MOLECULAR MECHANISMS REGULATING EOSINOPHIL MIGRATION AND AIRWAYS HYPERREACTIVITY IN MICE

By Ming Yang

Division of Molecular Bioscience
The John Curtin School of Medical Research
Australian National University

A thesis submitted for a PhD degree of
Australian National University

February 2003
STATEMENT OF AUTHORSHIP

I, Ming Yang declare that all of the investigations presented within this thesis are my own original work.

Ming Yang

Division of Molecular Bioscience
The John Curtin School of Medical Research
Australian National University

January 2003
Many people deserve great thanks during my Ph.D. I wish to thank Dr. Paul S. Foster very much, not only as my supervisor but also for being a good colleague and friend, who gave me so much support and assistance, and enlightened me with great ideas. Many thanks to my colleagues in the laboratory, Aulikki, Jason, Joerg, Kris, Luby andYeping for the great assistance. I also thank Dr’s Simon P. Hogan, Klaus I. Matthaei, Surendran Mahalingam, Dianne C. Webb and Professor Ian G. Young for their great input and assistance in this Thesis.

Thank you very much also to Dr. Debra Donaldson (Genetics Institute, Cambridge, MA), Dr. Bill W. Cowden (John Curtin School of Medical Research, Australian National University, Canberra, Australia) and Professor Marc E. Rothenberg (Children’s Hospital Medical Center, Cincinnati, OH) for the necessary reagents.

Also to Geoff Osborne, Sabine Gruniger, Ann Prins, Wayne Damevski and all the staff of animal service for their technical and animal support.

Finally, to my dear wife Jing for her support throughout my Ph.D, and I dedicate this thesis to my lovely son, Xinyuan Yang. I hope Xinyuan will be a great rugby player.
ABSTRACT

Asthma is recognized as a chronic inflammatory disease of the airways that is characterized by reversible airways obstruction in association with mucus hypersecretion, increased serum IgE, eosinophilia, remodeling of the airway wall and enhanced airways reactivity to inhaled non-specific spasmogens (Kay 1991; Bochner et al., 1994; Holgate 1997; Wills-Karp 1999). It is these pathogenic processes that are thought to promote airways obstruction in asthma, which predisposes to wheezing, shortness of breath and life-threatening limitations in airflow. Although the aetiology and inflammatory response are complex, aberrant CD4+ T helper 2 (Th2 cell) lymphocye responses to common environmental stimuli are thought to play a central role in the development and exacerbation of disease through the secretion of a wide range of cytokines (Bochner et al., 1994; Wills-Karp 1999). Indeed, the hallmark features of allergic asthma have all been linked to the effector functions of Th2 cytokines (e.g. interleukin [IL] -4, -5, -9, -10 and -13) and Th2 cells are obligatory for the development and expression of disease (Wills-Karp 1999). IL-4 plays a key role in Th2 cell development and IgE production (Finkelman et al., 1988; Corry et al., 1996; Cohn et al., 1997). IL-5 is an important regulator of eosinophil function (development, activation, migration and survival), cooperates with chemokines (such as eotaxin-1) to regulate migration of this leukocyte, and is a critical molecular switch for the induction of blood and tissue eosinophilia (Sur et al., 1995; Foster et al., 1996; Kopf et al., 1996; Hamelmann et al., 1997; Mould et al., 1997). Notably, IL-13 is a potent regulator of airways hyperreactivity [AHR], mucus hypersecretion and eotaxin-1 production (Grunig et al., 1998; Wills-Karp et al., 1998). Although extensive experimental and clinical studies have identified roles for Th2 cells, eosinophils and Th2 cytokines in pathogenic processes, the interplay between these cells and molecules for the development of disease needs to be further explored.

In this thesis, IL-5- and eotaxin-1- deficient BALB/c mice and mice deficient in both factors (IL-5/eotaxin-1) were employed to elucidate the role of these two molecules in the induction of AHR and eosinophilia in a mouse model of asthma (ovalbumin [OVA] sensitization model). IL-5 was shown to be crucial for the development of eosinophilia in the bone marrow and blood during allergic inflammation of the lung. However, eosinophil migration into the allergic lung occurred independently of IL-5. By contrast, eotaxin-1 played a key role in the recruitment of eosinophils into the lung, and this
processes occurred independently of IL-5 and the induction of a peripheral blood eosinophilia during allergic inflammation. In the absence of both IL-5 and eotaxin-1 eosinophil recruitment to the blood and lung tissue was abolished, highlighting the interplay between both systems for the regulation of eosinophil migration from the bone marrow to the inflamed airways. Only in mice deficient in both IL-5 and eotaxin-1 was AHR abolished. Investigation of Th2 cells derived from mice deficient in both IL-5 and eotaxin-1 identified an intrinsic defect in the production of cytokines from this cell. In particular, these Th2 cells were limited in their ability to produce IL-13, a key regulator of AHR. These investigations highlight a dynamic interplay between Th2 cells and eosinophil regulatory molecules for the regulation of allergic inflammation and the induction of AHR. They also indicate that the recruitment of eosinophils into tissues can occur independently of IL-5 and a pronounced blood eosinophilia during allergic inflammation, which has implications for therapeutic approaches to the treatment of asthma and allergic disorders.

Eotaxin-2, a member of the eotaxin family of CC chemokines shares similar biological function to eotaxin-1 by activating the CC chemokine receptor [CCR] 3. However, eotaxin-2's relationship with IL-5 in the development of disease processes associated with allergic inflammation remains unknown. Administration of recombinant eotaxin-2 to the airways of IL-5 transgenic [Tg] mice resulted in asthma-like features, including pulmonary eosinophilia, mucus hypersecretion and AHR. Eotaxin-2 induced AHR correlated with the enhanced production of pulmonary IL-13. Administration of eotaxin-2 (to the lung) and IL-5 (to the blood and lung) induced AHR, increased the numbers of eosinophils in the blood and lung, and promoted IL-13 production in the airways of wild type [WT] BALB/c mice. Intraperitoneal injection of anti-CCR3 receptor or anti-CD4 monoclonal antibodies [mAb] completely inhibited the development of these pathological responses. Eotaxin-2- and IL-5- induced AHR did not occur in IL-4Rα- or signal-transducer-and-activator-of-transcription [STAT] 6-deficient mice suggesting that enhanced reactivity was dependent on IL-13. In IL-5Tg/STAT6-deficient mice, eotaxin-2 treatment did not induce AHR or mucus hypersecretion in respiratory epithelium although enhanced levels of eosinophils and IL-13 were still observed in the lungs. By contrast to eotaxin-1, eotaxin-2 did not increase levels of circulating eosinophils when injected intravenously. Eotaxin-1 was also more potent at inducing eosinophil recruitment to lung tissue by comparison to eotaxin-2 but both molecules in association with IL-5 induced AHR. Collectively, these
data highlight the importance of the IL-4Rα/STAT6 pathway, downstream of eosinophils and IL-13, for the development of AHR and the central role of IL-5 in conjunction with eotaxin family members for the orchestration of eosinophilia and AHR.

To further investigate how IL-13 regulates AHR this cytokine was delivered to the airways of WT BALB/c mice. IL-13 induced asthma-like features in the lung including pulmonary eosinophilia, mucus hypersecretion and AHR. IL-13 induced AHR occurred in two phases; a pre-inflammatory phase [PIP] and an inflammatory phase [IP]. The PIP response peaked at 24 h and was disassociated from eosinophilia and mucus hypersecretion, whilst the IP response peaked at 48 h and was accompanied by a marked pulmonary eosinophilia and mucus hypersecretion. IL-13 induced responses occurred independently of endogenous IL-4/IL-13 and persisted (inflammation and mucus production but not AHR) for up to 8 days. Notably, IL-13 increased the number of resident pulmonary macrophages during both the PIP and PI phase suggesting a link to the induction of AHR through this cell. Examination of pulmonary mRNA expression after IL-13 administration showed that a range of inflammatory molecules were differentially regulated. The expression of eotaxin-1 and eotaxin-2 were dramatically increased, while the expression of thymus-expressed-chemokine [TECK], thymus-and-activation-regulated-chemokine [TARC], macrophage-derived-chemokine [MDC], monocyte-chemotactic-protein [MCP] (MCP-1, -2 and -3), macrophage-inflammatory-protein [MIP] (MIP-1α, -1β, -2 and -3α), matrix-metalloprotease [MMP]-12 and -13, mucin [Muc] -2 and -5AC were differentially effected. These molecules are reported to play important roles in the recruitment of pulmonary inflammatory cells, mucus hypersecretion and airways remodeling during allergic inflammation, and highlight the potency of IL-13 for inducing inflammatory cascades.

To further investigate how IL-13 promoted eosinophilia, AHR and mucus hypersecretion IL-13 was administered to a range of factor deficient mice and mice treated with pharmacological agents. IL-13 regulates physiological functions by binding to the IL-4Rα/IL-13Rα1 complex and thus, activates STAT6. IL-13 did not induce eosinophilia, AHR or mucus hypersecretion during the PIP and IP in IL-4Rα- or STAT6- deficient mice. Administration of IL-13 to Swiss nude mice (deficient in T cell populations) and those treated with the mast cell stabilizer, disodium chromoglycate [DSCG], did not significantly affect the development of AHR, mucus hypersecretion or
eosinophilia. Nitric oxide [NO] has been shown to be important for the regulation of smooth muscle constriction, and IL-13 may regulate the production of this molecule by macrophages (and macrophage numbers are increased by IL-13 treatment). Examination of pulmonary mRNA expression of molecules involved in NO-metabolism showed enhanced levels of arginase I but not arginase II, inducible nitric oxide synthase [iNOS], ornithine aminotransferase [OAT] or ornithine decarboxylase [ODC] in response to IL-13 administration to the lung. Increased levels of pulmonary arginase I mRNA directly correlated with the induction of IL-13 induced AHR. Arginase I mRNA levels were elevated during the PIP and IP but returned to normal by day 8 where AHR but not inflammation and mucus production, were absent. Activity of lung arginase I was also increased after IL-13 administration by greater production of urea in pulmonary abstract. L-arginine is the substrate of both arginase I and iNOS. Administration of L-arginine into IL-13 treated WT mice significantly enhanced IL-13 induced AHR. By contrast, supplementation of NO with the NO donor, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene [HOBAT], completely inhibited AHR but did not effect mucus hypersecretion or pulmonary eosinophilia in response to IL-13 stimulation. Moreover, IL-13 did not induce expression of lung arginase I mRNA in STAT6-deficient mice during the PIP response. Collectively, these data suggest that arginase I and NO in the lung play important roles in IL-13 induced AHR. Furthermore, IL-13 induced responses are critically dependent on the IL-4Rα and STAT6 signal transduction pathway.

There are increasing data that suggest that activation of STAT6 is important in the development of asthma by playing a pivotal role in Th2 regulated processes. To further investigate the relevance of responses observed with IL-13 to mechanisms underlying the development of allergic disease, STAT6-deficient mice were sensitized to OVA. OVA/OVA treated STAT6-deficient mice did not development pulmonary eosinophilia, mucus hypersecretion or AHR. Examination of the expression of pulmonary chemokines showed that eotaxin-1 and eotaxin-2 were absent in the lung after OVA/OVA treatment of STAT6-deficient mice and arginase I expression was also dramatically inhibited. However, expression of iNOS in the lung of OVA/OVA treated STAT6-deficient mice was not suppressed as seen in OVA/OVA treated WT mice. Moreover, the levels of serum NO metabolites were significantly higher in STAT6-deficient mice than in WT mice. Furthermore, inhibiting NO production by NG-methyl-L-arginine [L-NMA] in naïve STAT6-deficient mice restored basal airways reactivity similar to that of naïve WT mice. These data suggest important roles of arginase I and
NO in the pathogenesis of AHR in the allergic lung. Notably, a significant eosinophilia developed in the bone marrow from OVA/OVA treated STAT6-deficient mice, and the levels of circulating eosinophils in these mice were higher than that observed in OVA/OVA treated WT mice. This may reflect the absence of chemokine signals (that may be regulated by Th2 cells) in the inflamed lung of STAT6-deficient mice that results in the accumulation of eosinophils in the circulation. Neutralization of IL-5 by mAb treatment abolished eosinophilia in both bone marrow and blood compartment. However, depletion of CD4+ and CD8+ T-cells with mAbs did not reduce eosinophil numbers in either the bone marrow or blood. This suggests that other inflammatory cells (e.g. NK cells) also participate in the development of eosinophilia in bone marrow or blood by producing IL-5 and, thus this process is not exclusively controlled by Th2 cells.

