen retrieval buffer solutions the following method was identified to show CCR8 staining in our paraffin embedded biopsy specimens without causing destruction to the tissue architecture.
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Following de-paraffinisation, slides were immersed in a 10mM sodium citrate buffer (pH 6.0) (Lab Vision Corporation) contained within a heat resistant jar (PASCAL, Dako) and covered with a lid. The jar was then placed in a pressure cooker (PASCAL, Dako) with a small amount of water. Slides were heated to a temperature of 120°C and then held at this temperature for 20 seconds cooling down slowly to 90°C. The container was then removed from the pressure cooker and allowed to cool at room temperature for a further 20 minutes prior to staining.

2.3.7 Staining of sections

Sections were first demarcated on the slide with a PAP pen (Dako). Endogenous peroxidase was inhibited by applying 100µl of 0.1% sodium azide with 0.3% hydrogen peroxide to each section for 10 minutes at room temperature. Slides were then rinsed with PBS + 0.1% Tween 20 once. To prevent any non specific binding culture medium (Dulbecco’s Modified Eagles Medium, 20% fetal calf serum, 1% bovine serum albumin) was applied to sections for 20 minutes at room temperature and subsequently washed with PBS + 0.1% Tween 20. Primary antibodies at appropriate concentrations and relevant isotype controls were carefully applied to each section and allowed to incubate for 60 minutes. Anti CCR8 mAb and the isotype control antibodies were used at a concentration of 10µl/ml; CD3 (Clone SP7) rabbit monoclonal antibody (Lab Vision, UK) was used at a dilution of 1:200. Dilutions of the above antibodies were carefully titrated in preliminary experiments.

Slides were then washed three times with PBS + 0.1% Tween 20. Biotinylated secondary antibody was then added and allowed to stand for 30 minutes (biotinylated anti-mouse/anti-rabbit IgG (H+L) (Vector Laboratories, USA). Slides were then washed three times with PBS + 0.1% Tween 20. Streptavidin biotin-peroxidase complex (ABC complex) was then applied for 2 hours and then washed three times with PBS + 0.1% Tween 20. Slides were drained and AEC (3-Amino-9-Ethylcarbazole) solution was applied to sections for 5-10 minutes. Sections were then washed in running water for 5 minutes and then immersed in Mayers haematoxylin (5-10 minutes) and then finally
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washed in running tap water for 10 seconds. Slides were allowed to drain completely and Crystal Mount (Biomedia) was applied to sections and allowed to dry overnight at room temperature. Cover slips were placed using XAM (BDH Chemicals, UK) the next day.

2.3.8 Analysis of sections

Counterstain with hematoxylin allowed the sub-epithelium within the sections to be identified morphologically. Positive cells (CCR8+, CD3+, isotype control antibody) within the area of the sub-epithelium were enumerated at a magnification of X 400 and expressed as the number of positive cells per mm². The area covered by smooth muscle was excluded. A minimum of 0.1 mm² was analysed in each biopsy section. Image analysis software (Scion Image) was used to estimate the surface area.

2.4 Statistical analysis

Subject characteristics are described using descriptive statistics. Means are expressed together with SEM (standard error of mean). Median values were expressed with IQR (interquartile ranges). Statistical analysis was done with GraphPad Prism (GraphPad software Inc). Differences between groups were assessed using the Mann-Whitney U test for parametric and unpaired t tests for parametric data. For multiple group comparison (BAL cytology) the Kruskal-Wallis test was used to first identify any difference. Pearson’s correlation test was used to assess relationship between receptor expression and clinical parameters. A $p$ value <0.05 was considered statistically significant. This was an explorative study and power calculation had not been possible as the level and variation of the expression of these the receptors was not known. Sufficient numbers of subjects were included for the experiments on blood. The numbers of subjects used for the experiments with BAL were low, nevertheless, we did detect a difference between the two populations compared.
Figure 2-5
(a) Intracellular IFN-γ+ and IL-4+ T cells amongst T cells polarised with anti IL-4 and rhIL-12 (Cells were stimulated with PMA and calcium Ionophore for 6 hours prior to analysis). Isotype control plot is shown on the right.
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(b) Intracellular IFN-γ⁺, IL-4⁺ and IL-13⁺ T cells amongst T cells polarised with anti IFN-γ and rhIL-4. (Cells were stimulated with PMA and calcium Ionophore for 6 hours prior to analysis). Isotype control plot is shown on the right.
3 RESULTS: T CELLS AND ASTHMA

3.1 Subjects

Asthma is thought of as a T\(_h2\) biased disease. Although some studies have shown greater numbers of T\(_h2\) cells compared to T\(_h1\) cells in bronchial lavage samples and mucosal biopsies from asthmatics, others have failed to do so. Using flow cytometry we have studied intracellular cytokines within \textit{in vitro} activated peripheral blood mononuclear cells in 15 mild to moderate asthmatics, 14 severe asthmatics and 14 healthy controls. Severity of asthma was categorized as described in the previous chapter. Of the 15 patients with mild to moderate asthma 5 had mild asthma. All patients had intracellular IFN-\(\gamma\) measured. 14 severe asthmatics, 15 mild-moderate asthmatics and 13 healthy controls had intracellular IL-4 measured. 10 severe asthmatics, 11 mild-moderate asthmatics and 10 healthy controls had intracIL-13 measured. PBMC were activated using PMA and calcium ionophore for 6 hours. Characteristics of subjects used in this part of the study are shown in Table 3-1.

### Table 3-1: Characteristics of subjects studied for intracellular cytokines.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild-Moderate Asthma</th>
<th>Severe Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Age (median and range)</td>
<td>31 (21-56)</td>
<td>31 (20-60)</td>
<td>44 (33-66)</td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Atopic</td>
<td>5</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>IgE (kU/l)</td>
<td>22.4 (10)</td>
<td>1022 (495)</td>
<td>248 (79)</td>
</tr>
<tr>
<td>Blood Eosinophils (X10(^9)/l)</td>
<td>0.17 (0.12-0.3)</td>
<td>0.5 (0.2-0.7)</td>
<td>0.5 (0.2-1.8)</td>
</tr>
<tr>
<td>Pc20 (mg/ml)</td>
<td>(&gt;16)</td>
<td>1.1 (0.8)</td>
<td>0.57 (0.3)</td>
</tr>
<tr>
<td>Geo Mean (Log SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV(_1)%P</td>
<td>108 (85-112)</td>
<td>90 (75-105)</td>
<td>69 (61-90)</td>
</tr>
</tbody>
</table>
3.2 IFN-γ⁺/IL-4⁺ T cells in blood in asthma and normal subjects

T cells amongst PBMC were quantified amongst cells gated for lymphocytes, using CD3 staining. There was no difference in the percentages of CD3+ cells in blood between the three groups (figure 3.1A). There were generally small numbers of cytokine secreting T cells in the blood following stimulation, especially those that were IL-4⁺ and IL-13⁺. Isotype control plots were included for each subject to ensure that the small percentages were not due to non specific staining, and we believe the percentages do reflect a small but true IL-4⁺ and IL-13⁺ population of T cells. Sample tubes were not blinded and only sample was studied per subject which are limitations of this study.

The mean percentages of IFN-γ⁺ T cells in normal subjects were 3.3%±1.1 compared to 4.5% ± 0.9 in mild-moderate asthmatics and 4.1%±1.3 in the severe asthma group (figure 3.1B). IL-4⁺ T cells were 0.3%±0.07 in normal subjects compared to 0.8% ± 0.1 in mild-moderate asthmatics and 0.4%±0.1 in severe asthma subjects (figure 3.1C). IL-13⁺ T cells were 0.3%±0.07 in normal subjects compared to 0.4%±0.09 in mild-moderate asthmas and 0.4%±0.1 in the severe asthma group (figure 3.1D). There was no significant difference in any of the three cytokines between normal and the asthma groups. For comparison with previous published reports the median values and the interquartile ranges are also given (Table 3-2).

Table 3-2: Percentages of intracellular IFN-γ⁺, IL-4⁺ and IL-13⁺ T cells after stimulation of PBMC with PMA and calcium ionophore. In this table data is shown as median percentage with IQR.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild-Moderate Asthma</th>
<th>Severe Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>61% (56-63)</td>
<td>59% (42-53)</td>
<td>62% (46-47)</td>
</tr>
<tr>
<td>IFN-γ⁺ T Cells</td>
<td>1.8% (0.6-5.3)</td>
<td>2.8% (1.6-7.6)</td>
<td>2.6% (1.3-4.2)</td>
</tr>
<tr>
<td>IL-4⁺ T Cells</td>
<td>0.3% (0.1-0.6)</td>
<td>0.6% (0.4-1.1)</td>
<td>0.3% (0.2-0.8)</td>
</tr>
<tr>
<td>IL-13⁺ T Cells</td>
<td>0.3% (0.1-0.6)</td>
<td>0.4% (0.2-0.4)</td>
<td>0.3% (0.1-0.5)</td>
</tr>
</tbody>
</table>
RESULTS: T CELLS AND ASTHMA

Figure 3-1: Scatter plots of percentages of (A) CD3+ T cells in PBMC from normal (■), mild-moderate asthma (▲) and severe asthma (▼). (B) Percentages of IFN-γ+ T cells (C), IL-4+ T cells and (D) IL-13+ T cells in normal (■), mild-moderate asthma (▲) and severe asthma subjects (▼). PBMC were stimulated with PMA and calcium ionophore for 6 hours in the presence of brefeldin. Cytokine positive CD3+ cells are expressed as a percentage of the total CD3+ cells. The bars within the figure represent the mean percentages.

A

B

C

D
3.3 BAL cytology in asthma and normal subjects

BAL samples were obtained from 12 asthmatics and 7 normal subjects. Of the 12 asthmatics 10 had severe asthma one had mild asthma and the other moderate asthma. There was a significant difference in the percentage of BAL eosinophils in asthmatics (11.59%±5.8) compared to normal subjects (0.6%±0.2; p<0.05) (Figure 3-2). There was also a higher percentage of neutrophils and epithelial cells in the BAL of asthmatics, however this was not statistically significant. Median values are also included together with IQR (Table 3-3).

Figure 3-2: Differential cell count in BAL fluid from asthmatic and normal subjects. The bars represent mean percentages of each of the cell type. Error bars represent standard error of mean (SEM) *p<0.05 using Kruskal-Wallis and Mann Whitney test.
Table 3-3: Differential cell count in BAL fluid from asthma and normal subjects. Data is given in median percentages with IQR. *Krusksall Wallis and Mann Whitney test.