In summary, IL-5 cooperatively interacts with the CCR3-binding chemokines, eotaxin-1 eotaxin-2, to regulate eosinophil migration and the induction of AHR. However, eosinophils accumulate in tissues independently of either chemokines during allergic inflammation. Notably, eotaxin-1 can regulate the tissue accumulation of eosinophils in the absence of a blood eosinophilia. By regulating eosinophilia, IL-5 and eotaxin also promote IL-13 production in the lung. Notably, during allergic inflammation, in the absence of IL-5 and eotaxin-1, the production of IL-13 from Th2 cells is significantly impaired and this correlated with the inability of these mice to generate AHR in response to allergen challenge. IL-13 regulates AHR, eosinophilia and mucus hypersecretion through IL-4Rα/STAT6 pathways and potentially through arginase I activity.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Statement of authorship</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Table of contents</td>
<td>v</td>
</tr>
<tr>
<td>List of figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>vii</td>
</tr>
</tbody>
</table>

## Chapter 1 General introduction

### 1.1 Allergic inflammation and asthma pathogenesis

### 1.2 Inflammatory cells involved in pathogenesis of allergic diseases

#### 1.2.1 Eosinophils

1.2.1.1 General characteristics of eosinophils

1.2.1.2 Granular proteins

1.2.1.2.1 MBP

1.2.1.2.2 ECP

1.2.1.2.3 EDN

1.2.1.2.4 EPO

1.2.1.3 Eosinophils and allergy

1.2.1.4 Eosinophils as antigen-presenting cells [APC]

#### 1.2.2 T lymphocytes

1.2.2.1 CD4⁺ Th2 cells

1.2.2.2 Regulation of Th2 cell development

1.2.2.3 Transcriptional regulation of CD4⁺ T-cell differentiation

#### 1.2.3 Neutrophils

#### 1.2.4 Mast cells and basophils

#### 1.2.5 Macrophages

#### 1.2.6 Epithelial cells

#### 1.2.7 Smooth muscle

### 1.3 Inflammatory and proinflammatory factors

#### 1.3.1 IL-4

#### 1.3.2 IL-13

1.3.2.1 IL-13 receptors

#### 1.3.3 IL-5

#### 1.3.4 Other cytokines related to T helper lymphocyte responses

ix
1.3.4.1 IL-6  
1.3.4.2 IL-9  
1.3.4.3 IL-11  
1.3.4.4 IL-12  
1.3.4.5 IL-18  
1.3.4.6 IFN-γ  
1.3.4.7 TNF-α  

1.4. Chemokines

1.4.1 Eotaxins
   1.4.1.1 Eotaxin-1  
   1.4.1.2 Eotaxin-2  
   1.4.1.3 Eotaxin-3  

1.4.2 MCP-1, -2, -3, -4 and -5  

1.4.3 MIP-1α, -1β, -2 and -3α  

1.4.4 Other chemokines
   1.4.4.1 RANTES  
   1.4.4.2 MDC  
   1.4.4.3 TARC  
   1.4.4.4 TECK  

1.5 Summary and aims of this thesis  

Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

2.1 Introduction  

2.2 Materials and methods

2.2.1 Genetically manipulated mice  
2.2.2 Ovalbumin [OVA] treatment of mice  
2.2.3 Measurement of airways reactivity to methacholine  
2.2.4 Characterization of lung morphology  
2.2.5 Collection and analysis of peripheral blood, bone marrow and BALF  
2.2.6 Isolation of CD4+ T-cells from naive spleens  
2.2.7 Generation of Th2 cells from purified naive CD4+ T-cells  
2.2.7 Analysis of cytokines by ELISA  
2.2.8 Statistical analysis  

2.3 Results

2.3.1 Cooperation between IL-5 and eotaxin-1 for the regulation AHR  
2.3.2 Effect of IL-5 and eotaxin-1 on peripheral blood eosinophilia  
2.3.3 BALF eosinophilia is dependent on IL-5 and eotaxin-1  
2.3.4 Lung tissue eosinophilia is predominantly dependent on IL-5 and eotaxin-1  
2.3.5 Role of IL-5 and eotaxin-1 in the regulation eosinophilia in the bone
marrow
2.3.6 Role of IL-5 and/or eotaxin-1 in the regulation of CD4⁺ T-cell numbers in the spleen
2.3.7 Role of IL-5 and eotaxin-1 in cytokine-secretion by CD4⁺ Th2 cells

2.4 Discussion

Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

3.1 Introduction

3.2 Materials and methods

3.2.1 Mice
3.2.2 Induction of allergic phenotype by eotaxin-2
3.2.3 mAb depletion of CD4⁺ cells
  3.2.3.1 Generation of neutralizing antibodies
  3.2.3.2 Quantitation of ascites fluid
  3.2.3.3 Depletion of CD4 and CCR3 positive cells
3.2.4 Measurement of airways reactivity to methacholine
3.2.5 EPO activity assay
3.2.6 Measurement of IL-13 in BALF
3.2.7 Determination of eosinophil and mucus cell numbers in lung tissue
3.2.8 Comparison between eotaxin-1 and eotaxin-2 induced allergic phenotype
  3.2.8.1 Induction of peripheral blood eosinophilia
  3.2.8.2 Induction of AHR and pulmonary eosinophilia
3.2.9 Statistical analysis

3.3 Results

3.3.1 Characterization of eotaxin-2 induced peripheral blood and pulmonary eosinophilia in WT and IL-5 Tg mice
3.3.2 Characterization of the effects of eotaxin-2 on airways reactivity in WT and IL-5 Tg mice
3.3.3 Role of peripheral blood eosinophilia in the onset of eotaxin-2 induced pulmonary eosinophilia and AHR
3.3.4 Eotaxin-2 promotes the upregulation of pulmonary IL-13 levels and mucus secretion in IL-5 Tg mice
3.3.5 Role of IL-4Rα, STAT-6, IL-13 and eosinophil regulatory molecules in the induction of eotaxin-2 mediated pulmonary eosinophilia, AHR and increased IL-13 levels
3.3.6 Role of CD4⁺ T-cells and CCR3 receptor in the onset of eotaxin-2 induced pulmonary eosinophilia and AHR
  3.3.6.1 Role of CD4⁺ T-cells
  3.3.6.2 Role of CCR3 receptor
3.3.7 Eotaxin-2 induced pulmonary eosinophilia and IL-13 production but not mucus hypersecretion or AHR in IL-5Tg/STAT6-deficient mice
3.3.8 Comparison of the potency of eotaxin-1 and eotaxin-2 for the induction of AHR, pulmonary eosinophilia and IL-13 production in the BALF

xi
tissue
5.2.5 Drug treatment
5.2.6 Measurement of airways reactivity to different spasmogens
5.2.7 Treatment with NO-modulating agents
5.2.8 RT-PCR analysis
5.2.9 Role of L-arginine in IL-13 induced AHR
5.2.10 Arginase activity assay
5.2.10 Statistical analysis

5.3 Results

5.3.1 Airways response to different spasmogens in IL-13 treated WT mice
5.3.2 Characterization of responses to IL-13 challenge in Swiss nude mice
5.3.3 Effect of DSCG on IL-13 induced AHR
5.3.4 Role of IL-4Rα and STAT6 in the onset of IL-13 induced AHR
5.3.5 Role of IL-4Rα and STAT6 in the induction of eosinophil recruitment and mucus hypersecretion induced by IL-13
5.3.6 Treatment with 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (HOBAT) completely abolishes IL-13 induced AHR but not pulmonary eosinophilia and mucus hypersecretion in WT mice
5.3.7 IL-13 induced expression of arginase I in lungs is dependent on STAT6
5.3.8 L-arginine supplementation enhances IL-13 induced AHR

5.4 Discussion

Chapter 6 Peripheral eosinophilia but not arginase I or AHR is induced in the absence of STAT6

6.1 Introduction

6.2 Materials and methods

6.2.1 Mice
6.2.2 OVA treatment of mice
6.2.3 Measurement of airways reactivity to methacholine
6.2.4 Characterization of lung morphology
6.2.5 Collection and analysis of peripheral blood, bone marrow and BALF
6.2.6 Depletion of CD4⁺, CD8⁺ cells and IL-5
   6.2.6.1 Generation of neutralizing antibodies
   6.2.6.2 Procedure for the depletion of CD4⁺- and CD8⁺- cells and IL-5
6.2.7 RT-PCR analysis
6.2.8 Assay of serum reactive nitrogen intermediate (RNI; nitrite and nitrate)
6.2.9 Treatment of with N⁶-methyl-L-arginine [L-NMA]
6.2.10 Statistical analysis

6.3 Results

6.3.1 Characterization of eosinophil expansion in the bone marrow, peripheral blood and BALF of STAT6-deficient mice
6.3.2 Eosinophil numbers and mucus production in the lungs of STAT6-deficient mice
6.3.3 mRNA expression of chemokines in the lungs of STAT6-deficient mice
6.3.4 AHR and serum RNI levels in STAT6-deficient mice
6.3.5 mRNA expression of NO-associated enzymes in the lungs of STAT6-deficient mice
6.3.6 Administration of L-NMA increased the basal airways reactivity of STAT6-deficient mice
6.3.7 Role of IL-5, CD4 and CD8 positive cells in eosinophil accumulation in peripheral blood and bone marrow of STAT6-deficient mice

6.4 Discussion

Chapter 7: General discussion and summary

7.1 Pathophysiology of asthma
7.2 Interplay between IL-5 and eotaxin-1, eotaxin-2 for the regulation of eosinophilia, mucus secretion and AHR
7.3 IL-13 induced pulmonary eosinophilia and two phases of AHR
7.4 Supplementation of NO completely inhibits IL-13 induced AHR
7.5 Role of STAT6 in a mouse model of allergic airways disease
7.6 Conclusion

References
LIST OF FIGURES

Chapter 2

| Figure 2.3.1 | The development of AHR in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice | 43 |
| Figure 2.3.2 | Eosinophil levels in the peripheral blood of WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice | 45 |
| Figure 2.3.3 | BALF leukocytes in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice | 48 |
| Figure 2.3.4 | Eosinophil numbers in lung tissue from OVA/OVA treated WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice | 50 |
| Figure 2.3.5 | Eosinophil levels in the bone marrow of WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice | 52 |
| Figure 2.3.6 | CD4⁺ T-cells in spleen of WT and IL-5 Tg mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice | 54 |
| Figure 2.3.7 | Intrinsic defect in IL-4 and IL-13 production by naïve splenic CD4⁺ Th2 cells in the absence of IL-5 and eotaxin-1 | 57 |

Chapter 3

| Figure 3.3.1 | Effect of eotaxin-2 on the levels of eosinophils in the peripheral blood, BALF and pulmonary tissue and on lung EPO activity in WT and IL-5 Tg mice | 72 |
| Figure 3.3.2 | Airways reactivity to methacholine in eotaxin-2 treated WT and IL-5 Tg mice | 74 |
| Figure 3.3.3 | Role of IL-5 in eotaxin-2 mediated pulmonary eosinophilia and AHR in WT and IL-5 Tg mice | 76 |
| Figure 3.3.4 | Effect of eotaxin-2 on IL-13 levels in BALF and on airway epithelial mucus secretion in WT and IL-5 Tg mice | 79 |
| Figure 3.3.5 | Effect of eotaxin-2 on the development of AHR in WT mice and IL-4Rα-, IL-13-, STAT6- and IL-5/eotaxin-1- deficient mice | 82 |
| Figure 3.3.6 | Effect of eotaxin-2 on eosinophil accumulation and IL-13 levels in BALF of WT mice and IL-4Rα-, IL-13-, STAT6-, and IL-5/eotaxin-1- deficient mice | 84 |
| Figure 3.3.7 | Role of CD4 and CCR3 positive cells in airways reactivity induced by eotaxin-2 | 86 |
| Figure 3.3.8 | Role of CD4 and CCR3 positive cells in the production of BALF IL-13 and induction of pulmonary and peripheral blood eosinophilia by eotaxin-2 | 87 |
| Figure 3.3.9 | Eotaxin-2 induces pulmonary eosinophilia and IL-13 production but not mucus hypersecretion or AHR in IL-5 Tg/STAT6-deficient mice | 90 |
| Figure 3.3.10.1 | Comparison of the effect of eotaxin-1 and eotaxin-2 on the levels of peripheral blood eosinophils | 93 |
| Figure 3.3.10.2 | Comparison of the effect between eotaxin-1 and eotaxin-2 on airways reactivity | 94 |
| Figure 3.3.10.3 | Histological features of lung from eotaxin-1, eotaxin-2 and vehicle treated IL-5 Tg mice | 95 |
| Figure 3.3.10.4 | Comparison of the effect between eotaxin-1 and eotaxin-2 on | 97 |
Chapter 4

Figure 4.3.1  Characterization of the temporal development of IL-13 induced AHR 111
Figure 4.3.2a  Histological features of WT lungs exposed to IL-13 113
Figure 4.3.2b  Kinetic characterization of IL-13 induced eosinophil infiltration 115
Figure 4.3.3a  Mucus hypersecretion in the lungs of IL-13 treated WT mice 116
Figure 4.3.3b  Kinetic characterization of IL-13 induced mucus hypersecretion 118
Figure 4.3.4  Characterization of leukocyte numbers in the peripheral blood and BALF of IL-13 and PBS treated mice 120
Figure 4.3.5  Effect of IL-13 on respiratory chemokine mRNA expression 123
Figure 4.3.6  IL-13 induced expression of respiratory matrix proteinase and mucin genes 125
Figure 4.3.7  IL-13 induced AHR in IL-4/IL-13-deficient mice 128
Figure 4.3.8  IL-13 induced eosinophil accumulation and mucus hypersecretion in IL-4/IL-13-deficient mice 130
Figure 4.3.9  IL-13 induced AHR in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice 132
Figure 4.3.10 IL-13 induced eosinophil accumulation and mucus secretion in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice at 48 h 135