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>Normal</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>59% (24-86)</td>
<td>83% (54-91)</td>
<td>NS</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2.4% (1.2-10.5)</td>
<td>0.5% (0.0-1.2)</td>
<td>* P&lt;0.05</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7.2% (1.7-22.7)</td>
<td>2.3% (0.8-7.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.5% (0.8-5.0)</td>
<td>7.0% (1.2-11.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>3.3% (1.2-10.0)</td>
<td>1.8% (0.6-13.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>
3.4 IFN-γ⁺/IL-4⁺ T cells in BAL in asthma and normal subjects

Intracellular cytokines were only studied in 6 of the asthmatic subjects due to the low numbers of lymphocytes isolated from the BAL fluid. There were generally more cytokine positive T cells in BAL compared to blood. Furthermore, there were more cytokine positive T cells in asthmatics compared to normal subjects. There were statistically significant differences in IFN-γ and IL-13⁺ T cells in asthmatics compared to normal subjects (Figure 3-3). Median values and IQR are given in Table 3-4.

Figure 3-3: Mean percentages of intracellular IFN-γ⁺ (black), IL-4⁺ (clear) and IL-13⁺ (shaded) T cells in BAL following stimulation with PMA and calcium ionophore. Error bars represent SEM. *p<0.05 and **p<0.01 using Mann Whitney test.

Table 3-4: The median percentages and the IQR of IFN-γ⁺, IL-4⁺ and IL-13⁺ BAL T cells from asthmatic and normal controls following stimulation. Differences were analyzed using Mann Whitney test.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Asthma</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ⁺ T Cells</td>
<td>6.5% (5.8-16.6)</td>
<td>49.6% (32.7-57.7)</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>IL-4⁺ T Cells</td>
<td>0.8% (0.3-1.6)</td>
<td>2.1% (0.8-4.1)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-13⁺ T Cells</td>
<td>0.9% (0.4-1.6)</td>
<td>2.9% (1.1-4.7)</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>
3.5 T Cells in bronchial mucosa in asthma and normal subjects

Mucosal biopsies were obtained from 8 asthma and 5 normal subjects during bronchoscopies and were embedded in paraffin. All 8 asthma subjects had severe asthma according to the previously described criteria. CD3$^+$ cells were enumerated within the sub-epithelium (smooth muscle areas and glandular tissue were excluded) and expressed as cells/mm$^2$. There was a statistically significant difference in CD3$^+$ cells in the sub-epithelium of asthmatics compared to normal subjects (Figure 3-4). The mean number of CD3$^+$ cells in asthmatics was 392±39 and in normal subjects 169±42. Median and IQR for CD3$^+$ cells/mm2 in the sub-epithelium in asthmatics was 413 (310-477) and in normal subjects 181 (77-256). An example of a section with CD3$^+$ cells within the sub-epithelium from an asthmatic and healthy control is shown in Figure 3-5.

Figure 3-4: CD3$^+$ cells in the sub-epithelium of bronchial biopsies from asthmatic (●) and normal subjects (▲). Bars represent the median. *p<0.05 using Mann Whitney test.
RESULTS: T CELLS AND ASTHMA

Figure 3-5: An example of CD3+ cells in the sub-epithelium of the bronchial mucosa from (A) asthmatic and (B) Normal subject. Black arrows identify CD3+ cells.

(A)

(B)
3.6 Invariant NKT cells and asthma

Invariant NKT cells (iNKT) are a recently characterized group of cells that were shown to play an important role in animal models of asthma. A recent human study reported that up to 60% of CD4 T cells found in BAL of moderate-severe asthmatics were iNKT cells and these were enriched in BAL compared to blood (<1%). Furthermore they also demonstrated that most of these cells were able to produce T\(_H\)2 cytokines [77]. The scale of this finding was surprising and given the differing properties of these cells from conventional CD4 T cells this had important implications in understanding the pathophysiology of asthma and in developing future treatment strategies. We therefore measured the percentage of iNKT cells in the blood and BAL of 6 severe asthmatics. We have estimated the percentage of iNKT cells as a percentage of the total CD3\(^{+}\) cells in the sample. All 6 patients were also included in the severe asthma group in Table 4-1. Subject characteristics of these patients are given in Table 3-5. We used the mAb against invariable TCR chains V\(_{\beta}\)11 and V\(\alpha\)24 in all six patients. In 3 of the 6 we also used the 6B11 (targeting the V\(\alpha\)24 chain in a germ line rearrangement with J\(\alpha\)18).

Table 3-5: Characteristics of subjects who took part in the iNKT cells study. *mean (SEM); ** Geo mean (Log SEM); ++ median (range).

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(^{++})</td>
<td>51 (26-58)</td>
</tr>
<tr>
<td>Total IgE (kU/ml) *</td>
<td>801 (368)</td>
</tr>
<tr>
<td>Blood Eos (X10(^9)/l)*</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>Pc20**</td>
<td>0.4 (0.3)</td>
</tr>
</tbody>
</table>

Only small percentages of iNKT cells were seen in BAL and in blood. In BAL the mean percentages of iNKT cells were 3.0%±0.9, 0.9%±0.4 and 0.3% ± 0.2 using V\(_{\beta}\)11, V\(\alpha\)24 and 6B11 antibodies respectively. In blood these were 0.9%±0.3, 0.6%±0.03 and 0.3% ± 0.1 respectively. Results are shown graphically in Figure 3-6.
RESULTS: T CELLS AND ASTHMA

Figure 3-6: Presence of \( \alpha_24 \), \( \beta_{11} \) and 6B11 T cells in blood (○) and BAL (△) of 6 asthmatic subjects. Bars represent median percentages. No significant differences were detected between blood and BAL iNKT cell percentages.

We had also collaborated with researchers at the MRC centre for infection and inflammation in Birmingham who had also collected data on iNKT cells in the BAL from asthmatics and non asthma controls. These patients however, did not have their blood iNKT cells assessed. A method similar to that described in this work had been used by the MRC centre in Birmingham to collect BAL samples and quantify iNKT cells. A combined total of 11 asthmatics (2 mild and 9 moderate to severe asthmatics of which 4 had refractory asthma according to the ATS criteria) were compared with 5 controls (Four patients with sarcoidosis and one with eosinophilic bronchitis). The mean (SEM) age of the asthmatics and control group was 53.8±4.7 and 45.3±3.7 years and the FEV\(_1\)
RESULTS: T CELLS AND ASTHMA

was 2.29L±0.2 and 2.69L±0.7 respectively. In the asthmatic group the geometric mean (logSEM) $Pc_{20}$ (mg/ml) was 0.68 (0.3) and the sputum eosinophil percentage cell count was 4.6%±0.2. The combined data is shown in figure 3.7.

Figure 3-7: iNKT cells in the BAL of refractory asthmatics (○), moderate-severe asthmatics (▲) and non asthma controls (●). Bars represent median percentages of iNKT cells. No significant difference was detected between the groups. Data shown in this figure also includes data collected at the MRC centre for infection and inflammation in Birmingham.
3.7 Discussion

In this study there were very few cytokine expressing T cells seen in the blood of asthmatic and normal subjects. In asthmatics between 2.6-2.8% of the T cells were IFN-γ+ following stimulation with PMA and calcium ionophore, compared to ~1.8% of the blood T cells in healthy controls. This difference was not statistically significant. The T_{H2} cytokines (IL-4 and IL-13) were expressed by only ~0.6% of blood T cells in both asthmatic and normal subjects with no appreciable difference between the two. There were generally higher percentages of cytokine secreting T cells in the BAL fluid compared to blood in both asthmatic and normal subjects. There were also significant increases in the percentages of IFN-γ+ and IL-13+ T cells in the BAL of asthmatic subjects compared to normal subjects.

In this study the percentages of cytokine positive T cells (IFN-γ and IL-4) were lower compared to recent studies which have compared single cell cytokines in asthmatics and healthy controls [43, 63, 210]. This is because these studies have expressed cytokine producing T cells as a percentage of CD4+ T cells excluding CD8+ T cells and NKT cells, whilst in this study cytokine producing T cells are expressed as a percentage of all T cells. A trend towards increased expression of IFN-γ in blood T cells from asthmatics has been described in a number of previous studies. Increased levels of IFN-γ and TNF-α were detected in PBMC cultures supernatants derived from asthmatics [65]. At a single cell level increased IFN-γ+ T cells were shown following in vitro stimulation of blood from asthmatics compared to normal subjects [63, 210]. Impaired T_{H1} is implied by the T_{H2} hypothesis of asthma inflammation. It would be difficult to make conclusions on the presence of T_{H2} cells in our subjects as the percentages of IL-4+/IL-13+ T cells in our study will also include CD8+ T cells (Tc2). Furthermore cells were single stained with anti IL-4 or anti IFN-γ, and some of the IL-4+ T cells may also have been IFN-γ+ (T_{H0} cells). Nevertheless, we had not been able to detect higher percentages of IL-4+ T cells nor reduced percentage of IFN-γ+ T cells in blood from asthmatics.
RESULTS: T CELLS AND ASTHMA

It could be argued that the small percentages of IL-4+ and IL-13+ T cells identified in this study may not reflect a true population of cytokine+ T cells as the numbers identified are approaching the limit of the instrument’s resolution (flow cytometer). Furthermore, in this study sample tubes were not blinded and only one sample was taken from each subject. These are limitations of this study. Studies of IL-4 and IL-13 at a single cell level in asthma have generally reported very small percentages of T cells that are IL-4+ or IL-13+, which makes the study of these cells very difficult. In this study we have used isotype control plots to eliminate samples showing nonspecific staining. We believe that the percentages we report reflect a small but true population of IL-4+ and IL-13+ T cells. Although subjects were tested only once, the asthmatic and control groups had included large numbers of subjects (data from PBMC) and still, we had not been able to detect a significantly higher percentage of IL-4+ or IL-13+ T cells in asthmatics compared to normal controls.

Higher percentages of cytokine expressing T cells were seen in the BAL compared to blood in both asthma and normal subjects. In asthmatics ~50% of the BAL T cells were IFN-γ+, ~2% were IL-4+ and ~3% were IL-13+. The difference in cytokine expressing T cells in BAL compared to blood is expected as the lung is likely to have an increased percentage of antigen primed effector T cells compared to peripheral blood [116]. Higher percentage of cytokine expressing T cells in BAL compared to blood has been shown in previous studies [65, 161]. Compared to normal subjects asthmatics had a significantly higher percentage of both IFN-γ+ and IL-13+ T cells in the BAL. Although there was also a greater percentage of IL-4+ BAL T cells in asthmatics compared to normal it fell short of reaching statistical significance. The difference in IFN-γ+ T cells between blood and BAL in the asthmatics (~ 9 fold) was greater than that for IL-4+ or IL-13+ T cells (~ 3-5 fold). We had expected a greater fold difference in the IL-4/IL-13+ T cells in the BAL vs blood of asthmatics. The greater percentage of IFN-γ+ T cells may be in part due to the in vitro stimulation used to induce cytokine synthesis. T cell activation by TCR/allergen may have resulted in a greater percentage of IL-4/IL-13+ T cells. Support for this explanation comes from reports of increased Th2 cytokines and suppressed Th1 cytokines in BAL following allergen challenges [21, 211].
asthmatics from whom BAL samples were obtained had severe asthma. This phenotype is known to respond poorly to corticosteroids and it has been suggested that these patients may have T\textsubscript{H}1 biased disease [212]. Increased IFN-\(\gamma^+\) T cells in BAL has been described by another group before in which \(\sim 74\%\) of BAL T cells from asthmatics were shown to be IFN-\(\gamma^+\) compared to 43\% in normal controls [65].

We had measured the presence of T cells within the bronchial mucosa of asthmatics and compared this with normal subjects. There were approximately twice as many T cells/mm\(^2\) in the sub-epithelium of asthmatics compared to normal subjects. The numbers of T cells seen in asthma subjects (~ 400/mm\(^2\)) in this study were higher than most of the biopsy studies which have mostly included milder asthmatics. Some of these studies have used GMA embedded sections, which may give lower counts than paraffin embedded sections. In a study which included mild asthmatics no difference was seen in T cell numbers in the mucosa between asthma and normal subjects (~ 43/mm\(^2\)) [43]. In another study which included severe persistent asthmatics, there were significantly higher T cell numbers (~140-250/mm\(^2\)) in the asthmatics compared to normal controls [213]. In a descriptive study of severe of asthmatics ~1500 CD3+ cells/mm\(^2\) (0-1600) were shown in the bronchial mucosa [212].

In summary, it appears that there are a higher percentage of T cells in the airways of severe asthmatics compared to normal subjects. In this study we had not seen a higher percentage of IL-4\(^+\) or IL-13\(^+\) T cells in the blood of asthmatics compared to normal subjects. The importance of T\textsubscript{H}2 cells in asthma inflammation has been questioned in recent years. T\textsubscript{H}2 cytokines can indeed be linked to most if not all pathophysiological features seen in asthma. However, there is no evidence that T cells are the main source of the T\textsubscript{H}2 cytokines in the asthmatic. Mast cells and eosinophils have preformed stored T\textsubscript{H}2 cytokines and are able to secrete large quantities rapidly upon activation. However, T cells are the source for antigen inducible T\textsubscript{H}2 cytokine release. This was demonstrated in an early biopsy study of asthmatics where higher levels of IL-4 and IL-5 mRNA were seen mostly in T cells whilst the protein product was mostly localized to mast cells and
RESULTS: T CELLS AND ASTHMA

eosinophils [44]. T\textsubscript{H}2 cells and cytokines may be involved in some but not all stages of asthma inflammatory process. Furthermore, T cells may have other roles in addition to providing T\textsubscript{H}2 cytokines, for example they may provide a regulatory role and some of the T cells within the asthma airway may well be regulatory T cells.

In asthmatics only ~1% of blood T cells and 1-3% of the BAL T cells bear characteristics of iNKT cells, with no significant difference between the two. This is in marked contrast to the report by Akbari \textit{et al} who showed a similar percentage to ours in blood but ~60% of T cells in BAL [77]. However the low percentage we found in BAL are similar to the findings by other investigators who had studied milder asthmatics and children [78, 214]. We also collaborated with another group to study the presence of iNKT cells in non asthmatic subjects and there was no difference in the number of iNKT cells in the BAL between asthma and non asthma subjects [215]. One explanation for the previous discrepancy was that iNKT cells may be associated with more severe disease. Amongst the asthmatics included in the collaborative study, four had refractory asthma and still we had not been able find iNKT cells in the scale described by Akbari \textit{et al}. One explanation may be that Akbari \textit{et al} had used a tetramer whilst we had used antibodies to detect iNKT cells. The 6B11 antibodies used in our study targets the Va24-Ja18 region of the TCR which is very specific for iNKT cells. Although this study included small numbers our findings have since been confirmed in a larger study [216]. The consensus view now is that iNKT cells probably play a limited role in human asthma.
4 RESULTS: CCL1/CCR8 IN ASTHMA

4.1 Subjects

The CCL1-CCR8 axis has been shown to play a part in the pathogenesis of asthma in animal models. There is also evidence that CCL1-CCR8 may play a role in human asthma. We have acquired a monoclonal antibody claimed to have good specificity for the human CCR8 receptor and also compatibility with flow cytometry and immunohistochemistry (ICOS corporation; Clone 433H). We have therefore studied expression of CCR8 in asthmatics by analyzing blood, BAL and bronchial biopsy tissues. 38 subjects with asthma and 22 normal controls were studied in total. Subjects were characterized for atopy, blood eosinophil concentration, smoking status and lung function which are given in Table 4-1. Asthmatics were grouped into mild, moderate and severe according to criteria set out in the methods section. Half of the subjects with the asthma group were atopic, and as a group had higher blood eosinophils and serum IgE compared to the normal group. None of the asthmatics smoked.

Samples from some of the subjects in this part of the study were also used for the studies in the chapter 3 and chapter 4 (cells were analyzed for CCR8 and CRTh2). 14 normal subjects, 10 of the mild-moderate asthmatics, and 7 of the severe asthma samples from this part of the study were also included in chapter 3 (intracellular cytokine measurements made).

Not all subjects underwent bronchoscopy. The characteristics of those who underwent bronchoscopy are given in Table 4-2. Thirteen of the asthma subjects and 7 normal subjects had bronchoscopies. Of the 13 asthmatics who had bronchoscopy 10 had severe asthma, 2 had moderate asthma and one was a mild asthmatic. Amongst those who had a bronchoscopy the asthma group had higher percentage of lavage eosinophils and neutrophils compared to normal subjects. They also had slightly more obstructed airways than normal controls.
RESULTS: CCL1/CCR8 IN ASTHMA

Table 4-1: Subject characteristics. *mean (standard error of mean); **Geometric mean; ***median (Range)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild Asthma</th>
<th>Moderate Asthma</th>
<th>Severe Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>22</td>
<td>6</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Age **</td>
<td>29 (19-56)</td>
<td>28 (26-31)</td>
<td>35 (21-53)</td>
<td>43 (23-61)</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blood Eos (x 10⁹/L)</td>
<td>0.1 (0.03)</td>
<td>0.2 (0.07)</td>
<td>0.3 (0.06)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>IgE (kU/L)</td>
<td>17 (7.2)</td>
<td>727 (433)</td>
<td>615 (396)</td>
<td>339 (62)</td>
</tr>
<tr>
<td>Atopy (n)</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>FEV₁'%P</td>
<td>96 (8)</td>
<td>107 (4)</td>
<td>88 (11)</td>
<td>78 (4)</td>
</tr>
<tr>
<td>Inhaled CS* (µg/day)</td>
<td>0</td>
<td>0</td>
<td>582 (153)</td>
<td>1035 (93)</td>
</tr>
<tr>
<td>On OCS (n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Pc20**</td>
<td>16 (0)</td>
<td>3.1 (0.5)</td>
<td>1.86 (0.9)</td>
<td>0.83 (0.8)</td>
</tr>
</tbody>
</table>

Table 4-2: Subject characteristics of those who had a bronchoscopy.
*mean (standard error of mean); #10 out of the 13 patients had severe asthma; **median (range)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>13 #</td>
</tr>
<tr>
<td>Age **</td>
<td>24 (21-49)</td>
<td>46 (36-60)</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Atopy</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>On OCS</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Eosinophilis (Blood)</td>
<td>0.2 (0.09)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>IgE</td>
<td>101 (34)</td>
<td>277 (117)</td>
</tr>
<tr>
<td>FEV₁'%P</td>
<td>100 (6.3)</td>
<td>77 (5.9)</td>
</tr>
<tr>
<td>BAL Eosinophilis%*</td>
<td>0.5 (0.0-1.2)</td>
<td>2.4 (1.2-10.5)</td>
</tr>
<tr>
<td>BAL Neutrophils%*</td>
<td>2.3 (0.8-7.2)</td>
<td>7.2 (1.7-22.7)</td>
</tr>
</tbody>
</table>
4.2 CCR8 expression on in vitro polarised IL-4\(^+\) and IFN-\(\gamma\)^+ T cells in asthma.

We studied the effect of in vitro polarisation to IL-4\(^+\) and IFN-\(\gamma\)^+ T cells, on the expression of CCR8. IL-4\(^+\) T cells were expanded from PBMC obtained from asthmatic donors. This not only enabled us to see if CCR8 is associated with IL-4\(^+\) T cells but also provided an opportunity to test the selectivity of this new anti CCR8 mAb against the human CCR8 receptor in chemotaxis assays using polarised cells. The mean CCR8 expression on fresh PBMC CD3\(^+\) T cells was 5.3% ± 1.6. Expression was significantly increased in IL-4\(^+\) polarised T cells (21.9%±2.1) compared to baseline (p<0.001) and IFN-\(\gamma\)^+ polarised T cells (9.2±1.3) (p<0.01) (Figure 4-1). We also studied the effect of polarisation on the expression of two other T\(_h2\) associated chemokine receptors, CCR3 and CCR4. Although CCR4 expression increased 2 fold similar to CCR8, CCR3 failed to show an increased expression on IL-4\(^+\) T cells using our protocol (Figure 4-2). Against expectation a higher percentage expression of CCR3 was noted on IFN-\(\gamma\)^+ polarised T cells.
Figure 4-1: T cell CCR8 expression amongst fresh PBMC, in vitro polarised IFN-γ⁺ and IL-4⁺ cells. Graph shows data from 9 different experiments using PBMC from 9 different asthmatic subjects. (**P<0.01, using Mann-Whitney test).
Figure 4-2: Effect of in vitro polarisation in to IFN-γ⁺ and IL-4⁺ cells on the expression of (A) CCR4 and (B) CCR3 on T cells. Graph shows data from 3 different experiments using PBMC from 3 different asthma subjects.

A

B
4.3 Confirmation of specificity of 433H mAb for human CCR8

Previous commercially available mAb to CCR8 had been poor in quality with unacceptable levels of non specific binding. The specificity of the (clone 433H) CCR8 mAb had not been confirmed in ex vivo studies to date. Preliminary experiments using this mAb with flow cytometry and immunohistochemistry suggested good specificity for this receptor (data not shown). An example of 433H staining in vitro polarised IL-4⁺ cells is shown in Figure 4-3. We confirmed the specificity of this mAb further using a chemotaxis assay towards rhCCL1. Approximately 30% of the in vitro polarised IL-4⁺ T cells migrated towards CCL1 compared to 10% to the medium alone. This response was completely abrogated by pre-incubation of cells with the (clone 433H) CCR8 mAb but not the isotype control antibody (Figure 4-4). As further confirmation for the specificity of the 433H mAb we also demonstrated that the 433H antibody blocked binding of CCL1-F from binding to the CCR8 receptor. Conversely, pre-incubation of T cells with CCL1-F reagent prevented binding of the anti-CCR8 antibody (Figure 4-5).