Chapter 5

Figure 5.3.1  IL-13 induced airways responsiveness to spasmogens 148
Figure 5.3.2  IL-13 induced AHR in Swiss nude mice 149
Figure 5.3.3  IL-13 induced eosinophil accumulation and mucus secretion in Swiss nude mice 151
Figure 5.3.4  Onset of IL-13 induced AHR in WT mice treated with disodium cromoglycate (DSCG) 154
Figure 5.3.5  IL-13 induced AHR in WT mice and IL-4Rα- and STAT6-deficient mice 155
Figure 5.3.6  IL-13 induced eosinophil accumulation and mucus secretion in WT mice and IL-4Rα- and STAT6- deficient mice 157
Figure 5.3.7  Supplementation of NO abolished IL-13 induced AHR but not pulmonary eosinophil accumulation and mucus secretion in WT mice 159
Figure 5.3.8  Effect of IL-13 on mRNA expression of NO-associated respiratory enzyme 162
Figure 5.3.9  IL-13 induced arginase I expression is dependent on STAT6 163
Figure 5.3.10 L-arginine supplementation promotes IL-13 induced AHR 165

Chapter 6

Figure 6.3.1  Eosinophilia in BALF, peripheral blood and bone marrow in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice 179
Figure 6.3.2a Histological features of eosinophilia in the lungs of SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice

Figure 6.3.2b Mucus-staining-cell number in the lungs of SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice

Figure 6.3.3 Characterization of the numbers of eosinophils and mucus-staining cells in lung tissue in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice

Figure 6.3.4 Respiratory chemokine mRNA expression in lungs of SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice

Figure 6.3.5 Characterization of airways reactivity and serum RNI in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice

Figure 6.3.6 NO-associated respiratory enzyme mRNA expression in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice

Figure 6.3.7 L-NMA treatment enhanced basal airways reactivity of STAT6-deficient mice

Figure 6.3.8 Role of CD4⁺, CD8⁺ cells and IL-5 in the regulation of eosinophil numbers in the bone marrow and peripheral blood of OVA/OVA treated STAT6-deficient mice
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>airways hyperreactivity</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AP</td>
<td>activator protein</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ASM</td>
<td>airways smooth muscle</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DSCG</td>
<td>disodium cromoglycate</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
</tr>
<tr>
<td>EDX</td>
<td>eosinophil-derived neurotoxin</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-like immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>EPX</td>
<td>eosinophil protein X</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>hank's balanced salt solution</td>
</tr>
<tr>
<td>HOBAT</td>
<td>1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene</td>
</tr>
<tr>
<td>HPF</td>
<td>high-powered field</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IP</td>
<td>inflammatory phase</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>i.t.</td>
<td>intratracheal</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Jak</td>
<td>janus kinase</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>L-NMA</td>
<td>N(^6)-methyl-L-arginine</td>
</tr>
<tr>
<td>LTB(_4)</td>
<td>leukotriene B(_4)</td>
</tr>
<tr>
<td>LTC(_4)</td>
<td>leukotriene C(_4)</td>
</tr>
<tr>
<td>LTD(_4)</td>
<td>leukotriene D(_4)</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MBP</td>
<td>major basic protein</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemotactic protein</td>
</tr>
<tr>
<td>MDC</td>
<td>macrophage derived chemokine</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MLC</td>
<td>murine lymphocyte culture medium</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NF</td>
<td>nuclear factor</td>
</tr>
<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
</tbody>
</table>
OAT    ornithine aminotransferase
ODC    ornithine decarboxylase
OVA    ovalbumin
PAF    platelet activating factor
PB     peripheral blood
PBS    phosphate buffered saline
PE     phycoerythrin
Penh   Enhanced pause
PIP    pre-inflammatory phase
RANTES Regulated on activation, normal T-cell expressed and secreted
RNI    reactive nitrogen intermediate
RT-PCR reverse transcriptase-polymerase chain reaction
SCF    stem cell factor
SEM    standard error of the mean
STAT   signal transducer and activator of transcription
TARC   thymus and activation-regulated chemokine
TECK   thymus expressed chemokine
TGF    transforming growth factor
Th     T helper
Th2 cells T helper 2 cells
TIMP   tissue specific inhibitors of metalloproteinases
Tg     transgenic
TNF    tumor necrosis factor
TRF    T-cell replacing factor
TSM    tracheal smooth muscle cells
Tyk    tyrosine kinase
VCAM   vascular cell adhesion molecule
WT     wild type
-/-     deficient
CHAPTER 1

General introduction
1.1 Allergic inflammation and asthma pathogenesis

Asthma is a chronic inflammatory disorder of the lung and is clinically characterized by wheezing, coughing, chest tightness and difficulty in breathing that can be life threatening or even fatal (Nissler et al., 1970; Aubier 1995; Clark et al., 1999; Sistek et al., 2001). The prevalence of asthma in the western world has doubled in the last 20 years affecting 1 in 4 children in Australia, and 20% to 30% of children under 16 years old suffer at sometime from this condition (Seaton et al., 1994; Woolcock et al., 2001).

The hallmark clinical features of asthma are airways inflammation, airways obstruction, mucus hypersecretion, enhanced serum immunoglobulin [Ig] E and increased bronchial reactivity to spasmogens (airways hyperreactivity [AHR]) (Kay 1991; Bochner et al., 1994; Holgate 1997; Wills-Karp 1999). The infiltration and activation of CD4+ T helper 2 lymphocytes (Th2 cells), eosinophils, bronchoaveolar macrophages and mast cells are key features of the inflammatory response and these cells are thought to play critical roles in the pathogenesis of asthma (Djukanovic et al., 1990; Robinson et al., 1992; Robinson et al., 1993; Bochner et al., 1994; Seminario et al., 1994; Wills-Karp 1999).

1.2 Inflammatory cells involved in pathogenesis of allergic diseases

1.2.1 Eosinophils

The eosinophil has been identified as an important effector cell in asthma and allergic disorders (Gleich 1986). Investigators have found that eosinophils mediate inflammatory processes and cytotoxic events that are associated with allergic diseases such as asthma, rhinitis and urticaria (Gleich et al., 1993; Kroegel et al., 1994). Eosinophil accumulation in the lung and blood are hallmark features of both allergic and nonallergic asthma (Fukuda 1993; Venge 1994; Hamelmann et al., 2001). Clinically, the presence, number and activation status of eosinophils have been shown to strongly correlate with disease severity (Bousquet et al., 1990; Skedinger et al., 1995). At sites of allergic disease eosinophils become activated, displaying enhanced surface expression of receptors, increased production of membrane lipids and intracellular cytokines and the release of highly toxic granular proteins (Tsuda et al., 1993; Kroegel et al., 1994; Bandeira-Melo et al., 2002). In vitro, eosinophil granular proteins have been shown to be toxic to helminths and mammalian cells (e.g. human
respiratory epithelial cells) (Frigas et al., 1986). Eosinophil cationic proteins [ECP], eosinophil peroxidase [EPO], major basic protein [MBP], eosinophil protein X [EPX] and eosinophil-derived neurotoxin [EDN] are key constituents of eosinophil granules with enzymatic activity (Motojima et al., 1989; Hakansson et al., 1990; Venge et al., 1991; Zimmerman 1994). The release of these granules during allergic inflammation is thought to be a key mechanism that underpins damage to lung tissue such as respiratory epithelium desquamation (Frigas et al., 1986; Frigas et al., 1991; Hirata et al., 1996).

Notably, sputum samples from asthmatic patients contain high levels of MBP (Frigas et al., 1981). Furthermore, instillation of MBP into the lungs of monkeys induces AHR to methacholine (Gleich et al., 1993), which correlates with damage to the airway epithelium (Beasley et al., 1989).

1.2.1.1 General characteristics of eosinophils

Eosinophils have been found in many mammalian and nonmammalian species and are derived from bone marrow progenitor cells (Wendler 1968). Under healthy conditions the eosinophil has a bi-lobed nucleus that is filled with partially condensed chromatin, however the number of lobes can increase to four or more in some disorders (Kroegel et al., 1992; Hamanaka et al., 1993; Stockert et al., 1993). Eosinopoeisis occurs predominantly in the bone marrow but maturation can occur in the spleen, thymus and in lymph nodes (Parish et al., 1977). Bone marrow consists of approximately 3% eosinophils of which 37% are mature nondividing granulocytes, and the remainder is a mixture of promyelocytes/myelocytes (37%) and metamyelocytes (26%) (McEwen 1992). At any given time, approximately 16% of myelocytes are estimated to be undergoing S-phase, which lasts for approximately 12 hours. Eosinophils are generated from small populations of self-regenerating, hematopoietic stem cells. However, it is still not clear how the progenitor cells differentiate into mature eosinophils and what regulates their subsequent survival and expansion in the bone marrow and in tissue. A number of cytokines have been shown to play key roles in eosinophil maturation.

Interleukin [IL] -3, IL-5, IL-6, IL-11, IL-12, granulocyte colony-stimulating factor [G-CSF], stem cell factor [SCF], granulocyte and monocyte colony-stimulating factor [GM-CSF] and leukemia inhibitory factor [LIF] have been linked to regulating eosinophil development and/or survival (Wiktor-Jedrzejczak 1993; Yamaguchi 1998;
Robinson et al., 1999). Notably, of these cytokines IL-5 is the most selective factor for regulating growth, migration and survival of eosinophils (Ohnishi et al., 1993; Bozza et al., 1994). Importantly, IL-5 provides a critical signal for eosinophil expansion in the bone marrow and subsequent migration into the blood in response to allergic inflammatory stimuli and some parasite infections (el-Cheikh et al., 1991; Kopf et al., 1996; Foster et al., 2001). IL-5 plays a key role in the migration of eosinophils by regulating the expression of adhesion molecules on the surface of the cell and by modulating β2 integrin function (Walsh et al., 1990; Resnick et al., 1993; Palframan et al., 1998). The functional significance of these integrins for eosinophil migration has been demonstrated by using monoclonal antibodies [mAb] against β2 and α4 integrins (Palframan et al., 1998). Blockade of β2 integrin function suppresses eosinophil mobilization induced by IL-5, while attenuation of α4 integrin increases eosinophil release in response to IL-5 (Hamann et al., 1996).

Mature eosinophils and progenitors are found in the circulation. The eosinophil has a half-life of approximate 18 hours and a transit mean time of 26 hours once it has appeared in the circulation, moreover, the half-life is prolonged when cytokines, such as IL-5, are increased in the circulation (Wiktor-Jedrzejczak 1993; Yamaguchi 1998; Robinson et al., 1999). Eosinophils account for approximately 2 to 10% of the peripheral leukocyte population in most mammal and once eosinophils enter tissue it is thought that they do not re-enter the circulation (Wiktor-Jedrzejczak 1993; Yamaguchi 1998). Eosinophils primarily reside in the gastrointestinal tract, lung, skin and uterus (Frigas et al., 1986; Gaga et al., 1991; Mishra et al., 1999; Rothenberg et al., 2001).

1.2.1.2 Granular proteins

1.2.1.2.1 MBP: MBP, a 13.8 kDa polypeptide, accounts for approximately 50% of the granule protein in guinea pig eosinophils (Gleich et al., 1973). MBP is a strong cytotoxin and highly toxic to helminths and some bacteria (Wassom et al., 1979; Lehrer et al., 1989). MBP binds to anionic domains on target cells and parasites that result in increasing the membrane permeability leading to cell death (Wasmoen et al., 1988). MBP has also been shown to promote Ca\(^{2+}\) mobilization in cultured bovine tracheal smooth muscle cells [TSM], and augment TSM contractility to acetylcholine (Wylam et al., 1998). The effect of MBP on smooth muscle may be one way this protein promotes AHR. Smooth muscle contraction is known to be regulated by muscarinic receptors
(M2 and M3) and parasympathetic nerves. (Yost et al., 1999; Jacoby et al., 2001). Interestingly, MBP acts as an antagonist of the neuronal M2 muscarinic receptor resulting in an increase in the release of acetylcholine (Evans et al., 1997), and thus enhanced contractility of smooth muscle. Virus induced AHR and M2 muscarinic receptor dysfunction was inhibited by neutralizing antibody to MBP in a guinea pig model of asthma (Adamko et al., 1999). Notably, sputum samples from asthmatic patients contain high levels of MBP (Frigas et al., 1981). Furthermore, instillation of MBP into the lungs of monkeys induced AHR to methacholine (Gleich et al., 1993).

1.2.1.2.2 ECP: ECP, is a member of a family of RNase A multigenes and has a molecular weight between 16-21.4 kDa (Kalayci et al., 2000). Two forms of eosinophil cationic protein, ECP-1 and ECP-2, have been identified immunologically (Gleich et al., 1986). ECP-1 was found expressed in the granules of resting eosinophils (EG1+ cells), and ECP-2 was found in the granules of activated eosinophils (EG2+ cells). The individual roles of ECP-1 and ECP-2 remain unknown. ECP also possess other characteristics: toxicity to bacteria and helminths and this protein promotes degranulation of mast cells (Barker et al., 1989; Rosenberg 1995). The cytotoxicity of ECP is not dependent on its RNase activity but on creating channels that transverse the plasma membrane of target cells (Young et al., 1986).

1.2.1.2.3 EDN: EDN is another eosinophilic granular protein, which belongs to the RNase A multigene family (Rosenberg et al., 1989; Domachowske et al., 1998). Basophils and neutrophils may also secret EDN (Abu-Ghazaleh et al., 1992). The RNase activity of EDN is approximately 125-fold higher than that of ECP (Gullberg et al., 1986). EDN also induces neurotoxicity and causes the Gordon phenomenon (a syndrome manifested by ataxia, muscular rigidity, paralysis, and tremor that may lead to death), when injected intraventricularly into guinea pigs or rabbits (Fredens et al., 1985; Newton et al., 1994).

1.2.1.2.4 EPO: EPO catalyzes the peroxidative oxidation of halides, which are localized to the matrix of the eosinophil’s secondary granules (Zabucchi et al., 1990; Sanz et al., 1997; Krug et al., 1999). EPO accounts for approximately 5% of the total granule protein. EPO contains two subunits, a heavy chain of 50-57 kDa and a light chain of 11-15 kDa. EPO converts leukotriene C4 [LTC4] to all-trans isomers of leukotriene B4 [LTB4], and inactivates peptido-leukotrienes (Henderson et al., 1982). In the presence
of peroxide and bromide, EPO can catalyze the formation of hypobromous acid and highly reactive singlet oxygen (Slungaard et al., 1991). EPO also possesses the capacity to kill bacteria (Hirai 1997).

1.2.1.3 Eosinophils and allergy

Marked eosinophil infiltration into the lung is a prominent feature of asthma (Frigas et al., 1986; Irie et al., 1990; Cui et al., 1997). Clinically, bronchial eosinophilia is a hallmark feature of allergic and nonallergic asthmatics (Godard et al., 1982; Frigas et al., 1991; Frangova et al., 1996). Asthma severity is also strongly associated with the degree of eosinophil accumulation and activation in the lung (Gleich 1990; Fujisawa et al., 1993; Venge 1994). Activated eosinophils induce disease by releasing many factors. These factors include the toxic granule proteins, oxygen free radicals, cytokines and growth factors which may enhance bronchial smooth muscle contraction and vascular permeability, damage the respiratory epithelium, promote inflammation and induce AHR (Gleich et al., 1983; Durham et al., 1989; Zimmerman 1994; Skedinger et al., 1995; Kalayci et al., 2000). Notably, eosinophil toxic granule contents (MBP, ECP, EDN) may damage the bronchial structure, inducing epithelial cell shedding, which may enhance the sensitivity of sensory neurons that regulate smooth muscle responsiveness. Elastase and metalloproteases are also released by activated eosinophils and these proteases may induce pulmonary fibrosis by stimulating fibroblasts and may contribute to airways remodeling (Riise et al., 1996; Rochester et al., 1996).

1.2.1.4 Eosinophils as antigen-presenting cells [APC]

Eosinophils possess phagocytic capacity and express various adhesion molecules, major histocompatibility complex [MHC] class I and II and T-cell costimulatory molecules (CD80 and CD86), which allow this granulocyte to present antigen and engage T-cells (Del Pozo et al., 1992). There has been emerging evidence that eosinophils may regulate T-cell function, bridging the innate and adaptive immune responses (Shi et al., 2000; MacKenzie et al., 2001). Airway eosinophils, recovered after antigen inhalation in mouse models of asthma, have been shown to stimulate antigen-specific CD4+ T-cells to proliferate within the paratracheal lymph node (Shi et al., 2000). Furthermore, antigen loaded eosinophils also enhance the ability of CD4+ Th2 cells to release IL-4,
IL-5 and IL-13 in culture and induced allergic disease of the lung after being transferred into naïve mice (MacKenzie et al., 2001).

1.2.2 T Lymphocytes

Numbers of T lymphocytes are elevated in the mucosal area of asthmatics (Corrigan et al., 1988; Harmanci et al., 1998; Kraft et al., 1999). The major infiltrating T lymphocyte are CD4+ cells, however, CD8+ cells are also found in some asthmatics (Kochman et al., 1987; Corrigan et al., 1991; Kroegel et al., 1995). The number of activated CD4+ T-cells, like eosinophils, directly correlates with the severity of asthma (Walker et al., 1991). CD4+ T-cells are thought to orchestrate the development of inflammation in asthma through the secretion of proinflammatory cytokines (Wills-Karp 1999). In response to antigen stimulation, the CD4+ T-cell population rapidly expands and promotes the recruitment of other inflammatory cells to the site of inflammation through the release of proinflammatory factors (Kay 1992; Hogan et al., 1998; Wills-Karp 1999; Lee et al., 2001). According to the profile of cytokines secreted, CD4+ T-cells can be divided into several subsets. CD4+ Th1 cells produce IL-2, interferon [IFN]-γ and tumor necrosis factor [TNF]-β, which play important roles in delayed hypersensitivity reactions and antiviral immunity; CD4+ Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 and play predominant roles in regulating allergic inflammation and IgE production (Romagnani 1991; Del Prete 1992; Maurer et al., 1993; Karupiah 1998; Rabinovitch 1998). CD4+ Th0 cells produce IL-4 and IFN-γ (Dieli et al., 2000; Marchant et al., 2001). CD4+ Th3 cells primarily produce transforming growth factor [TGF]-β with various amounts of IL-4 and IL-10 (Weiner 2001; Giomi et al., 2002). CD4+ Th3 cells are thought to promote the production of IgA and suppress the development of Th1 and Th2 cell responses. In particular, CD4+ Th2 cells and their cytokines are thought to orchestrate the development of atopic diseases such as asthma, eczema and allergic rhinitis. The role of CD4+ Th2 cells in asthma and allergic diseases has been elucidated from experiment models in animals and allergen challenge studies in the human (Robinson et al., 1992; Kay 1996; Benson et al., 2001; Lordan et al., 2002; Smart et al., 2002).
1.2.2.1 CD4⁺ Th2 cells

CD4⁺ Th cells and their cytokines play key roles in the development of allergic and non-allergic asthma (Corrigan et al., 1995; Ying et al., 1995; Cohn et al., 1999). In response to allergen challenge, increased numbers of CD4⁺ Th cells and their secreted cytokines are found in the asthmatic lung (Cohn et al., 1999; Magnan et al., 2000). CD4⁺ Th2 cell cytokines (e.g. IL-4, IL-5 and IL-13) have been detected in asthmatic bronchoalveolar lavage fluid (BALF), airway biopsies and serum (Ying et al., 1995; Cohn et al., 1999; Lee et al., 2001). These cytokines may regulate asthma by modulating specific immune and physiological responses (Mazzarella et al., 2000; Maeda et al., 2001). IL-5 plays an important role in eosinophil differentiation and maturation (Foster 1999). IL-13 (with IL-4) regulates B-cell IgE production, primes smooth muscle for enhanced airways responsiveness to cholinergic stimuli and regulates the production of mucus (de Vries 1998; Corry 1999). IL-4 drives CD4⁺ Th cell differentiation, growth and IgE production by B cells (Savelkoul et al., 1996; Asselin et al., 1997).

Notably, in mouse models of asthma, CD4⁺ Th2 cells and not CD4⁺ Th1 cells, have been shown to play a critical role in the induction of airways inflammation, AHR, eosinophilia, mucus hypersecretion and IgE production (Hansen et al., 1999; Li et al., 1999; Randolph et al., 1999; Mathew et al., 2001). The selective overexpression of Th2 cell cytokines (IL-4, IL-5, IL-9 and IL-13) in transgenic [Tg] mice induces many of the immune and pathogenic features common to asthma (pulmonary eosinophilia, mucus overproduction, collagen deposition in the airways, mucus cell hyperplasia and metaplasia, enhanced IgE production and AHR) (Anderson et al., 1994; Cohn et al., 1999; Zhu et al., 1999; Fallon et al., 2001; Kishikawa et al., 2001; Temann et al., 2002). Collectively, these studies have supported the paradigm that Th2 cells and Th2 cell cytokines are sufficient to induce asthma-like inflammation and pathologic changes. However, it is likely that asthma results from a complex interplay between many cells. Eosinophils, mast cells, CD8⁺ T-cells and γδ T-cells have all been implicated in the development of allergic airways disease in animal models (Kung et al., 1995; Erb et al., 1996; Hogan et al., 1997; Zuany-Amorim et al., 1998).
1.2.2.2 Regulation of Th2 cell development

Dendritic cells have been shown to promote the polarization of Th2 cells in the airways (Kapsenberg et al., 1999; Foucras et al., 2000; Liu et al., 2000). Dendritic cells take up and process inhaled antigens and migrate to local draining lymph nodes where they present the processed antigen to naïve (and memory) CD4+ T-cells. Polarization to the Th1 or Th2 phenotype may be influenced by the interaction of costimulatory molecules and MHC class II with their ligands on the cell surface. Studies have shown that CD40-CD40L and CD80/CD86-CTLA-4/CD28 interactions are important in the generation of Th cells and recall Th responses (Kapsenberg et al., 1999; Hammad et al., 2001). However, signals exhibited by specific cytokines play a central role in influencing naïve CD4+ T-cells to the Th1 or Th2 cell phenotype (Mazzarella et al., 2000; Moser et al., 2000; Romagnani 2000). In the presence of IL-12, Th1 cells develop when they are activated by APC. By contrast, the Th2 cells develop in the presence of IL-4 and APC.

1.2.2.3 Transcriptional regulation of CD4+ T-cell differentiation

Development of the Th1 or Th2 cell subpopulation is ultimately controlled at the level of cytokine gene transcription. In particular, the signal-transducer-and-activator-of-transcription [STAT] 6 protein has been shown to play an important role in Th differentiation (Minty et al., 1997; Oki et al., 2000; Zhu et al., 2001). The binding of IL-4 and IL-13 to their receptors induces phosphorylation of STAT6 by janus kinases [Jak] -1 and -2. Jak-1 and Jak-2 belong to a unique class of tyrosine kinases that associate with cytokine receptors (Heim 1999). Studies in IL-4 knockout mice have revealed that IL-13 itself can stimulate the phosphorylation of STAT6 (Herrick et al., 2000). However, IL-13 does not induce CD4+ Th2 cell polarization as this cell lacks the IL-13 receptor complex (Minty et al., 1997). In murine models of asthma models, STAT6 deficient mice have marked attenuation of pulmonary eosinophilia, mucus production, AHR and serum IgE levels in response to antigen challenge (Kuperman et al., 1998).

The transcription factor GATA-3 has also been identified as a Th2 cell differentiation factor and a hematopoietic factor (Manaia et al., 2000; Das et al., 2001). The expression of GATA-3 is dramatically increased in cells differentiating to the Th2 cell lineage and decreased in that of the Th1 phenotype (Zheng et al., 1997). Naïve CD4+ T-cells
express low levels of GATA-3 mRNA (Zheng et al., 1997). Interestingly, GATA-3 binding sites have been identified in the promoter of the IL-5 gene suggesting that GATA-3 plays an important role in Th2 cell cytokine gene expression (Siegel et al., 1995). GATA-3 also binds to several regions within the IL-4/IL-13 locus including the IL-4 promoter region (Ranganath et al., 1998; Lee et al., 2001; Lavenu-Bombled et al., 2002). Overexpression of GATA-3 in Th1 cells resulted in a significant suppression of the expression of the IL-12 receptor β2 subunit and of IFN-γ production (Nawijn et al., 2001). Increased expression of GATA-3 in the lungs of asthmatics or in the allergic airways of mice is associated with increased production of IL-5, elevated numbers of eosinophils and the development of AHR (Nakamura et al., 1999; Finotto et al., 2001; Justice et al., 2002).

Nuclear factor of activated T-cells [NF-AT] and c-Maf are other nuclear transcription factors that are also thought to be important in the regulation of Th2 gene expression. Studies with c-Maf deficient mice showed that this transcription factor played a critical role in IL-4 gene expression (Ho et al., 1996). c-Maf deficient mice have impaired IL-4 production in CD4+ T-cells but normal production of IL-5 and IL-13 (Kim et al., 1999). NF-AT exists in both Th1 and Th2 cells but has been demonstrated to directly promote IL-4 gene expression (Rincon et al., 1997). NF-ATc deficient mice have a pronounced defect in IL-4 production (Glimcher et al., 1999). Other transcription factors such as nuclear factor [NF] -κB and activator protein [AP] -1 are also important for Th2 cell cytokine production (Das et al., 2001; Yamazaki et al., 2002). These investigations may indicate that Th2 cell-specific transcription factors, such as GATA-3 and c-Maf, are critical for tissue specific cytokine production, while other transcription factors (NF-ATc, AP-1 and NF-κB) are necessary for the optimal expression of Th2 cell cytokine genes (Barnes et al., 1998).

Transcriptional repressor BCL-6 has been identified as a suppressor of Th2 cell responses (Dent et al., 1997; Ye et al., 1997). BCL-6 deficient mice showed enhanced IgE levels, eosinophil infiltration and inflammatory responses in animal models of allergic disease (Dent et al., 1999; Harris et al., 1999). It still remains unclear as to whether BCL-6 suppresses Th2 cell responses by inhibiting the STAT6 binding site (Harris et al., 1999). Collectively, these studies show that Th2 cell responses are mediated by a network of transcription factors.
1.2.3 Neutrophils

Neutrophils are found in increased numbers in the sputum of asthmatics, although levels of this cell are in low in the BALF and in bronchial biopsies (Jeffery et al., 1989; Bradley et al., 1991; Lacoste et al., 1993). In lung samples taken from patients with fatal asthma, nocturnal asthma and individuals with long-term asthma, increased levels of neutrophils are often observed (Martin et al., 1991; Carroll et al., 1996). The role of neutrophils in the pathogenesis of asthma is unclear. Several studies have suggested that neutrophils may regulate the development of grain-dust induced bronchoconstriction (Park et al., 1998; Jung et al., 1999). Immunochemical data obtained from bronchial mucosal biopsies taken from patients 24 hours after challenge with grain-dust showed that the levels of mast cells and neutrophils are significantly higher than that observed in allergic asthma (Busse 1993). These investigations suggest that activated neutrophils may regulate asthma development and aspects of pathogenesis under some conditions.