Figure 4-3: Cytospin preparation of in vitro polarised IL-4⁺ cells stained with anti CCR8 (433H) mAb and its isotype control (IgG2a). (X400)
Figure 4-4: Chemotaxis of in vitro polarised IL-4+ cells towards rhCCL1. SDF-1 was used as the positive control. Migration towards CCL1 was blocked by pre-incubating cells with antiCCR8 mAb (433H). Graph shows data from 6 different experiments. Columns represent mean percentages and error bars standard error of mean. *P<0.05 using Mann-Whitney test.

Figure 4-5: Binding of anti CCR8 mAb is blocked by fluorescent labelled CCL1. (A) cells are incubated with CCR8 mAb (433H) then labelled CCL1. (B) Cells are incubated with labelled CCL1 first then CCR8 mAb.
4.4 CCR8+ T cells in blood in asthma and normal subjects

CCR8 expression in blood T cells was confined to a small percentage of CD3+ cells in both asthmatic and normal subjects (normal controls 3.05±0.4, mild-moderate asthma 4.1±0.6 and severe asthma 5.2±0.6). The percentage of CCR8+ T cells was significantly higher in severe asthmatic subjects compared to normal controls (p<0.01) (Figure 4-6). CCR8 expression was confined to CD3+ cells amongst the PBMC population. The majority of CCR8+ T cells were CD45R0+ (77%±4.3; n=3) and CD4+ T cells (82.3±2.8; n=6) (Representative plots are shown in Figure 4-7). 15%±2.1 of CD8+ T cells expressed CCR8 (n=6). Animal models of asthma have shown that CCR8 and its ligand CCL1 are associated with T\textsubscript{H}2 response and eosinophil recruitment to the lung. We therefore explored whether there were any association of CCR8 expression with indices of allergy (IgE and eosinophils) in both asthmatics and normal controls. There was no significant association between the percentage of CCR8+ T cells and serum IgE ($r^2=0.14$; p=0.4) or blood eosinophil count ($r^2=0.267$; p=0.09).
RESULTS: CCL1/CCR8 IN ASTHMA

Figure 4-6: Scatterplot of CCR8 expression on blood T cells in severe asthmatic (○), mild-moderate asthmatics (▲) and normal subjects (●). **P<0.01 using Mann-Whitney test. Bars represent the mean percentages.

Figure 4-7: Representative FACS dot plots showing the association of CCR8 expression with (A)CD3, (B) CD4 and CD8 and (C) CD45R0. The numbers within plots B and C represent percentages.
4.5 CCR8 expression on IFN-γ⁺/IL-4⁺ blood T cells.

We studied the association of CCR8 with IL-4⁺ T cells by analyzing their expression on IFN-γ⁺, IL-4⁺ and IL-13⁺ T cells following stimulation. We had confirmed that stimulation did not affect CCR8 on the surface significantly (materials and methods). In subjects with asthma 29.8%±3.5 and 36%±3.5 of the IL-4⁺ and IL-13⁺ blood T cells expressed CCR8 whilst only 4.3%±1.0 of IFN-γ⁺ cells expressed CCR8 (p<0.001) (Figure 4-9). A similar degree of enrichment of CCR8⁺ cells on IL-4⁺ and IL-13⁺ T cells relative to IFN-γ⁺ T cells was seen in the blood from normal subjects (Figure 4-10). The percentages of T cells that were cytokine positive were low and on average 30000-40000 PBMC were analyzed from each sample. Sample plots from an asthmatic subject is given in Figure 4-8. Samples were not replicated (n=1) and were not blinded. Isoytpe controls were included for all tests. Samples showing non specific binding with isotype control (>0.1%) were eliminated from analysis.
Figure 4-8: Representative FACS dot plots of CD3 gated cells co-stained for CCR8 and intracellular (A) IL-4, (B) IL-13, (C) IFN-γ and the (D) isotype control plot from an asthmatic subject. Cytokine positive cells are expressed as a percentage of CD3+ cells and those that are CCR8+ (Q2-1) and CCR8- (Q4-1) are noted within the plots. Figures within parenthesis are absolute events (number of cells).
Figure 4-9: Scatterplot of CCR8 expression on IFN-γ⁺ (♦), IL-4⁺ (●) and IL-13⁺ (▲) PBMC derived CD3⁺ cells from asthmatic subjects. Cells were incubated with PMA and calcium ionophore for 6 hours prior to staining with anti IFN-γ, anti IL-4 or anti IL-13. ***p<0.001 using Mann-Whitney test. Bars represent the mean.

Figure 4-10: Scatterplot of CCR8 expression on IFN-γ⁺ (♦), IL-4⁺ (●) and IL-13⁺ (▲) PBMC derived CD3⁺ cells from normal subjects. Cells were incubated with PMA and calcium ionophore for 6 hours prior to staining with anti IFN-γ, anti IL-4 or anti IL-13. ***p<0.001 using Mann-Whitney test. Bars represent the mean percentages.
4.6 CCR8+ T cells in BAL in asthma and normal subjects

Previous flow cytometry based studies of CCR in BAL have shown higher percentages of CCR4, CCR2, CCR6 and CXCR6 in lung CD3 cells compared to blood, however none have shown a difference between asthmatic and normal controls. CCR8 had not been studied to date due to the unavailability of suitable mAb. We studied BAL samples from 13 asthmatics and 7 normal subjects for CCR8 expression. There were significantly more CCR8 expressing T cells in BAL from asthmatics compared to normal controls (mean percentage 8.6%±0.8 vs 3.9±0.7) (p<0.01) (Figure 4-11). There was also a significant increase in the percentage of CCR8 positive T cells in BAL compared to blood in matched samples from subjects with asthma (9.03%±0.9 vs 5.69%±0.94) (p<0.01) (Figure 4-12). This was not observed in normal subjects.

Figure 4-11: CCR8+CD3+ cells in BAL samples from asthmatic (●) and normal subjects (▲).**P<0.01 using Mann-Whitney test. Bars represent mean percentages.
Figure 4.12: CCR8+CD3+ cells in matched BAL (△) and blood (□) samples from the same subject in (a) asthma and (b) normal subjects. **P<0.01 using Mann whitney test. Lines connect samples from the same subject. Mean percentages are given at the top of the figure.
RESULTS: CCL1/CCR8 IN ASTHMA

4.7 CCR8 Expression on IFN-γ+/IL-4+ T cells in BAL

As with blood T cells CCR8 was enriched on IL-4+ T cells in BAL. In asthma CCR8 was expressed by 30.3±3.0 and 28.9±7.8 of the IL-4+ and IL-13+ producing BAL CD3+ cells respectively. In contrast only 4.9±2.0 of IFN-γ+ BAL CD3+ cells expressed CCR8 (Figure 4-13). A similar pattern of CCR8 expression was seen on IL-4+ and IL-13+ producing BAL CD3+ cells from normal subjects. The percentages of T cells that were cytokine positive were low and on average 30000-40000 PBMC were analyzed. Samples were not replicated (n=1) and were not blinded. Isoytype controls were included for all tests and those samples showing non specific binding were eliminated from analysis.

Figure 4-13: CCR8 expression on IFN-γ+ (♦), IL-4+ (●) and IL-13+ (▲) BAL CD3+ cells from (a) asthma subjects and (b) normal subjects. Cells were first stimulated with PMA and calcium ionophore. **P<0.01, ***p<0.001 using Mann-Whitney test. Bars represent mean.

(a)       (b)
4.8 Cytokine$^+$ cells amongst BAL CCR8$^+$ T cell population

Amongst IFN-γ$^+$ and IL-4$^+$ BAL T cells, CCR8 was expressed on a greater proportion of the IL-4$^+$ T cells than IFN-γ$^+$ T cells (Figure 4-13). The other side of the coin is, of the CCR8$^+$ T cells what percentage produce each of these cytokines? The number of IL-4 and IFN-γ positive cells as a proportion of the CCR8$^+$ T cells in BAL is shown in Figure 4-14 (asthma vs normal). There was a significantly greater percentage of CCR8$^+$ BAL T cells that were either IL-4$^+$ or IFN-γ$^+$ in asthmatics (14%±6 and 15%±4) than in normal controls (2.5%±0.8 and 3.7%±1.1) (p<0.01) (Figure 4-14). The data suggests that there was a higher percentage of both cytokine positive CCR8$^+$ T cells in asthmatics compared to normal subjects, and there was equal percentage of CCR8$^+$ T cells that are IL-4$^+$ and IFN-γ$^+$. The equal percentages of IL-4$^+$ and IFN-γ$^+$ cells amongst the CCR8$^+$ T cells, despite preferential expression of CCR8 on IL-4$^+$ T cells, can be explained by the higher percentage of IFN-γ$^+$ T cells compared to IL-4$^+$ T cells in the BAL of asthmatics.

Figure 4-14: CCR8$^+$ BAL CD3$^+$ cells that were IL-4$^+$ in asthma and normal subjects (A) CCR8$^+$ BAL CD3$^+$ cells that were IFN-γ$^+$ in asthma and normal subjects (B). Columns represent mean percentages. Error bars represent SEM. **p<0.01 using Mann whitney test.
4.9 CCL1 in the BAL fluid of asthmatics and normal subjects

We found a higher percentage of CCR8+ T cells in the BAL of asthmatic subjects compared to normal controls. We investigated whether the ligand for CCR8 (CCL1) is also found in greater quantities in the BAL fluid of asthmatics compared to normal controls. Preliminary studies showed that CCL1 was not detectable in BAL fluid but, was detectable following concentration. We used ultracentrifugation filters to concentrate BAL fluid samples 20 fold. The mean concentration of CCL1 in 20X BAL fluid from asthmatic subjects was significantly higher than in normal subjects (35±6 pg/ml vs 12.9±7 pg/ml; p<0.05) (Figure 4-15).

Figure 4-15: Concentration of CCL1 in 20 fold concentrated BAL fluid from asthmatic (▲) and normal subjects (○). *P<0.05 using Mann-Whitney test. Bars represent mean concentrations.
4.10 CCR8 positive cells in bronchial mucosa

In addition to blood and BAL fluid, we have also investigated the presence of CCR8+ T cells within the bronchial mucosa of asthmatic and normal subjects. Biopsies were obtained from 5 normal and 8 severe asthmatics. The 433H anti CCR8 mAb only showed staining in paraffin embedded specimens. The thickness of sections produced using paraffin embedded specimens did not allow co-localisation of CD3+ and CCR8+ cells to be accurately performed. Most if not all blood CCR8+ were CD3+ on flow cytometry studies so those cells that were CCR8+ are probably all T cells. The mean±SEM of CCR8+ cells seen within the sub-epithelium of the bronchial biopsies taken from asthmatics was 93±11 cells/mm² compared to 31±16 cells/mm² in normal subjects (p<0.05) (Figure 4-16). CCR8+ cells were seen mostly intra-epithelium and in the sub-epithelium. Median values with IQR were 95 (71-117) in asthma and 14 (0-70) in normal subjects. Examples of bronchial epithelial sections stained for CCR8 from an asthma and normal subject are shown in Figure 4-17.