1.2.4 Mast cells and basophils

Like eosinophils, mast cells are also thought to play an important role in asthma (Bradley et al., 1991). Mast cells are thought to induce AHR and pathogenic features of asthma by releasing granular proteins, proinflammation molecules and spasmogens (Bingham et al., 2000). Mast cell activation is thought to play a key role in the early asthmatic response and provide factors that regulate the late-phase response (Hart 2001). Interestingly, mast cells may also release factors that may have anti-inflammatory effects such as heparin and heparan sulfate, which have the capability to modulate tissue homeostasis and wound healing (Bowler et al., 1993; Diamant et al., 1996).

Basophils, like their tissue counterpart mast cells, are also thought to play a critical role in the development of allergic asthma (Kimura et al., 1968). Basophils are very rare in both blood and tissue (KleinJan et al., 2000), however, significant numbers of basophils are recruited to regions around the bronchial mucosa in response to allergen provocation in allergic asthmatic patients (Nouri-Aria et al., 2001). After allergen challenge, basophils are recruited to the sites of inflammation in allergic patients as early as 1 hour and levels peak by 24 hours and this cell may persist in tissues for up to a week (Ying et al., 1999; KleinJan et al., 2000).
Mast cells and basophils are also rich cellular sources of inflammatory mediators such as cytokines (e.g. IL-4 and IL-13), leukotrienes (e.g. LTC₄ and LTD₄), histamine and eicosanoids (Drazen et al., 1987; Plaut et al., 1989; Shichijo et al., 1997; Schroeder et al., 2001; Weiss et al., 2001). IgE, influenza A virus and eotaxin-1 stimulate basophils to release histamine and cytokines (e.g. IL-4) (Gaddy et al., 1986; Clementsen et al., 1988; Devouassoux et al., 1999). Inhaled LTC₄ and LTD₄ induce potent bronchoconstriction in both normal and asthmatic subjects (Adelroth et al., 1986). Thus, mast cells and basophils may participate in the pathogenesis of asthma and importantly regulate Th2 cell responses.

1.2.5 Macrophages

Like many inflammatory cells increased activation and infiltration of macrophages have been shown to directly correlate with the severity of asthma (Gant et al., 1992; Lane et al., 1994). Inhalation of antigen induces alveolar macrophage activation, which subsequently may regulate airways inflammation through the release of factors such as eicosanoids, platelet-activating factor [PAF], cytokines, oxygen free radicals and mucus secreagogues (Lane et al., 1994; Mugnai et al., 1997). Investigations on alveolar macrophages suggest that they may be responsible for airways remodeling through the secretion of various cytokines and growth factors (e.g. platelet-derived growth factor, basic fibroblast growth factor or TGF-β) that promote pulmonary fibrosis (Gibson et al., 1998; Magnan et al., 1998; Alexis et al., 2001).

1.2.6 Epithelial cells

Airway epithelial cell shedding is a predominant feature of asthma (Chiappara et al., 2001). These cells may also actively participate in the asthmatic response and disease progression through the secretion of a wide range of proinflammatory mediators after being activated by various stimuli (Mogel et al., 1998; Fox et al., 2001; Rodgers et al., 2002). Several molecules released by leukocytes residing in the lung may mediate damage to the epithelium. Proteases (e.g. collagenase, elastase and cathepsin) and oxidants (e.g. H₂O₂) have been shown to play a critical role in mediating epithelial cell detachment (Mendis et al., 1990). Bronchial epithelial cells also release a wide range proinflammatory molecules after stimulation by IgE (Tomee et al., 1998; Cook et al., 2001), viruses (Becker et al., 1992; Noah et al., 1993; Olszewska-Pazdrak et al., 1998;
Seo et al., 2001), air borne pollutants (Devalia et al., 1993; Van Winkle et al., 1997; Hong et al., 2001), cytokines (Levine 1995; Bianco et al., 1998; Li et al., 1999) and various proinflammatory mediators (Harris et al., 1993; Yanni et al., 1999) and may act in concert with other cells to induce disease.

In asthma, bronchial epithelial cells actively express many mediators, including cytokines (e.g. IL-6, IL-8 and GM-CSF) (Noah et al., 1993), chemokines (e.g. eotaxin-1, RANTES and MCP-1) (Li et al., 1999; Momoi et al., 1999), growth factors (e.g. TGF-α and -β) (Korfhagen et al., 1994; Kumar et al., 1996), extracellular matrix protein (e.g. plasminogen activator inhibitor-1 and fibronectin) (Jakowlew et al., 1997) and matrix metalloproteases [MMP] (e.g. MMP-2 and MMP-9) (Yao et al., 1998; Legrand et al., 1999; Kunugi et al., 2001), which induce airways inflammation, remodeling and obstruction. Adhesion molecules (Oertli et al., 1998; O'Brien et al., 1999), endothelin-1 (Deprez-Roy et al., 2000; Samransamruajkit et al., 2000) and nitric oxide synthase (Inaba et al., 1984) are also expressed at greater levels in bronchial epithelial cells in response to antigen/pathogen challenge. Bronchial epithelial cells may also take an active role in airways remodeling processes by releasing fibronectin, insulin growth factor and TGF-β, which regulate fibroblast proliferation and collagen deposition (Kim et al., 1997; Parekh et al., 1998; Hastie et al., 2002). The cytokines derived from bronchial epithelial cells may also promote the recruitment, activation and survival of inflammatory cells in the lung. Thus, although bronchial epithelial cell shedding is an important characteristic of asthma, this cell may also actively contribute to pathogenic processes through the secretion of proinflammatory mediators that activate many cellular responses.

1.2.7 Smooth muscle

Abnormal contractile responses of airway smooth muscle (ASM) has traditionally been thought to play a central role in the pathogenesis of asthma (Johnson et al., 1997). Hyperplasia and/or hypertrophy of ASM is a predominant feature of airways remodeling in asthma, which may underpin altered lung function (Seow et al., 1998; Martin et al., 2000). Thickening of the ASM band is an important structural abnormality that is thought to be responsible for excessive airway lumen narrowing (Black et al., 1996). Recent data also indicate that ASM is highly responsive to many inflammatory
mediators, growth factors and extracellular matrix components (Belvisi et al., 1998; Foda et al., 1999; Coutts et al., 2001; Moore et al., 2002).

Several studies on isolated ASM indicate that these cells can secrete proinflammatory factors (Johnson et al., 1997). Co-culturing ASM with cytokines (e.g. IL-1, TGF-β1 and TNF-α) resulted in enhanced production and release of chemokines (e.g. RANTES, eotaxin-1 and IL-18) (Elias et al., 1997; John et al., 1997; Ghaffar et al., 1999; Ammit et al., 2000; Pang et al., 2001), which may induce recruitment and activation of inflammatory cells. ASM cells have increased expression of adhesion molecules in response to various inflammatory stimuli, which could promote direct interaction with leukocytes (Barks et al., 1997; Lavie et al., 1999). Thus, ASM may actively contribute to inflammatory cascades in asthma, which subsequently effects the contractile response.

1.3 Inflammatory and proinflammatory factors

1.3.1 IL-4

IL-4, a 15-kDa protein with pleiotropic effects on many other cell-types, has an important role in immune responses (Paul 1997). The results from experimental models consistently show that CD4+ Th2 cells are very important cellular sources of IL-4 in vivo in particular under allergic inflammatory conditions (Mosmann et al., 1986; Demeure et al., 1995). IL-4 is also produced from other T-cell subpopulations (e.g. CD4+ NK1.1, CD4+ Mel-14low memory T-cells, γδ T-cells and a low level from naïve T-cells) but to a much lesser extent (Bradley et al., 1991; Flesch et al., 1997; Gerber et al., 1999; Yagi et al., 2002). Mast cells, basophils and eosinophils are also able to produce IL-4 after appropriate activation (Moqbel et al., 1995; Bjerke et al., 1996; Ricci et al., 1997; Ying et al., 1997). In one study, 83% of eosinophils in skin biopsies taken from atopic subjects produced IL-4 (Moqbel et al., 1995). Interestingly, IL-5 may promote biosynthesis and/or storage of IL-4 in eosinophils (Nakajima et al., 1996). IL-4 predominantly binds to the IL-4 receptor complex (Nelms et al., 1999). The IL-4 receptor consists of two subunits, an α subunit which IL-4 binds with high affinity and which is able to induce signal transduction, and a common γc subunit that is also found in the IL-2 receptor, which may be responsible for amplifying signal transduction (Galizzi et al., 1990; Russell et al., 1993). IL-4 receptors are expressed on
hematopoietic cells and non-hematopoietic cells, including of T-cells, B-cells, epithelial cells, endothelial cells, liver cells and fibroblasts (Ohara et al., 1987; Lowenthal et al., 1988; Murata et al., 1998). In particular, IL-4 drives CD4\(^+\) T-cell differentiation to the Th2 cell subtype (Ricci et al., 1997). Indeed, IL-4 is very important in antigen-specific IgE production (Finkelman et al., 1988; Pene et al., 1988).

Clinical studies have shown enhanced expression of IL-4 mRNA in peripheral blood lymphocytes and in sputum taken from asthmatic patients (Borger et al., 1999; Olivenstein et al., 1999). In IL-4 deficient mice mature CD4\(^+\) T-cells failed to express normal levels of Th2 cell cytokines after \textit{in vitro} stimulation (Kanagawa et al., 1993). Studies with IL-4 Tg mice have shown that overexpression of IL-4 in the lung induces elevated serum IgE and pulmonary inflammatory and histopathologic features similar to that observed in asthma (Tepper et al., 1990). Furthermore, antigen-specific IgE was only produced in IL-4 deficient mice when reconstituted with IL-4-producing T-cells but not IL-4-producing non-T-cells (Schmitz et al., 1994). Thus, T-cell derived IL-4 plays an important role in the induction of CD4\(^+\) Th2 cell responses, which orchestrate the pathogenic features of allergic disease.

1.3.2 IL-13

IL-13 is an important Th2 cell cytokine, which is able to induce proinflammatory and anti-inflammatory immune responses (Tanaka et al., 1996). IL-13 is a member of the short chain cytokine family, which includes IL-2, IL-3, IL-4 and GM-CSF (Corry 1999). CD4\(^+\) Th2 cells orchestrate allergic responses through the secretion of a range of cytokines and these cells have been identified as the predominant cellular sources of IL-13 (Corry 1999; Bourreau et al., 2001). Other T-cell populations including NK T-cells, NK cells, macrophages and mast cells/basophils have been shown to express IL-13 (Huang et al., 1995; Hancock et al., 1998; Johansson et al., 2000).

IL-13 shares 25% homology with IL-4 and possesses two of the three disulfides which are found in IL-4 (McKenzie et al., 1993). The structure of IL-13 suggests that IL-13 has similar biological effects to that of IL-4 (Lefort et al., 1995). Indeed, many of the overlapping biological effects of IL-4 and IL-13, such as the regulation of antibody production and inflammatory processes, have been explained by the utilisation of a common receptor pathway (Corry 1999). However, IL-13 and IL-4 also possess
independent biological functions. IL-4 plays a more important role in the development of CD4+ Th2 cells and in isotype switching in B cells (Cohn et al., 1997). By contrast, IL-13 by signalling through the IL-4Rα has been shown to be important in the induction of AHR and mucus hypersecretion (Gavett et al., 1997; Grunig et al., 1998). The inability of IL-13 to directly regulate T-cell function, by contrast to IL-4, is due to restricted expression of the IL-13Rα1 chain (Murata et al., 1998).

The expression of IL-13 is increased in the serum and peripheral blood tissue lesions and airways of patients with bronchial asthma (Izuhara 2001; Lee et al., 2001), atopic dermatitis (Katagiri et al., 1997; Takamatsu et al., 1998), allergic rhinitis (Li et al., 1998) and allergic conjunctivitis (Yokota et al., 1998). BALF recovered from asthmatic and rhinitic patients showed a significant increase in the level of both IL-13 mRNA and protein (Huang et al., 1995; Jaffar et al., 1999). Studies in vitro have revealed that IL-13 is sufficient to enhance IgE secretion from cultured human but not murine B-cells (Van der Pouw Kraan et al., 1998). Results from antigen sensitized IL-13-deficient mice show that IgE responses are reduced compared to normal mice (McKenzie et al., 1998). Studies with mice deficient in IL-13 have also shown that these mice are not able to clear helminth infections or induce mucus overproduction in goblet cells (Barner et al., 1998; Urban et al., 1998). Repeated administration of recombinant IL-13 into the airways of naïve mice was sufficient to induce IgE production, airways inflammation, mucus production and AHR (Grunig et al., 1998; Walter et al., 2001). Overexpression of IL-13 in mice results in significantly increased IgE levels in the serum (Fallon et al., 2001), and this effect is independent of IL-4. IL-13 also induces synthesis of proinflammatory cytokines (e.g. IL-6 and GM-CSF) (Doucet et al., 1998; Frost et al., 2001), regulates the production of collagen, the expression of α1 integrins and vascular cell adhesion molecule-1 [VCAM-1] (Doucet et al., 1998; Bessis et al., 1999), and the secretion of chemokines (Marfaing-Koka et al., 1995; Jordan et al., 1997; Li et al., 1999; Zhu et al., 2002). All of these molecules are thought to play important roles in the allergic inflammatory response. Overexpression of IL-13 in the lung results in the induction of fibrosis, airways remodeling, mucus hypersecretion, airways inflammation and AHR (Emson et al., 1998). Collectively, these data indicate that IL-13 may induce many of the airways abnormalities seen in asthma.
1.3.2.1 IL-13 receptors

IL-13 potentially elicits its biological action through two receptor subunits (IL-13Rα1 and IL-13Rα2) that are expressed on target cells (Murata et al., 1997; Donaldson et al., 1998). These receptor subunits are expressed in both the mouse and humans (de Vries 1998). IL-13 can modulate macrophage function in culture (Doherty et al., 1993). Investigations have shown by immunohistochemistry that IL-13Rα1 is expressed on CD38⁺ B-cells, and also on monocytes (Graber et al., 1998). CD4⁺ T-cells and CD8⁺ T-cells express IL-13Rα1 mRNA and release a soluble form of IL-13Rα1, however, this subunit is not found on the membrane of T-cells (Graber et al., 1998). Investigations have shown that fibroblasts, “mast-like cells”, submucosal glands in the nasal mucosa and ciliated respiratory epithelial cells express these IL-13 receptor units (Akaiwa et al., 2001). IL-13 utilizes both the IL-13Rα1- and the IL-4Rα -subunit to transduce intracellular signals (Hart et al., 1999; Osawa et al., 2000; Schmidt-Weber et al., 2000). These receptor subunits associate with tyrosin kinases and janus kinases (Malabarba et al., 1996; Orchansky et al., 1997). The phosphorylation of these kinases is important in the activation of STAT6 (Wang et al., 1995; Wurster et al., 2000). STAT6 is a critical intracellular molecule for IL-4 and IL-13 to transduce their intracellular signals (Hart et al., 1999; Yoshidome et al., 1999; Schmidt-Weber et al., 2000). IL-13Rα2 subunit has a short cytoplasmic domain and is thus unlikely to transduce intracellular signals (Wu et al., 2002). The biological function of IL-13Rα2 subunit remains unclear, however, this protein may act as a decoy receptor to capture IL-13 (Donaldson et al., 1998). Furthermore, IL-13Rα2 was not required for IL-13-induced signal transduction by the STAT6 pathway (Kawakami et al., 2001).

1.3.3 IL-5

IL-5 was first shown to induce differentiation of B-cells into immunoglobulin-secreting cells, and was originally named as T-cell-replacing factor [TRF] (Takatsu et al., 1988). Activated CD4⁺ T-cells are a predominant source of IL-5, however mast cells and eosinophils also produce this cytokine (Lalani et al., 1999). The IL-5 receptor consists of a βc subunit and an α subunit (Adachi et al., 1998). Although the βc subunit does not have the capacity to bind the ligand, phosphorylation of the βc subunit is critical for signal transduction that regulates the viability, proliferation and survival of cells.
(Yoshimura et al., 1996; Ogata et al., 1997; Caldenhoven et al., 1999; Bates et al., 2000) and is also important for membrane ruffling, oxidative burst and glucose transport (Sato et al., 1994; Bracke et al., 2000). The IL-5 receptor is expressed predominantly on eosinophils, basophils and B-cells (Katoh et al., 1992; Takatsu 1995), and not found on neutrophils and monocytes (Kotsimbos et al., 1997). CD34+ eosinophil progenitors express the IL-5 receptor and these cells are found in increased numbers in bronchial biopsies from asthmatic patients (Robinson et al., 1999).

Murine IL-5 has been shown to promote B-cell growth, differentiation and immunoglobulin production (Takatsu et al., 1987). The effect of human IL-5 on B-cell function is controversial (Takatsu et al., 1994). In particular, the effect of IL-5 on eosinophils has been extensively investigated (Walsh et al., 1990; Chihara et al., 1993; Mould et al., 2000; O'Byrne et al., 2001). IL-5 plays an important role in the terminal differentiation of eosinophils and in the activation and survival of mature eosinophils (Lopez et al., 1992; Miyajima 1992; Goodall et al., 1993). Indeed of all the cytokines so far described, IL-5 is the most eosinophil-specific factor. IL-5 inhibits apoptosis, promotes degranulation, production of leukotrienes and superoxides, stimulates cytokine synthesis, up-regulates adhesion molecules and promotes cytotoxicity, chemotaxis and expression of MHC II molecules on eosinophils (Fabian et al., 1992; Stern et al., 1992; Carlson et al., 1993; Takatsu 1993; Zangrilli et al., 1995; Lalani et al., 1999; Weltman et al., 2000; Maeda et al., 2001). IL-5 also stimulates basophils to release inflammatory mediators such as histamine and leukotrienes (Sarmiento et al., 1995). Furthermore, IL-5 plays a critical role in the expansion of the eosinophil pool in both bone marrow and blood in response to allergic stimuli and in concert with the CCR3 signaling system to induce airways inflammation and AHR (Collins et al., 1995; Mould et al., 1997; Foster et al., 2001).

Both IL-5 protein and mRNA have been found increased in the lungs of asthmatics (Mori et al., 1996). Levels of serum IL-5 correlate with the severity of asthma (Ikeda et al., 1997). Studies with IL-5 deficient mice have shown that these mice do not generate a pronounced eosinophilia in response to inflammatory stimuli even though there is basal production of eosinophils (Kopf et al., 1996). However, this basal level of eosinophils does not appear sufficient for normal host defense against several helminth infections (Matthaei et al., 1997). Overexpression of IL-5 in CD2+ cells results in a pronounced blood eosinophilia without a concominant increase in tissue levels of this
cell or induction of pathological responses (Dent et al., 1990). Moreover, overexpression of IL-5 in the lung result in many of the pathological features observed during allergic inflammation (Lee et al., 1997). Investigations show that IL-5 in concert with eotaxin-1 regulates cytokine production (in particular IL-13) from CD4+ Th2 cells (Mattes et al., 2002), and this is possibly through the interaction between activated eosinophils and CD4+ T-cells (MacKenzie et al., 2001). Collectively, IL-5 regulates many aspects of allergic inflammation and host defense through a network of inflammatory cells and factors, in particular by acting on eosinophils.

1.3.4 Other cytokines related to T helper lymphocyte responses

1.3.4.1 IL-6

IL-6, originally identified as B-cell differentiation factor, binds to a receptor complex, which has α and β subunits (the latter one is responsible for signal transduction) (Liang et al., 1998). IL-6 is produced by wide range of cells (e.g. T-cells, macrophages, fibroblasts, endothelial cells, epithelial cells and bone marrow stromal cells) (Hodgkin et al., 1988; Podor et al., 1989; Mattoli et al., 1991; Lanfrancone et al., 1992; Jarvis et al., 1995; Liang et al., 1998). IL-6 regulates the function of many cell types such as hepatocytes, thymocytes, T-cells and B-cells (Ishihara et al., 2002). Studies have suggested that IL-6 plays an important role in humoral immune responses and Ig secretion by primary and secondary B-cells (Quentmeier et al., 1992). IL-6, potentially derived from APCs, has also been found to potentiate naïve CD4+ T-cells to the Th2 cell phenotype by promoting IL-4 production in CD4+ T-cells (Rincon et al., 1997). However, Th differentiation in vivo has been shown not to be directly dependent on IL-6 and this cytokine induces lymphoproliferation through the induction of IL-2 receptor expression (La Flamme et al., 1999).

Clinical investigation showed that allergic asthmatic patients have higher plasma levels of IL-6 than non-asthmatics controls (Wong et al., 2001). Depletion of IL-6 in mice results in impairment of immunoglobulin production and proliferative responses of a range of cells (e.g. CD4+ and CD8+ T-cells and B-cells) (Kopf et al., 1995; Hope et al., 2000). However, recent studies have shown that deficiency of IL-6 results in enhanced IL-4 production and reduced IFN-γ production from T-cells (Tanaka et al., 2001). Thus,
although IL-6 was shown to be a pro-inflammatory factor, its effects on immune responses needs to be further investigated.

1.3.4.2 IL-9

IL-9, a pleiotropic Th2 cell cytokine, acts as the growth factor for certain T-lymphocytes and mast cells (Van Damme et al., 1992; Houssiau et al., 1993; Stassen et al., 2001). IL-9 is predominantly produced by CD4+ Th2 cells (Schmitt et al., 1991), however other cells including naïve T-cells, eosinophils, mast cells and epithelial cells also secret this cytokine (Schmitt et al., 1994; Longphre et al., 1999; Gounni et al., 2000). The IL-9 receptor complex consists of two subunits, γc and α chain (the latter one is responsible for early T-cell development) (Kimura et al., 1995; De Smedt et al., 2000). IL-9 acts on many cells (e.g. T-cells, B-cells, mast cells, eosinophils, neutrophils and epithelial cells) (Soussi-Gounni et al., 2001).

Enhanced expression of IL-9 mRNA has been detected in the bronchial tissue of atopic asthmatic patients (Shimbara et al., 2000). Cultured human eosinophils were demonstrated to synthesize and release IL-9, which may integrate with other immune effect cells (Gounni et al., 2000). Studies with IL-9-deficient mice have shown a crucial role for this cytokine in the induction of pulmonary goblet cell hyperplasia and mastocytosis in response to antigen challenge, however, eosinophilia and granuloma formation were not effected (Townsend et al., 2000). Overexpression of IL-9 in the lung of mice results in an asthma-like response characterized by lymphocyte and eosinophil infiltration, airway epithelial cell hypertrophy with mucus overproduction and mast cell hyperplasia, which are completely blocked by the neutralization of IL-13 activity (Temann et al., 2002). This may suggest that IL-9 induces an asthma-like response by promoting other cytokines such as IL-13. The immune and pathological responses of IL-9 suggest that this cytokine may play an important role in the pathogenesis of asthma.

1.3.4.3 IL-11

IL-11 has pleiotropic actions, which stimulates bone marrow stem cells to proliferate and protects the intestinal mucosal from injury (Keith et al., 1994). In the airways, IL-11 is produced by structural cells and infiltrating eosinophils in response to stimuli (e.g.
MBP, TGF-β and virus) (Rochester et al., 1996; Tang et al., 1996; Zheng et al., 2001). The IL-11 receptor complex consists of two subunits, an α and β chain (Neddermann et al., 1996; Cork et al., 2002). IL-11 promotes antigen-specific antibody responses (Yin et al., 1993) and plays an important role in the development of disease associated with viral infections of the lung (Tang et al., 1996). Asthma patients show an increased level of IL-11 mRNA in the airways and these levels are associated with severity of disease (Minshall et al., 2000). Airway epithelial cells and MBP+ eosinophils have elevated levels of IL-11 mRNA, suggesting a role for this cytokine in the development of asthma. Furthermore, overexpression of IL-11 in the lung of mice results in nodular mononuclear infiltration, airway wall remodeling with subepithelial fibrosis, obstruction and AHR (Zheng et al., 2001). IL-11 may be an important factor in the events that regulate airways remodeling in chronic asthma.

1.3.4.4 IL-12

IL-12 operates as a growth factor for T-cells and NK cells, increases NK cell activity and enhances the expression of IFN-γ by resting human peripheral blood mononuclear cells (Schoenhaut et al., 1992). IL-12 is primarily produced by APCs (e.g. B-cells, monocytes and macrophages) (Trinchieri et al., 1995; Gately et al., 1998). The IL-12 receptor complex consists of two subunits, designated p40 and p35 (Presky et al., 1998). The IL-12 receptor is predominantly expressed on the surface of T-cells, NK cells and DC cells (Rogge et al., 1997; Yin et al., 2000; Latour et al., 2001). Intraperitoneal injection of IL-12 increases NK cell lytic activity, infiltration of CD8+ T-cells and monocytes into the liver, and enhances IFN-γ production (Gately et al., 1994). Significant tumor regression has also been observed in mice after systemic administration of IL-12 (Nastala et al., 1994). Depletion of CD4+ and CD8+ T-cell populations diminished this effect and anti-IFN-γ antibody treatment almost abolished the tumor suppression effect of IL-12 (Nastala et al., 1994). These data suggest that IL-12 stimulates CD4+ and/or CD8+ T lymphocytes to produce IFN-γ which subsequently promotes tumor regression. IL-12 is more viewed as a key promoter of Th1-driven cell immunity and a suppressor of Th2 cell-regulated response.

In a mouse model of asthma, treatment with recombinant IL-12 resulted in down-regulation of IL-4, IL-5 and IL-10 production but an increased level of IFN-γ in BALF (Sur et al., 1996). IL-12 regulated suppression of eosinophil infiltration into the allergic
Chapter 1: General introduction

lung in response to antigen inhalation is thought to be mediated by its capacity to enhance IFN-γ production and subsequently inhibition of IL-5 production (Wynn et al., 1995; Sur et al., 1996; Russell et al., 1997). IL-12 is an important modulator of Th1 mediated immune responses and IL-12 deficient mice have enhanced Th2 cell immune responses (Wills-Karp 2001). However, in response to viral infection, the production of IFN-γ and IgG2a were unimpaired when these responses were compared between IL-12-deficient and WT mice (Xing et al., 2000). Furthermore, depletion of IL-18 with mAb inhibited the release of IFN-γ and the infiltration of lymphocytes into the lung (Xing et al., 2000). Overall, IL-12 is recognised as an important regulator of Th1 responses by enhancing the production of IFN-γ. IL-18 may be required to optimize IL-12 regulated antiviral immune response by promoting the release of IFN-γ.