Figure 4-16: CCR8+ cells/mm² within the bronchial mucosa of asthma (n=8) (▲) and normal control subjects (n=5) (●). *P<0.05 using Mann-Whitney test. Bars represent the mean cells/mm².
Figure 4-17: Bronchial biopsy section from an asthmatic subject stained with (A) anti CCR8 mAb (433H) (black arrows indicate CCR8+ cells) and (B) IgG2a isotype control.
4.11 Discussion

In this study we have shown for the first time that there are increased numbers of CCR8⁺CD3⁺ T cells in the BAL of asthmatics compared to normal subjects. Moreover, in paired samples there were more CCR8 expressing BAL T cells than blood T cells in asthmatics but not in normal subjects. This difference could be due to recruitment of CCR8 expressing T cells from blood to lung, a possibility supported by the increased concentration of CCL1 in BAL fluid from asthmatics compared to normal subjects. This study therefore provides the first evidence for a potential role for CCR8 in T cell recruitment to the lung in clinical asthma.

The investigation of the role of CCR8 in human disease has been hampered by the lack of reliable reagents to detect CCR8 expression on the cell surface. We were fortunate in obtaining a well characterised, specific, blocking, monoclonal antibody from ICOS Corporation which could be used in both flow cytometry and immunohistochemistry. As well as the characterization undertaken by investigators at ICOS Corporation we also found that this antibody showed good specificity to the CCR8 receptor. In chemotaxis assays using in vitro polarised IL-4⁺ cells, we found that the antibody effectively inhibited CCL1 mediated T cell migration. In addition CCL1-F gave the same amount of staining for CCR8 as the anti-CCR8 antibody and furthermore pre-incubation with the labelled CCL1 blocked subsequent binding of the anti-CCR8 antibody to T cells. Anti-CCR8 also blocked binding of the CCL1-F to T cells. We are therefore confident that this antibody recognizes CCR8.

Our data supports the findings from human in vitro studies of CCR8 expression by T H2 cells. After two weeks of in vitro culture in T H2 polarising conditions, percentage of CD3+ cells expressing CCR8 was almost similar to that expressing CCR4, T H2 cell associated chemokine receptor. The other T H2 related chemokine receptor, CCR3 on the other hand, showed no up regulation upon polarisation and this is in keeping with other groups who have also failed to demonstrate CCR3 expression on polarised T H2 cells [217]. We have also demonstrated the association of CCR8 expression with IL-4⁺ T cells.
RESULTS: CCL1/CCR8 IN ASTHMA

ex vivo using blood and BAL T cells by simultaneous intracellular cytokine and surface CCR8 measurement. There was a ~6 fold enrichment of CCR8 expression on IL-4+ T cells compared to IFN-γ+ T cells. The percentages of IL-4+/IL-13+ T cells in our study also included CD8+ T cells (Tc2). Furthermore, cells in our study were single stained with anti IL-4 or anti IFN-γ, and some of the IL-4+ T cells may also be IFN-γ+ (Th0 cells). This would also apply to the in vitro polarised T cells. Given these limitations we would not be able to suggest a definite association of CCR8 with Th2 cells using our data. Nevertheless CCR8 expression was seen on significantly higher percentage of IL-4+ and IL-13+ T cells compared to IFN-γ+ T cells amongst blood and BAL T cells.

Studies of IL-4 and IL-13 at a single cell level in asthma have generally reported very small percentages of T cells that are IL-4+ or IL-13+, which makes the study of these cells very difficult. In this study the numbers of IL-4+ or IL-13+ T cells identified are approaching the limit of the instrument’s resolution (flow cytometer). By using isotype control plots we have eliminated samples showing nonspecific staining and we believe that the percentages we report reflect a small but true population of IL-4+ and IL-13+ T cells. Although we had only used one sample from each subject and therefore have difficulty demonstrating reproducibility, the study (data from PBMC) had included a large number of subjects, and the pattern of CCR8 expression on IFN-γ+, IL-4+ or IL-13+ T cells had been very consistent. The small numbers of patients included for BAL analysis is a limitation of the study and reflects the difficulty in obtaining these samples.

Our findings on the expression of CCR8 on peripheral blood cells were similar to the recently reported findings of Soler et al (2007) who used CCL1-F to identify CCR8 expressing cells in human peripheral blood [88]. They noted predominant expression on CD4+CD45RO+ T cells with an approximately six fold enhancement of expression on Th2 cytokine producing T cells. They also found increased expression of CCR8 on FOXP3 expressing Treg cells accounting for ~60% of this group of Treg cells. Amongst CCR8+ T cells ~20% were shown to express FOXP3. In this study we had noted a significant increase of CCR8 expression on peripheral blood T cells from patients with severe asthma which has not been observed for other chemokine receptors.
Our most striking finding was the greater percentage of BAL T cells expressing CCR8 in asthmatics compared to normal controls. Previous studies have demonstrated higher percentages of CCR2, CCR4, CCR5, CCR6, CXCR3 and CXCR6 amongst lung T cells compared to blood. However, there had been no difference in any of the T cell expressed chemokine receptors between asthmatics and normal subjects including the T_h2 cell associated receptors CCR3 and CCR4, so what we have observed appears to be specific for CCR8 [118, 119, 154, 161]. This may be because previous studies of chemokine receptor expression in asthma have been on mild steroid naïve asthmatics whereas our subjects, who underwent bronchoscopy had more severe disease. It is always possible that the increased expression of CCR8 compared to normal subjects was due to treatment, in particular steroids. However, as we found no difference in the expression of CCR8 in blood or BAL in patients taking oral steroids versus those only on inhaled steroids we feel this is unlikely.

In BAL, CCR8 was preferentially expressed by IL-4^+ T cells (~ 30%) compared to IFN-γ^+ T cells (<5%). Interestingly, amongst the CCR8 expressing T cells found in BAL there were equal proportions of IL-4 and IFN-γ expressing cells. However, there were significantly more IFN-γ^+ T cells in the BAL than IL-4^+ T cells in asthma. As there were more CCR8^+ T cells in the BAL in asthma, this could suggest a potential mechanism for the recruitment of IL-4^+ T cells to the lung in asthma.

It is not possible to say whether the increased CCR8 expression was due to activation of BAL T cells or increased recruitment of CCR8 expressing T cells from the blood. However in support of the latter mechanism we did see an increase in CCL1 concentrations in BAL fluid. This was at low concentrations, well below the amount required for chemotaxis. However, it is difficult to extrapolate from concentration in BAL, the site of release and action in the lung. One other group has also reported increased CCL1 in BAL fluid in asthmatics although another group was not able to detect CCL1 in allergen challenged asthmatics [186, 218]. CCL1 was not detected in bronchial biopsies following allergen challenge in one study [148]. These findings may indicate
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that the source of CCL1 may be alveolar macrophages and not the bronchial epithelial cells. There have also been reports of CCL-17 (found at higher levels in asthma BAL compared to normal controls) being a ligand for CCR8 although, this observation was not supported by another group [173, 174]. The extent to which there is a chemotactic signal for CCR8+ cells in asthmatic airways therefore needs further investigation.

Two reports have been made of CCR8 expression in asthma [148, 219]. In the first of these reports CCR8 expression was studied using a goat polyclonal antibody in bronchial biopsies after allergen challenge [148]. The specificity of this antibody has not been well established and we were unable to detect staining of peripheral blood T cells by flow cytometry using this reagent (data not shown). The exceptionally high numbers of T cells in the bronchial mucosa after allergen challenge reported in this study (~ 4400/mm²) was not comparable to other studies which have shown only a modest increase [20]. The study also reported very high levels of T cell IL-4+ and CCR4 expression both at baseline (~ 40% of T cells were IL-4+) and after allergen challenge (~ 100% were IL-4+), which is inconsistent with other reports both in BAL fluid and bronchial mucosal biopsies [19, 149, 154, 161, 220]. These studies had shown no significant difference in the numbers of T cells expressing CCR4 between asthma and normal subjects both in clinical disease and after allergen challenge. In addition, cytokine expression by T cells cannot usually be detected by immunohistochemistry firstly as these cells do not store the mediators and secondly T cells do not generally express IL-4 or IFN-γ unless stimulated. In any case, the numbers of T cells expressing IL-4 in asthmatics even after stimulation with calcium ionophore and PMA in other studies had been less than 5% of the total T cells. Overall therefore the results of this study are difficult to interpret.

The second very recent report (published after the completion of this study) had studied CCR8 mRNA in bronchial biopsies using in-situ hybridization [219]. 10 asthmatics were compared to 7 normal controls. Increased CCR8 mRNA was shown in the asthmatic biopsies compared to normal controls (27.3 vs 7.4 cells/mm²). ~70% of CD4 T cells in the asthmatic mucosa were shown to be CCR8+. This study had also shown increased staining for CCL1 in the bronchial biopsies of asthmatics. Interestingly they had also
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shown that CCL1 was major chemokine secreted by mast cell lines following FceR1 activation. A mast cell driven recruitment of T_{H2} cells to the asthmatic airway was proposed by the authors. Increased mRNA does not always correlate with the translated product, nonetheless in this study we did see an increase in CCR8 expression in our bronchial biopsies from asthmatics compared to controls further supporting our BAL data.

Although there was an approximately 3 fold difference in CCR8 expressing cells in the bronchial mucosa of asthmatics compared to normal subjects, we had also seen a 2-fold difference in the number of total T cells in asthmatic biopsies. As we had not co-stained tissues for CCR8 and CD3 it is difficult to accurately comment on whether the percentage of CCR8^+ T cells was higher in the asthmatics. However if we were to assume all the CCR8+ cells are also CD3+, combining figures from chapter 3 and 4 gives a percentage CCR8+ T cells of ~24% in asthma and ~18% in normal subjects. Although the percentage appears higher in asthmatics it is only marginal and is difficult to make comment on its significance.

CCL1 mRNA is upregulated in murine lung following allergen sensitisation and challenge in the mouse model of T_{H2} mediated lung inflammation. Although neutralisation of CCL1 had no effect on T_{H2} cell recruitment to the lung in mice, one study did show reduction in eosinophil recruitment to the lung following CCL1 blockade [171]. Direct instillation of CCL1 in to allergen sensitised challenged mice also induced recruitment of eosinophils. Gene knockout (KO) studies had been non conclusive. One study showed reduced T_{H2} responses and eosinophil recruitment in the absence of CCR8 [170], however, two subsequent studies had not been able to reproduce these findings [183, 184]. Animal studies have therefore been inconclusive but this model is at some distance from clinical disease and cannot be taken as a guide for the effects of CCR8 antagonists in asthma.

The exact contribution made by CCR8 expressing T cells would need further detailed study, especially so with the development of CCR8 antagonists that may have potential clinical use [221]. Current evidence suggests that CCR8 expressing T cells may have
multiple roles. It is possible that a significant proportion of CCR8 expressing cells are Tregs and CCR8 may play a role in the trafficking of these cells [87, 88]. Its role in T cell homing to the skin in atopic dermatitis has been described [110]. Interestingly CCR8+ cells which also expressed cutaneous lymphocyte antigen (CLA) were shown to have equal frequencies of $T_{H1}$ and $T_{H2}$ cells whereas $T_{H2}$ cells were several folds enriched in the CLA- CCR8+ population suggesting that CCR8 may be one of a combination of receptors guiding T cells to particular sites [88]. This study provides evidence that CCR8 may be involved in human asthma and may contribute to its pathogenesis through its role in trafficking T cells to the lung. As will be discussed later CCR8 compares favourably as a marker of IL-4+ T cells when compared with the other $T_{H2}$ associated chemokine receptors. CCR8 therefore may be involved in recruitment of $T_{H2}$ cells to the lung in asthma and therefore is a potentially important target for therapeutic intervention.
CRTh2 is a receptor which has been suggested to be a reliable marker for Th2 cells. Despite this, its role in asthma had not been described in any great detail. An orally bioavailable antagonist is already in use and therefore it would be useful to explore the role of this receptor in asthma. Using a commercially available mAb we set out to study this receptor in blood and BAL samples of asthma and normal subjects with the objective to see if it played any part in recruiting Th2 cells to the asthmatic lung. A total of 24 asthma and 20 normal subjects were studied. Samples from some of these subjects were also included in studies described in chapters 3 and 4 (dual staining with CCR8). 18 of the asthmatics and 9 of the normal subjects from this study were also included in experiments described in chapter 3 for intracellular cytokine measurements. Asthma severity was classified as described in the methods section. Clinical characterization of subjects included in the following experiments are given in Table 5-1. All but one patient with asthma who were studied with bronchoscopy in chapter 4 were also included in this study and their subject characteristics are given in Table 4-2.
RESULTS: CRTh2 AND ASTHMA

Table 5-1: Characteristics of all subjects who took part in the CRTh2 study. * mean (SEM); ++ Geometric mean (SD); ** Median (range).

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild Asthma</th>
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<td>20</td>
<td>5</td>
<td>7</td>
<td>12</td>
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<td>35 (21-53)</td>
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<td>5</td>
<td>9</td>
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<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0.2 (0.07)</td>
<td>0.3 (0.06)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>IgE (kU/L)*</td>
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<td>727 (433)</td>
<td>615 (396)</td>
<td>339 (62)</td>
</tr>
<tr>
<td>Atopy (n)</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>FEV₁%P*</td>
<td>94 (9)</td>
<td>100 (3)</td>
<td>73 (11)</td>
<td>79 (4)</td>
</tr>
<tr>
<td>Inhaled CS* (µg/day)</td>
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<td>0</td>
<td>390 (165)</td>
<td>1091 (77)</td>
</tr>
<tr>
<td>On OCS (n)</td>
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<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Pc20++</td>
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<td>3.9 (0.2)</td>
<td>0.9 (0.9)</td>
<td>0.7 (0.3)</td>
</tr>
</tbody>
</table>
RESULTS: CRTh2 AND ASTHMA

5.2 CRTh2\(^+\) T cells in blood in asthma and normal subjects.

Amongst PBMC, CRTh2 expression was seen mostly on non T cells (~ 3%) (Figure 5-2). The non T cells were confirmed as basophils using Ig\(\varepsilon\)R1 (results not shown). Only a small number of CRTh2\(^+\) T cells were seen in blood in asthma (mild-moderate asthma 2.2\(%\pm0.7\); severe asthma 2.8\(%\pm0.7\)) and in normal subjects (1.6\(%\pm0.2\)) with no significant difference between the groups (Figure 5-1). The majority were CD4\(^+\) T cells (62.8\(%\pm2.1\)). CRTh2\(^+\) T expression was seen on 30.9\(%\pm1.5\) of CD8 T cells. 68\(%\pm9.8\) were CD45RO\(^+\). Representative FACS plots are shown in Figure 5-2.

CRTh2 is expressed on eosinophils, basophils as well as T\(_{H2}\) cells. Given its association with allergy we investigated whether T cell CRTh2 expression was associated with the serum allergy markers. We assessed the percentage of CRTh2\(^+\) T cells in blood against serum IgE and eosinophil numbers. CRTh2\(^+\) T cell numbers did not show any significant correlation with serum IgE levels (r\(_2=0.2\); p=0.28) or blood eosinophil numbers (r\(_2=0.1\); p=0.5).

Figure 5-1: Scatter plot of the percentages of CRTh2\(^+\) T cells in blood of normal (●), mild-moderate asthma (▲) and severe asthmatics (○). Bars represent the mean. No significant difference in the expression of CRTh2 on T cells was seen between the groups.
Figure 5-2: Representative FACS dot plots showing association of CRTh2 with (A) CD3, (B) CD4 and CD8 and (C) CD45RO. Cells in plot B and C were gated through Gate 2.

Gate 1: CD3-CRTh2+

Gate 2: CD3+CRTh2+

5.3 CRTh2 expression on IFN-γ⁺/IL-4⁺ T cells in blood.

The association of blood T cell CRTh2 expression with intracellular cytokine was made following in-vitro stimulation with PMA and calcium ionophore for 6 hours. The percentages of T cells that were cytokine positive were low and on average 30000–40000 PBMC were analyzed. Samples were not replicated (n=1) and were not blinded. Isotype controls were included for all tests and those samples showing non specific binding were eliminated from analysis. CRTh2 was expressed by a higher percentage IL-4⁺ and IL-13⁺ T cells than IFN-γ⁺ T cells (Representative FACS plots are shown in Figure 5-3). In asthma, 26.5%±4.5 and 35.5%±3.3 of the IL-4⁺ and IL-13⁺ producing T cells also expressed CRTh2 whilst only 2.4%±0.5 of IFN-γ⁺ cells also expressed CRTh2 (p<0.001). (Figure 5-4). A similar pattern of expression of CRTh2 on IL-4⁺ and IL-13⁺ T cells relative to IFN-γ⁺ T cells was also seen in blood of normal subjects (Figure 5-5).
RESULTS: CRTh2 AND ASTHMA

Figure 5-3: Representative FACS dot plots of CD3 gated cells co-stained for CRTh2 and intracellular (A) IFN-γ, (B) IgG, (C) IL-4 and IL-13 from an asthmatic subject. Cytokine+ cells are expressed as a percentage of CD3+ cells. Percentage of cells that are CRTh2+ (Q2) and CRTh2- (Q4) are noted within the plots. Figures within parenthesis are absolute events (number of cells).
RESULTS: CRTh2 AND ASThma

Figure 5-4: Scatter plot showing CRTh2 expression on (♦) IFN-γ⁺, (●) IL-4⁺ and (▲) IL-13⁺ T cells derived from PBMC from asthma subjects. Cells were stimulated for 6 hours with PMA and calcium ionophore. ***P<0.001 using Mann-Whitney test. Bars represent mean percentages.

Figure 5-5: Scatterplot showing CRTh2 expression on (♦) IFN-γ⁺, (●) IL-4⁺ and (▲) IL-13⁺ T cells derived from PBMC from normal subjects. Cells were stimulated for 6 hours with PMA and calcium ionophore. ***P<0.001 using Mann-Whitney test. Bars represent mean percentages.
RESULTS: CRTh2 AND ASTHMA

5.4 CRTh2 expression on in vitro polarised IFN-γ+/IL-4+ T cells.

We explored the association of the CRTh2 receptor with IL-4+ cells by measuring their expression on in vitro polarised cells IL-4+ cells. Against expectation we had not been able to increase the percentage of cells expressing CRTh2 by IL-4 polarising conditions (Figure 5-6).

Figure 5-6: Effect of IL-4 polarising conditions on the expression of CRTh2. Graph shows data from 8 independent experiments using blood from 8 different asthmatic donors. The different symbols describe different donors. No difference in CRTh2 expression was seen between IFN-γ and IL-4 polarising conditions.
RESULTS: CRTh2 AND ASTHMA

5.5 CRTh2+ T cells in BAL from asthmatic and normal subjects.

Although BAL was taken from 12 asthma subjects one sample had be eliminated due to very poor quality and nonspecific staining. There was a low percentage of CRTh2+ T cells in the BAL fluid in asthmatics (~3%). The mean percentage CRTh2 positive T cells in the BAL in asthma subjects was 2.3%±0.6, and 0.3%±0.1 from normal subjects with a significant difference between the groups (Figure 5-7). We have also studied the expression of CRTh2 in paired blood and BAL samples from the same subject. The percentage of CRTh2+ T cells in blood was 2.0%±0.4 vs 3.5%±0.8 amongst BAL T cells in asthma, with no difference between the two. There was a significant difference in the CRTh2+ T cells in the BAL compared to blood in normal controls (Figure 5-8).

Figure 5-7: CRTh2 expression on BAL CD3+ cells in asthmatic (●) and normal (▲) subjects. The was significant difference between asthmatic and normal controls. *P<0.05 using Mann-whitney test. Bars represent mean percentages.
RESULTS: CRTh2 AND ASTHMA

Figure 5-8: CRTh2+CD3+ cells in paired blood (□) and BAL (△) samples from (A) asthma and (B) normal subject. Lines connect samples from same subject. A significant difference was seen in the percentage of CRTh2+CD3+ T cells between BAL and blood T cells in the normal group only. *P<0.05 using Mann-Whitney test.
RESULTS: CRTh2 AND ASTHMA

5.6 CRTh2 expression on IFN-γ⁺/IL-4⁺ T cells in BAL.

Intracellular cytokine content in BAL T cells was studied in 6 out of the 13 subjects who had BAL samples taken due to lack of sufficient cell numbers in all samples. Cells were stimulated with PMA and calcium ionophore for 6 hours. The percentages of T cells that were cytokine positive were low and on average 30000-40000 PBMC were analyzed. Samples were not replicated (n=1) and were not blinded. Isotype controls were included for all tests and those samples showing non specific binding were eliminated from analysis. In asthmatic subjects CRTh2 was expressed by 17.3%±5.3 of the IL-4⁺ cells, 16%±6.4 of the IL-13⁺ cells. In contrast only 1.2%±0.1 of IFN-γ⁺ cells expressed CRTh2 (Figure 5-9). The expression of CRTh2 on BAL T cells from normal subjects was too small for this assessment to be made accurately.