1.3.4.5 IL-18

IL-18, an 18kDa protein, induces the production of IFN-γ, which acts as a growth and differentiation factor for Th1 cells (Kawakami et al., 1997). IL-18 has been initially detected in epidermal cells, activated macrophages and mononuclear phagocytes (Stoll et al., 1997; Kashiwamura et al., 1998; Gerdes et al., 2002). Endothelial cells, smooth muscle cells and mononuclear phagocytes all express the IL-18R α and β chains (Gerdes et al., 2002). T-cells, B-cells and NK cells respond to IL-18 stimulation suggesting they also express IL-18 receptors (Yoshimoto et al., 1998; Sarneva et al., 2000). IL-18 also induces eotaxin-1 expression in bronchial epithelial cells and macrophages (Campbell et al., 2000). IL-18 can induce the production of a range of factors such as cytokines (e.g. IL-6 and IL-13), chemokines (IL-8), intracellular adhesion molecules (ICAM)-1, and MMPs indicating an important role for this cytokine in the early stage of immune responses (Gerdes et al., 2002).

The level of IL-18 increases in asthmatic patients and correlated with the increased levels of IFN-γ (Tanaka et al., 2001). In IL-18-deficient mice IFN-γ production is markedly reduced and NK cell activity and Th1 responses are significantly attenuated (Takeda et al., 1998). IL-18-deficient mice also have impaired antibacterial host responses (Lauw et al., 2002). Recombinant IL-18 instilled into the lungs induces peribronchial eosinophil accumulation (Campbell et al., 2000). Surprisingly, administration of IL-18 and ragweed to naïve mice resulted increased total serum IgE
Chapter 1: General introduction

level, Th2 cell cytokine production, eosinophil accumulation and mucus hypersecretion (Wild et al., 2000). Thus, IL-18 may have a critical role in the pathogenesis of asthma by promoting Th2 cell responses, and amplify many immune responses through the production of cytokines, chemokines and other proinflammatory response molecules.

1.3.4.6 IFN-γ

IFN-γ, a pleiotropic cytokine, plays key roles in host defense against a range of pathogens (Boehm et al., 1997). The predominant cellular sources of IFN-γ are activated NK cells, Th1 cells and CD8⁺ cytotoxic cells and endothelial cells (Lee et al., 2000; Wei et al., 2000; Derby et al., 2001; Thornton et al., 2001). NK cells are very important cellular sources of IFN-γ (Perussia 1996). The IFN-γ receptor is a single membrane crossing protein, which binds IFN-γ with high affinity and induces activation of STAT1 (Sedo et al., 1996; Bach et al., 1997). The IFN-γ receptor is predominantly expressed on T-cells, B-cells, dendritic cells, macrophages, epithelial cells and endothelial cells (Bach et al., 1997).

IFN-γ plays a pivotal role in the development of Th1 responses, is also a predominant cytokine secreted by Th1 cells and is associated with cell-mediated immunity (Bradley et al., 1996; Chen et al., 2001). IFN-γ is thought to enhance Th1 differentiation by inducing the expression of the IL-12 receptor on naïve T-cells (Smeltz et al., 2002). IFN-γ upregulates the expression of MHC class I and II molecules (Lombard-Platet et al., 1997; Tsuyuki et al., 1998; Kim et al., 2002). The co-stimulation molecules B7-1 and B7-2 are also upregulated by IFN-γ (Nikcevich et al., 1997; Banu et al., 1999). These data suggest a direct role for this cytokine in regulation of antigen presentation. IFN-γ regulates B-cell production of IgG2a and IgG3, which is enhanced by IL-1 and IL-2 (Snapper et al., 1996). IFN-γ is also a strong antagonist of IL-4-mediated IgE production, while IL-4 inhibits IgG2a production (Hasbold et al., 1999; Christie et al., 2000). The antagonistic actions between IFN-γ and IL-4 may arise from direct competition between IFN-γ-activated STAT1 and IL-4-activated STAT6 for binding to common DNA transcription elements (Ohmori et al., 1997). Furthermore, a study shows that up-regulation of silencer of cytokine signaling 1 [SOCS-1] by IFN-γ also contributes to the inhibition IL-4 induced STAT6 activation (Venkataraman et al., 1999).
Data from asthmatics has shown reduced production of IFN-\(\gamma\) in the airways (Koning et al., 1997; Leonard et al., 1997). Corticosteroid treatment increases the expression of IFN-\(\gamma\) in airways of asthmatics (Bentley et al., 1996), while corticosteroid resistant patients show reduced production of IFN-\(\gamma\) (Leung et al., 1995). Administration of IFN-\(\gamma\) into airways of mice inhibited pulmonary eosinophilia and AHR in response allergen challenge (Iwamoto et al., 1993; Lack et al., 1996). Depletion of IFN-\(\gamma\) in mice prolongs airway eosinophilia (Coyle et al., 1996). Collectively, these data suggest that IFN-\(\gamma\) may play an inhibitory role in the pathogenesis of asthma.

1.3.4.7 TNF-\(\alpha\)

TNF-\(\alpha\) is a multifunctional cytokine with a wide range of proinflammatory activities, which play a critical role in innate and adaptive immune responses (van Der Veen et al., 2000). Activated macrophages are the predominant sources of TNF-\(\alpha\) (Pass et al., 1995), however, many other cells (e.g. T-cells, mast cells and epithelial cells) also produce this cytokine (Spriggs et al., 1992). Two TNF-\(\alpha\) receptor subunits, designed p55 and p75, have been identified (Nophar et al., 1990). Stimulation of the TNF-\(\alpha\) receptor complex induces the activation of transcription factors, NF-\(\kappa\)B and AP-1 (Baker et al., 1996; Hsu et al., 1996; Xu et al., 1997). TNF-\(\alpha\) receptors are expressed on nearly all cells but not resting T lymphocytes and red blood cells (Naismith et al., 1998; MacEwan 2002).

After allergen challenge, nasal biopsies from atopic subjects have increased levels of TNF-\(\alpha\) mRNA (Ying et al., 1991). Investigations have shown TNF-\(\alpha\) induces vasoconstriction by regulating the release of endothelin [ET] -1 (Wagner 2000). Selectively blocking the endothelin receptor, with an ET-1 antagonist, successfully attenuated TNF-\(\alpha\) induced bronchial vasoconstriction (Wagner 2000). TNF-\(\alpha\) also stimulates eotaxin-1 release from cultured human ASM cells, which can be abolished by \(\beta2\)-agonists and steroids (Pang et al., 2001). TNF-\(\alpha\) can modulate eosinophil function and adhesion molecule expression. Human eosinophils express higher levels of CD4 ligands after TNF-\(\alpha\) stimulation (Tsukadaira et al., 2001). TNF-\(\alpha\) also promotes cultured human eosinophils to release IL-9 (Gounni et al., 2000). TNF-\(\alpha\) significantly enhances ICAM-1 expression on cultured human bronchial epithelial cells (Krunkosky et al., 1996). The induction of ICAM-1 expression in response to TNF-\(\alpha\) is attenuated
by stimulation of cAMP with forskolin (Panettieri et al., 1995). In culture, TNF-α has been shown to damage the structure of human bronchial epithelial cells and increase the level of nitric oxide [NO] production and the rate of glucose oxidation (Kampf et al., 1999). TNF-α regulates inducible NO synthase [iNOS] activity as this cytokine promotes mRNA and protein production and elevated levels of NO in human smooth muscles (Lau et al., 1995; Li et al., 2000). Collectively these data suggest that TNF-α is an important immune modulator of allergic and non-allergic immune responses.

1.4. Chemokines

Chemokines are classified into two super-families, CXC (α) and CC (β) according to the proximity of the first two of four highly conserved cystein residues (Kaplan 2001). Macrophage-inflamatory-proteins [MIP] -1α, -1β and -3α (Wolpe et al., 1988; Kim et al., 1998; Czaplewski et al., 1999; Aquaro et al., 2001), monocyte-chemotactic-protein [MCP] -1, -2, -3, -4 and -5 (Garcia-Zepeda et al., 1996; Bonini et al., 1997; Sarafi et al., 1997; Van Coillie et al., 1997; Barczyk et al., 2001), macrophage-derived-chemokine [MDC] (Godiska et al., 1997), eotaxin-1, -2, -3 (Kitaura et al., 1996; Forssmann et al., 1997; Kitaura et al., 1999), regulated-on-activation-normal-T-cell-expressed-and-secreted [RANTES] (Belperio et al., 2000), thymus-and-activation-regulated-chemokine [TARC] (Imai et al., 1997) and thymus-expressed-chemokine [TECK] (Vicari et al., 1997) belong to the CC chemokine family. These molecules have chemotactic activity on monocytes, basophils, eosinophils and some T lymphocyte subpopulations but not on neutrophils. Among these molecules, MCPs, eotaxins and RANTES regulate eosinophil recruitment and activation. IL-8 and MIP-2 belong to the CXC family of chemokines, which can predominantly regulate migration and activation of neutrophils (Giovannelli et al., 1998). Understanding how these molecules regulate leukocyte functions will improve our understanding of the pathogenesis of atopic and non-atopic allergic disease.

1.4.1 Eotaxins

1.4.1.1 Eotaxin-1

Eotaxin-1 selectively regulates eosinophil migration both in vivo and in vitro through the CC chemokine receptor [CCR] 3 (Rothenberg et al., 1995; Gao et al., 1996; Ponath
et al., 1996). Eotaxin-1 was discovered in the BALF of allergen challenged guinea-pigs, and induced a selective eosinophil accumulation into the sites of administration (Griffiths-Johnson et al., 1993). The cloning of the human eotaxin-1 gene indicated an association to MCP family of genes (Ponath et al., 1996). Eotaxin-1 and MCP gene families are clustered on human chromosome 17q11 (Van Coillie et al., 1997). This region also includes of other CC chemokines (e.g. MIP-1α and RANTES) (Rothenberg et al., 1995).

Eotaxin-1 is broadly expressed in many tissues, however, mucosal tissues have the highest constitutive levels of eotaxin-1 (Pullerits et al., 2000). In the lung, eotaxin-1 mRNA is predominantly found in epithelial cells and macrophages, and to a lesser extend in T-cells and eosinophils [Lamkhioued, 1997 #350;Cook, 1998 #1505:]. Human fibroblasts and smooth muscle cells are also able to synthesis this chemokine (Ghaffar et al., 1999; Jundt et al., 1999). Eosinophils and basophils express high levels of CCR3 (Heath et al., 1997; Uguccioni et al., 1997; Grimaldi et al., 1999). When rhesus macaques were challenged with simian immunodeficiency virus, CCR3 was found to be expressed on the surface of macrophages and T-cells (Zhang et al., 1998). Cultured human Th2 cells were also found to express the CCR3 receptor, however the functional significance of this observation to Th2 cell immunity in vivo is yet to be fully explored (Gerber et al., 1997). A large proportion of CD3⁺ T-cells extracted from inflamed skin were identified as CCR3 positive and these cells co-localized with eosinophils. The CCR3⁺ T-cells were absent from tissues lacking eosinophils (Gerber et al., 1997). This may imply that there is a cooperative relationship between eosinophils and CD3⁺ T-cells, potentially with Th2 cells to regulate allergic inflammation. Additionally, CCR3 was also found to be highly expressed on neurons from the central and peripheral nervous systems of rhesus macaques. This may imply that CCR3-mediated responses extend beyond the eosinophil. However, in the mouse a specific CCR3 mAb suggests that CCR3 is primarily expressed on eosinophils, as immunohistochemistry failed to detect this receptor on stem cells, dendritic cells or cells from the thymus, lymph nodes or spleen of normal mice (Grimaldi et al., 1999). Furthermore, murine Th2 cells were found not to express CCR3 or respond to eotaxin-1 (Grimaldi et al., 1999). Eotaxin-1 promotes IL-4 production from purified antigen [Ag] -priming basophils by activating CCR3 (Devouassoux et al., 1999). Eotaxin-1 also induces actin polymerization, the release of reactive oxygen species and a transient rise of cytosolic Ca²⁺ concentration in eosinophils (Elsner et al., 1998). Actin polymerization and Ca²⁺ transients regulate cell
migration and modulate respiratory burst (Wiles et al., 1995; Bengtsson et al., 1996). Reactive oxygen species from eosinophils are thought to induce tissue damage at sites of allergic inflammation and promote parasite clearance (Elsner et al., 1996).

High concentrations of eotaxin-1 have also been found in respiratory fluids recovered from atopic asthmatic patients and elevated levels of eotaxin-1 mRNA and protein were observed in the epithelium and submucosa of their airways by contrast to control individuals (Lamkhioued et al., 1997). Eotaxin-1 is important for maintaining the basal levels of tissue eosinophils (Matthews et al., 1998). Over-expression of eotaxin-1 in the gastrointestinal tract results in a marked accumulation of eosinophils in the gastrointestinal mucosa but not in the bone marrow or peripheral blood (Mishra et al., 2002). Although depletion of eotaxin-1 partly reduces tissue eosinophilia (in particular at early stage of recruitment) in a mouse model of asthma (Rothenberg et al., 1997), a study shows that mice deficient in this chemokine do not exhibit a significant reduction in the recruitment of eosinophils to the lung after multiple antigen challenges (Yang et al., 1998). However, together with IL-5, eotaxin-1 generates fundamental signals that regulate eosinophil migration and activation and contribute to the development of AHR (Mould et al., 2000). Thus, eotaxin-1, by signalling through CCR3 and interplaying with other factors (e.g. IL-5) results in eosinophil migration and activation, which is important in allergic and parasite immune responses. Collectively, these data indicate that eotaxin-1 plays a key role in a range of eosinophil functions but may also act on other cellular systems.