Figure 5-9: Scatter plot of the percentage of CRTh2⁺CD3⁺ BAL cells from asthmatic subjects expressing IFN-γ, IL-4 and IL-13. **P<0.01 using Mann-Whitney test. IL-4 (●), IL-13(▲), IFN-γ (♦). Bars represent mean percentages.
RESULTS: CRTh2 AND ASTHMA

5.7 PGD$_2$ in BAL of asthmatic and normal subjects.

Although there was no difference in the percentage of CRTh2$^+$ T cells between BAL and blood in asthma there appeared to be a higher percentage of these cells in asthmatics compared to normal controls. We have therefore measured the ligand for CRTh2, PGD$_2$ in BAL. This was estimated in 11 out of 12 asthma subjects studied (one had an insufficient sample) and all of the normal subjects. Although a significant difference was not seen between the two groups, a bimodal distribution of PGD$_2$ levels similar to that seen with CRTh2 (Figure 5-7) was observed within the asthma group (Figure 5-10). We therefore attempted to correlate the percentage of CRTh2$^+$ T cells found in the BAL to the BAL-PGD$_2$ levels from the same subject. Both asthma and normal subjects were included. Due to the small numbers, Spearmans correlation test was used. No significant association was seen ($r=0.4; p=0.07$).

Figure 5-10: Levels of PGD$_2$ in BAL fluid from asthmatic (●) and normal subjects (▲). Bars represent mean percentages. No significant difference was seen between the two groups.
RESULTS: CRTh2 AND ASTHMA

5.8 Co-expression of CCR3, CCR4, CCR8 AND CRTh2 in PBMC

There are 4 chemokine receptors that are reported to show preferential expression on T\(\text{H}2\) cells. It would be of interest to see if these receptors are co-expressed, which would allow more accurate identification of T\(\text{H}2\) cells. Using multicolour flow cytometry we investigated the pattern of co-expression of the T\(\text{H}2\) cell associated chemokine receptors CCR8, CRTh2, CCR3 and CCR4 in PBMC with samples from both asthmatic and normal subjects. We found that all CCR8\(^+\)/CCL-F\(^+\) T cells in blood were also CCR4\(^+\). ~20% of CCR4\(^+\) expressing T cells also expressed CCR8/binding sites for CCL1-F. None of the CCL1-F\(^+/\)CCR8\(^+\) cells were CCR3\(^+\). CCR3 and CCR4 expression were almost mutually exclusive. All CRTh2\(^+\) T cells in blood were also CCR4\(^+\). ~5% of CCR4\(^+\) T cells were CRTh2\(^+\). None of the CRTh2\(^+\) T cells were CCR3\(^+\). Approximately 30% of CCR8 expressing T cells in blood also expressed CRTh2 (some representative FACS plots are shown in Figure 5-11).

Figure 5-11: Representative FACS plots showing CD3 gated PBMC co-stained for various combinations of chemokine receptors. (Not all combinations are shown). Plot on the right shows isotype control.
RESULTS: CRTh2 AND ASTHMA

5.9 Discussion

The inflammatory process in asthma is considered to be driven at least in part by cytokines derived from Th2 lymphocytes recruited into the airway. The mechanisms controlling migration of Th2 cells into the airway are therefore of interest. CRTh2 has been suggested to be the best marker for Th2 cells [222]. It binds to the ligand PGD2 known to be released in large quantities by mast cells in response to allergen exposure [188]. PGD2 has therefore attracted considerable interest due its potential for recruiting Th2 cells to the asthma lung. We believe that this is the first study to report expression of CRTh2 on T cells in asthma compared to normal subjects. We found that CRTh2 is expressed by a greater percentage of IL-4+ T cells than IFN-γ+ T cells. However, we found only very low levels of expression of CRTh2 in both blood and BAL T cells; nevertheless a significant difference was seen in CRTh2+ BAL T cells in asthmatics compared to controls. Asthmatics had a greater percentage of CRTh2+ T cells in their BAL than normal controls.

We found that CRTh2 was expressed by only a small percentage of blood T cells in asthma (~ 2.5%) and there was no difference in the percentage of these cells in asthma compared to normal subjects. The lack of any difference in the percentage of CRTh2+ T cells between asthma and normal subjects in the blood may reflect the absence of any difference in IL-4+ T cells in the blood of asthmatics compared to normal subjects (chapter 3). The percentages of IL-4+IL-13+ T cells in our study also included CD8+ T cells (Tc2). Furthermore, cells in our study were single stained with anti IL-4 or anti IFN-γ, and some of the IL-4+ T cells may also be IFN-γ+ (Th0 cells). Given these limitations it would be difficult to suggest a definite association of CRTh2 with Th2 cells. In this study we show some evidence that they may be associated with Th2 cells. CRTh2 was expressed on ~ 8-10 fold higher percentage of IL-4+ T cells than IFN-γ+ T cells. However against expectation we had not been able to demonstrate a change in the percentage of CRTh2+ T cells after culture in IL-4 polarising conditions. The reasons for this are not clear. This may be related to the particular method used for polarisation.
RESULTS: CRTh2 AND ASTHMA

Limitations of the CCR8 part of the study would also apply to this part of the study. The percentages of IL-4+ or IL-13+ T cells that are CRTh2+ are very small. We also have difficulty demonstrating reproducibility as only one sample was taken from each subject. However the data from PBMC had included a large number of subjects, and the pattern of CRTh2 expression on IFN-γ+, IL-4+ or IL-13+ T cells had been very consistent. The small numbers of patients included for BAL analysis is a limitation of the study and once again reflects the difficulty in obtaining these samples.

Differences in both IFN-γ+ and IL-4+ T cells were seen in the asthma lung compared to normal subjects (Chapter 4). This difference in IL-4+ T cells in the BAL of asthmatics seems to be mirrored by a difference in CRTh2+ T cells in the BAL of asthmatic compared to normal controls. The expression of CRTh2 on BAL T cells in asthma has the appearance of being bimodal (Figure 5-7). Although analysis of BAL fluid PGD2 levels and the percentage of BAL CRTh2+ T cells showed no significant correlation between the two, the 5 subjects within the group who had low numbers of CRTh2+ T cells in BAL (<2%) (Figure 5-7) also had lower levels of PGD2 (<500pg/ml) (Figure 5-10) and furthermore, all 5 of these subjects were on oral maintenance corticosteroids. This may indicate that the PGD2 may be playing a role in recruiting CRTh2+ T cells to the lung in some asthmatics, and that corticosteroids might affect production of PGD2 in the lungs and possibly the recruitment of CRTh2+ T cells.

Against expectation, we had not been able to see a significant increase in CRTh2+ T cells in BAL compared to blood in asthma subjects. Infact, in the normal group there was a lower percentage of CRTh2 expressing T cells in the BAL compared with blood. It is possible that this could be due to down regulation of the receptor due to interaction with PGD2 in the lung or could be related to more activated cells being present in the lung. However, only low levels of PGD2 would be expected to be present in the normal lung and we have shown that short term activation with PMA and calcium ionophore did not effect expression of CRTh2 (chapter 3).
RESULTS: CRTh2 AND ASTHMA

PGD$_2$ has been shown to be able to modulate T cells locally and encourage T$_{H2}$ cell polarisation through interaction with the DP1 and CRTh2 receptor [191]. DP1 receptor is expressed mostly by T$_{H1}$ cells and its interaction with PGD$_2$ results in the inhibition of cytokines by these cells. CRTh2 on the other hand has been shown to be involved in the induction of cytokines by T$_{H2}$ cells [201]. CRTh$_2^+$ T cells were shown to be able to produce more PGD$_2$ providing an autocrine amplification mechanism. It has been suggested that PGD$_2$, by acting on DP1 and CRTh2 receptors could drive a state of T$_{H2}$ polarisation within the tissue [201]. However given the small number CRTh$_2^+$ cells we had observed amongst BAL T cells, our data suggests that CRTh2 probably does not have a significant biological functional role in modulating T cells in asthma pathogenesis.

Despite this CRTh2 may still have a significant contribution to asthma pathology through its actions on non T cells such as eosinophils and basophils. The role of CRTh2 and basophils in asthma has not been explored in any great detail to date. There is an abundance of evidence that eosinophils play a major role in asthma pathology [223]. There are several lines of evidence that CRTh2 plays a significant part in eosinophil recruitment and its activation in allergic diseases [191]. The selective CRTh2 agonist DK-PGD$_2$ induced eosinophil recruitment to the airway in mice. The moderate CRTh2 antagonist Ramatroban has shown some effect in treating eosinophil mediated diseases. The clinical implications of CRTh2 antagonist on eosinophils in asthma still remains to be explored in further detail.

CRTh2 has been suggested to be the most reliable marker for circulating human T$_{H2}$ cells [222]. In our study ~26% of IL-4$^+$ T cells and ~35% of IL-13$^+$ T cells in blood expressed CRTh2 compared to ~2% of IFN-$\gamma^+$ T cells indicating a degree of specificity for IL-4$^+/IL-13^+$ T cells. In a previous study ~40% of IL-4$^+$ T cells were shown to express CCR4, however in the same study ~15% of IFN-$\gamma^+$ T cells also expressed CCR4 which may suggest that CCR4 is less specific than CRTh2 as a marker for IL-4$^+$ T cells. However, CRTh2 expression was only seen in ~17% of IL-4$^+$ T cells in the BAL of asthmatics. CCR8 expression on the other hand was seen on ~30% of IL-4$^+$ T cells in the
RESULTS: CRTh2 AND ASTHMA

BAL of asthmatics, which suggest that CCR8 may be better able to detect IL-4⁺ T cells in the BAL of asthmatics. Moreover CCR8 was also expressed on significantly higher percentage of IL-4⁺ T cells compared to IFN-γ⁺ T cells in blood and BAL indicating specificity for IL-4⁺/IL-13⁺ T cells. Our data indicate that CCR8 may be a better marker than CRTh2 for IL-4⁺ T cells in asthma.

In summary, we have found that CRTh2 was expressed by a significantly higher percentage of IL-4⁺/IL-13⁺ T cells compared to IFN-γ⁺ T cells, however, seems to identify less IL-4⁺ T cells in the BAL of asthmatics when compared to CCR8. We have found a difference in the percentage of CRTh2⁺ T cells in the lung in asthmatics compared to normal subjects. However, the percentage of these cells in BAL is very low and therefore it is difficult to conclude whether they have a significant biological role in asthma pathogenesis. There was no evidence of enrichment of CRTh2 within the lung compared to blood in asthmatics or normal subjects and furthermore, we had not been able to demonstrate a difference in the level of PGD₂ in the BAL fluid between asthmatics and normal controls. Our data therefore does not provide evidence to support CRTh2 playing a role in recruiting T cells to the lung.
6 SUMMARY AND DISCUSSION

Despite the multiple potential roles that T cell could have in the causation and maintenance of asthma, to date there had not been much convincing evidence that they are increased in numbers in the asthmatic lung. There are many more studies showing no increase in T cell numbers in the lung than there are studies showing an increase. However this may be because most studies have used asthmatics with milder disease due to the potential risks with bronchoscopy. In recent years bronchoscopy had been used more often to study severe asthma and findings from these studies appear to indicate that the pathophysiology within this group may be distinct from that of milder disease [224]. In this study lavage and biopsies were obtained from mostly severe asthmatics. These patients had increased airway eosinophilia, poor lung function and airway hyperresponsiveness compared to the control subjects. We have shown that in this group of asthmatics there appears to be a significant increase in the number of T cells compared to non asthma healthy controls.

Contrary to the popular hypothesis of the last two decades, we had not been able to demonstrate an increased presence of IL-4$^+$ T cells at the expense of IFN-γ$^+$ T cells in the blood or lavage fluid from asthmatics subjects. Instead, we saw higher percentages of both IFN-γ$^+$ and IL-13$^+$ T cells in the BAL of asthmatics compared with normal controls, with a marked increase in IFN-γ$^+$ T cells in the asthmatic BAL compared to normal controls. This may be due to the agents used in this study to stimulate cytokine production, which provides the cell with non specific stimuli. Similarly, compared to normal controls the asthmatics studied also had a trend towards increased neutrophils in their airway as well as significant eosinophilia. Our finding of higher percentage of cells expressing IFN-γ in asthma compared to normal controls is consistent with reports by some other groups who had also shown increased IFN-γ in the blood and airways of asthmatics. In terms of the percentages of T cells expressing IFN-γ, IL-4 and IL-13 the data from this study does not indicate a dominant T\textsubscript{H}2 profile at the expense of the T\textsubscript{H}1 arm in the asthmatics. However it is possible that the biological potency of IFN-γ$^+$ and IL-4$^+$ differ. It is therefore difficult to draw conclusions about the T\textsubscript{H}1/T\textsubscript{H}2 imbalance.
SUMMARY AND DISCUSSION

based on comparisons of numbers or percentages of IL-4+ and IFN-γ+ cells in isolation. Furthermore, one of the limitations with our study is that the IL-4+ and IL-13+ T cells described in this study also included Th0 and Tc2 cells. Selecting for CD4+ T cells and double staining with anti IFN-γ and anti IL-4 would have allowed accurate identification of the Th2 cells.

CCR8 had been a poorly studied chemokine receptor in human diseases due to the non availability of good antibodies. Therefore its role in T cell recruitment to the lung had not been very clear. One previous study on CCR8 in asthma had produced results that are difficult to interpret due to the poor quality of antibody used [148]. A recent study using CCR8 mRNA had showed increased CCR8 in asthma bronchial mucosa compared to healthy controls [219]. In this study we have demonstrated that the anti CCR8 (433H) monoclonal antibody has very good specificity. Using this mAb we have demonstrated that CCR8 may have a role in T cell recruitment to the lung. There was a higher percentage of CCR8+ T cells in the BAL compared to blood (~10% vs 4%) in asthmatics only. There were also a higher percentage of CCR8+ T cells in the lavage of asthmatics compared to normal controls. This would indicate that CCR8 may have a role in recruiting T cells from the blood into the lung and in asthma. The absence of a higher percentage of CCR8+ T cells in the normal subjects would suggest that the CCR8-CCL1 combination may not be involved in T cell movement in to the lung in homeostasis. This is further supported by the absence of any difference in CCR8+ T cells in dispersed T cell population obtained from lung resection specimens (COPD/Carcinoma of the lung) compared to blood (appendix). CCL1, the ligand for CCR8 is known to be produced by a number of resident lung cells and recently mast cells have been suggested to be a dominant source in the lung [219]. In support for the role of CCR8 in recruiting T cells to the lung in asthma, we had also shown that the concentration of CCL1 is higher in the lavage fluid from asthmatics than in normal controls. We have also shown that CCL1 can induce the migration of ~20% of a population of cells that were 21.9% CCR8+ (Figure 4-4) (Section 4.2).
SUMMARY AND DISCUSSION

The chemokine receptor CCR8 was expressed on IL-4+ T cells identifying between 25-30% of the IL-4+ T cells in blood and BAL of asthmatics and normal subjects. There was a higher percentage of CCR8+ T cells in the lung of asthmatics. There was also a correspondingly higher percentage of IL-13+ T cells in the asthma BAL compared to normal BAL, however no difference was found for IL-4. The small number of subjects is a weakness of the study. However, the only two reports on CCR8 in asthma had included 6 and 10 mild to moderate asthmatics respectively [148, 219]. In this study we have used bronchial biopsies from 8 severe asthmatics as well as BAL samples from 13 asthmatics 10 of whom had severe asthma. In our study we had also used blood samples to study the association of CCR8 with IL-4+ and IL-13+ T cells. Our data therefore provides more compelling evidence for CCR8 being involved in recruiting T cells to the lung in asthma.

CCR8 may also be responsible for the recruitment of cells other than Th2 cells to the lung. A significant percentage of Tregs have been shown to express CCR8. However given their inhibitory effect on immune processes including Th2 cells [91], one would expect their numbers to be decreased in asthma. On the other hand the percentage of Treg cells in blood are known to be increased with the use of inhaled and oral glucocorticoids. All patients who had taken part in the bronchoscopic part of the study were taking high doses of inhaled steroids and some were also taking oral corticosteroids.

Although there was higher percentage of CRTh2+ T cells in the BAL of asthmatic compared to normal subjects the percentages were very small (~ 2%). There was no significant difference in PGD2 levels in BAL of asthmatics compared to normal controls. Overall there was little evidence that CRTh2 is involved in the recruitment of T cells to the lung in asthma. The small percentage of CRTh2+ cells in the lung suggests that CRTh2+ T cells are unlikely to be of significant functional importance. However, the CRTh2 receptor may have an important role in asthma, via its expression on eosinophils. As a marker for Th2 cells, CRTh2 did identify a higher percentage of IL-4+ T cells compared to IFN-γ+ T cells in blood and BAL of asthmatics. However they identified a lower percentage of IL-4+ T cells in the BAL of asthmatics when compared to CCR8.
The small percentages of IL-4+ and IL-13+ T cells found in the normal as well as the asthmatic subjects is a limitation of this study. Other ex vivo studies of these cytokines at a single cell level had also reported very small percentages of IL-4+ and IL-13+ T cells in asthma and normal subjects [43, 161]. We have had difficulty demonstrating reproducibility, as we had only studied a single sample from each subject. However, the data from PBMC had included a large number of subjects, and the patterns of CCR8 and CRTh2 expression on IFN-γ+, IL-4+ or IL-13+ T cells had been very consistent. The small numbers of patients included for BAL analysis is a limitation of the study and reflects the difficulty in obtaining these samples.

Recently there had been a lot of interest in iNKT cells and whether they could play a role in human asthma. Animal studies had shown some evidence that these cells could play a role in the pathogenesis of asthma. A recent human study [77] had claimed that iNKT accounted for ~ 60% of the CD4 T cells in BAL in asthma and that most were able to produce T\textsubscript{H}2 cytokines. We had collaborated with the MRC centre for infection and inflammation in Birmingham to study the possible role of iNKT cells in a small group of asthmatics and non asthma controls. We were not able to detect iNKT cells in the magnitude described by Akbari et al (2006) and instead only saw very few iNKT cells in BAL of asthmatics including severe asthmatics (~3%). Our conclusion is that it probably plays a limited role in human asthma.
7 FUTURE WORK

In this study a possible role for T cell recruitment to the lung by CCR8 was suggested in patients with severe asthma. It would be interesting to see if this occurs in patients with less severe disease including those who are steroid naïve and whether it occurs to the same extent. This study demonstrates that anti-CCR8 (H433) is an antibody that could be used in immunohistochemistry and flow cytometry. Using this antibody it is now possible to explore the association of CCR8 in other T cell subsets and immune cells. Recent reports have demonstrated CCR8 expression on Treg which is starting to emerge as an important group of cells with probably a significant involvement in asthma pathogenesis. Specific markers are now available to identify these cells easily. If CCR8 antagonists are to be used as a therapeutic agent in asthma it is important to first explore whether CCR8+ T cells accumulating in the lung is a beneficial phenomenon in asthma. CCR8 was shown to be associated with FOXP3+ Treg cells in in vitro asthma studies. Intracellular IL-10 and TGF-β in CCR8 expressing T cells will also need to be studied to see if CCR8 is related to the other types of Treg cells. It would be interesting to see if CCR8 also plays a role in the migration of Tregs. I am aware that the anti CCR8 (433H) mAb is no longer available to the wider scientific community and it may be difficult for other groups to confirm or refute any data using it. This may reduce the impact of any studies using this mAb.
APPENDIX

8 APPENDIX

CCR8 and CRTh2 on dispersed lung T cells

T cells are compartmentalized within the lung and may show different composition of subset within each of the different compartments (blood, interstitium, bronchial epithelium and alveolar spaces (bronchial lavage). We had measured CCR8 and CRTh2 on dispersed lung T cells. Results shown came from 8 different resection specimens from 8 different patients. These specimens were obtained from patients undergoing thoracic surgery for malignancy or as part lung volume reduction surgery in emphysema. These lungs were therefore representative of COPD lung. For comparison CD3, CCR8 and CRTh2 measurements from blood, BAL and mucosal biopsies (normal subjects) from previous chapters are included in Table 8-1. CD3 values are the percentage CD3+ cells within the lymphocyte gated population.

Table 8-1: CD3, CCR8 and CRTh2 from lung together with values from blood, BAL, lung and biopsies from normal subjects from previous chapters.* Median (IQR); ** Mean (SEM)

<table>
<thead>
<tr>
<th></th>
<th>CD3</th>
<th>CCR8</th>
<th>CRTh2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUNG</td>
<td>**47% (6.8)</td>
<td>**2.8% (0.3)</td>
<td>**1.1% (0.4)</td>
</tr>
<tr>
<td></td>
<td>*44% (32-68)</td>
<td>*2.7% (2.1-3.7)</td>
<td>*0.6% (0.3-1.5)</td>
</tr>
<tr>
<td>BLOOD</td>
<td>**61% (56-63)</td>
<td>**3.05% (0.4)</td>
<td>**1.6% (0.2)</td>
</tr>
<tr>
<td></td>
<td>*2.7% (1.7-4.6)</td>
<td>*1.3% (0.8-2.0)</td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>**47% (21-82)</td>
<td>**3.9% (0.7)</td>
<td>**0.3% (0.1)</td>
</tr>
<tr>
<td></td>
<td>*0.9% (2.1-6.4)</td>
<td>*0.3% (0.1-0.7)</td>
<td></td>
</tr>
<tr>
<td>BRONCHIAL EPITHELIUM</td>
<td>*181/mm² (77-250)</td>
<td>*14/mm² (0-70)</td>
<td></td>
</tr>
</tbody>
</table>

The prevalence of CCR8+ T cells in lung appears similar to that of blood and may indicate blood contamination of the specimen; this is one of the drawbacks of using resection specimens. The processing of the specimen may also interfere with the receptor
detected which adds to the difficulty. As such it is difficult to draw accurate conclusions regarding CCR8+ T cell frequencies in the interstitium from the data obtained.
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