1.4.1.2 Eotaxin-2

Eotaxin-2, a CC chemokine, consists of 78 amino acids that share only 39% homology with eotaxin-1 (Forssmann et al., 1997). Eotaxin-2 has also been detected in cytokeratin+ epithelial cells, CD31+ endothelial cells and CD68+ macrophages in the asthmatic lung and in the skin after allergen-induced late-phase responses of human atopic subjects (Ying et al., 1999; Ying et al., 1999). In mice, eotaxin-2 mRNA was detected in jejunum and spleen, whereas eotaxin-1 mRNA was detected more ubiquitously in the stomach, jejunum, lung and thymus (Zimmermann et al., 2000). Eotaxin-2 selectively binds to CCR3 (Forssmann et al., 1997). Antibodies raised against CCR3 inhibited eotaxin-2-induced responses, including the mobilization of Ca2+ and the release of reactive oxygen species from cultured human eosinophils (Elsner et al.,
1998). Eotaxin-2 has very similar functions to that of eotaxin-1 inducing actin polymerization, migration and activation of eosinophils (Elsner et al., 1998).

Notably, the expression of eotaxin-2 mRNA is higher than that of eotaxin-1 in human nasal polyps with a prominent eosinophilia (Jahnsen et al., 1999). Moreover, eotaxin-2 mRNA was highly induced in the lung of mice in response to both A. fumigatus and OVA sensitization and airway challenge (Zimmermann et al., 2000; Watanabe et al., 2002). Notably, eotaxin-1 was found associated with an early 6-hour phase of eosinophil infiltration and eotaxin-2 was associated with a later 24-hour phase of tissue recruitment of eosinophils (Ying et al., 1999). Therefore, although eotaxin-1 and eotaxin-2 predominantly bind to CCR3, they may induce recruitment and activation of eosinophils and other CCR3+ inflammatory cells at the different stage of allergic responses.

1.4.1.3 Eotaxin-3

Like many chemokines, eotaxin-3 was found by screening genomic DNA through sequence similarity (Kitaura et al., 1999). The mRNA for eotaxin-3 has been detected in a human vascular endothelial cell line (Shinkai et al., 1999) and in human heart and ovary (Kitaura et al., 1999). Eotaxin-3 is upregulated by both IL-4 and IL-13, but not by TNF-α, IL-1β and IFN-γ (Kitaura et al., 1999). Human endothelial cells release eotaxin-3 in response to IL-4-stimulation and this effect results in eosinophil transmigration, in vitro, which could be abolished by treatment with either anti-eotaxin-3 or anti-CCR3 (Cuvelier et al., 2001). Similar to eotaxin-1, eotaxin-3 also induces transient Ca2+ mobilization and chemotaxis of human eosinophils specifically through CCR3 (Kitaura et al., 1999). However, the potency of eotaxin-3 was found to be much less than that of eotaxin-1 (Shinkai et al., 1999).

A recent study on BALF cells and bronchial biopsies from asthmatics showed that mRNA for eotaxin-1 and eotaxin-2 was expressed at significantly higher levels than that observed in control subject after allergen challenge but levels did not further increase after 24 hours. By contrast, eotaxin-3 mRNA levels did not increase in the first 24 hours after antigen challenge but were markedly elevated thereafter (Berkman et al., 2001). Although these three chemokines bind to the same receptor, different spatial and
temporal aspects of their production influence their role in the regulation of eosinophil function and involvement in disease.

1.4.2 MCP-1, -2, -3, -4 and -5

MCPs, belong to CC chemokine family, and are pleiotropic factors that play important roles in both allergic and non-allergic inflammatory reactions (Dilloo et al., 1996). MCPs are primarily produced by monocytes and tissue macrophages, however, human epithelial cells located in small airways and eosinophils also express some family members (e.g. MCP-1) (Olszewska-Pazdrak et al., 1998; Ying et al., 1999). All MCPs bind to CCR2, while MCP-2, -3 and -4 also bind to CCR1 and 3 (Garcia-Zepeda et al., 1996; Gong et al., 1997; Fujisawa et al., 2000). MCP-3 binds CCR5 as well (Blanpain et al., 1999). Functionally, MCP-1 promotes the development of Th0 cells to Th2 cells in vitro, and indirectly contributes to IgE production through the up-regulation of IL-4 (Karpus et al., 1997). MCP-2, -3 and -4 potentially induce migration and activation of eosinophils, basophils, lymphocytes, and neutrophils (Nosó et al., 1994; Uguccioni et al., 1995; Garcia-Zepeda et al., 1996; Proost et al., 1996). MCP-3 also activates NK cells (Nosó et al., 1994) while MCP-4 and -5 regulate monocytes movement (Garcia-Zepeda et al., 1996; Sarafi et al., 1997).

Elevated mRNA levels of MCPs have been reported in a wide range of studies with asthmatic subjects (Powell et al., 1996; Humbert et al., 1997; Ying et al., 1999; Jahnz-Rozyk et al., 2000; Lamkhioued et al., 2000). Mucosal regions of allergic and non-allergic sinusitis patients have elevated levels of MCP-4 (Garcia-Zepeda et al., 1996). Thus, MCPs may widely participate in the pathogenesis of asthma by regulating a range of inflammatory cells.

1.4.3 MIP-1α, -1β, -2 and -3α

MIP-1α, MIP-1β and MIP-3α belong to the CC chemokine family (Wolpe et al., 1988; Kim et al., 1998), whilst MIP-2 is a major CXC chemokine (Wolpe et al., 1989). Monocytes and tissue macrophages are the primary source of MIPs (Driscoll 1994). Lymphocytes express both MIP-1α and -1β (Zipfel et al., 1989; Schall et al., 1992). Eosinophils and neutrophils also produce MIP-1α (Costa et al., 1993; Kasama et al., 1993). Expression of MIP-2 is strongly associated polymorphonuclear neutrophils
The major cellular sources of MIP-3α are monocytes, eosinophils, dendritic cells, epithelial cells and endothelial cells (Rossi et al., 1997; Sun et al., 2002; and neutrophils have also been reported to produce this chemokine (Sullivan et al., 1999). MIP-1α interacts with CCR1, CCR5 (Andjelkovic et al., 2000), while MIP-1β binds to CCR5 (Youn et al., 2001). MIP-2 operates with the CXC chemokine receptor (CXCR) 2 (Luan et al., 2001). CCR6 has been identified as the only receptor of MIP-3α (Liao et al., 1997). MIP-1α, -1β, -2 and -3α enhance cytotoxic T lymphocyte (CTL) and NK-mediating killing effects to target cells (Taub et al., 1995). MIP-1α is reported to suppress hematopoiesis colony formation (Gao et al., 1997). MIP-1α can also promote Th0 cells to Th1 cells, since this chemokine suppresses the production of IL-4 and increases the expression of IFN-γ (Karpus et al., 1997). MIP-1α shows a weak chemoattraction to eosinophils (Karpus et al., 1997). MIP-1α and MIP-1β induce the migration of T-cells and monocytes to inflammatory sites. MIP-2 is not only a chemoattractant for polymorphonuclear neutrophils to inflammatory sites but also promotes the growth and expansion of neutrophil progenitors in the bone marrow (Feng et al., 1995; Matzer et al., 2001). MIP-3α promotes the migration of CCR6+CD4+ T-cells, eosinophils and dendritic cells but not neutrophils and monocytes (Power et al., 1997; Sullivan et al., 1999). MIP-3α also activates NK cells (Al-Aoukaty et al., 1998) and B-cells also respond to this chemokine (Liao et al., 2002). MIP-3α in concert with TGF-β1 regulates the migration of langerhans cells into the epidermis under normal conditions (Sullivan et al., 1999) but also strongly attracts LC precursors into inflamed epithelial surfaces (Dieu-Nosjean et al., 2000).

MIP-1α and MIP-1β are significantly increased in the BALF and sputum recovered from asthmatics (Holgate et al., 1997; Capelli et al., 1999; Barczyk et al., 2001). There are high serum levels of MIP-1α and MIP-1β in individuals with atopic dermatitis (Kaburagi et al., 2001). MIP-1α was observed to be significantly increased after 4 hours and return to basal levels in the BALF of asthmatic patients in response to allergen challenge (Blease et al., 2000). The mRNA levels of MIP-2 were markedly elevated during the early acute phase of allergen challenge in the lung of rats (Shahan et al., 1998; Matikainen et al., 2000; Yamauchi et al., 2002). MIP-3α levels in the lungs of mice exposed to cockroach antigen increased rapidly within hours of challenge (Lukacs et al., 2001). Collectively, these data suggest that MIP-1α, -1β, -2 and -3α and their
respective receptors play important roles in the development, recruitment and activation of leukocytes, which are crucial for many immune and allergic inflammatory responses.

1.4.4 Other chemokines

1.4.4.1 RANTES

As a CC chemokine, RANTES displays high affinity for CCR1, CCR3 and CCR5 (Elsner et al., 2000). RANTES is expressed in cytokeratin+ epithelial cells, CD31+ endothelial cells and CD68+ macrophages (Ying et al., 1999). Human ASM cells also synthesis and release RANTES in response to IFN-γ stimulation (John et al., 1997). RANTES promotes the accumulation of monocytes, eosinophils and T-cells at sites of inflammation (Schroder 1995; Ying et al., 1995; Lukacs et al., 1996) and induces basophils to release histamine (Conti et al., 1997). Studies on asthmatic biopsies with immunohistochemistry, however, have shown that RANTES expression in the epithelium and submucosa is not significantly enhanced when compared to non-asthmatic controls (Fahy et al., 1997). This may suggest that RANTES is normally expressed in these regions, but is not significantly upregulated by local antigen challenge. Similar observations were obtained in a model of acute lung inflammation in the rat (Xing et al., 1994). Thus, RANTES may play a role in maintaining basal levels of inflammatory cell migration into tissues.

1.4.4.2 MDC

MDC, as a CC chemokine, is primarily expressed in mature macrophages and monocyte-derived dendritic cells but not in monocytes or NK cells (Godiska et al., 1997). Epithelial cells and B-cells also produce this chemokine (Schaniel et al., 1998; Chantry et al., 1999). MDC potently binds to CCR4 (Mantovani et al., 2000). NK cells express CCR4 and respond to MDC vigorously (Inngjerdingen et al., 2000). MDC also acts on dendritic cells and monocytes (Godiska et al., 1997). MDC operates specifically on a subset of memory CD4+ T-cells with a Th2 cell cytokine profile (Andrew et al., 1998). MDC+ cells and CCR4+ T-cells were found enriched in regions of both the dermis of inflamed skin and the T-cell area of lymph nodes (Katou et al., 2001), and the majority of CCR4+ T-cells were CD4+. Collectively, these data indicate that MDC may
play an important role in the interaction between APCs and T-cells, in particular promoting T-cell responses with a Th2 cell profile.

1.4.4.3 TARC

TARC is identified as CC chemokine, which binds to CCR4 (Imai et al., 1997; Abi-Younes et al., 2001). In the mouse TARC is constitutively expressed by CD11c+ cells in the lung, dendritic cells in the thymus and in the lymph nodes (Lieberam et al., 1999). In response to TNF-α and IFN-γ, but not IL-4, TARC was upregulated in human bronchial epithelial cells (Berin et al., 2001). TARC is a selective chemoattractant for T-cells that express CCR4. This chemokine also has a high affinity for CCR8 and plays an important role in the activation, migration and proliferation of lymphoid cells (Bernardini et al., 1998). However, TARC showed no chemoattractant activity towards naïve CD4+ T-cells (Lieberam et al., 1999). By contrast, TARC promoted the migration of OVA-primed CD4+ T-cells with a Th2 cell cytokine profile (Vestergaard et al., 2000). Thus, TARC may have an important role in the interplay of APC and memory T-cells.

1.4.4.4 TECK

TECK was initially discovered in the thymus of mice and humans (Vicari et al., 1997). Dendritic cells in the thymus are a primary source of TECK (Vicari et al., 1997). Expression of TECK has also been observed in epithelial cells (Wurbel et al., 2000). TECK showed chemoattract ability for immature CD4+CD8+ T-cells and mature CD4+ T-cells and CD8+ T-cells (Youn et al., 1999). A novel CC chemokine receptor, CCR9, shows high affinity for TECK (Zaballos et al., 1999). Thus, the interplay between TECK and its receptor CCR9 may have a significant role in the recruitment of developing thymocytes and mature T-cells to inflammatory compartments.

1.5 Summary and aims of this thesis

Enhanced airways reactivity to spasmogenic stimuli is a predominant feature of allergic airways disease and asthma. CD4+ Th2 cells and their cytokines primarily regulate the pathogenesis of AHR and inflammatory cell infiltration. Notably, IL-5 in concert with the CCR3-binding chemokines (e.g. eotaxin-1 and eotaxin-2) modulates the activation
and recruitment of eosinophils. IL-13, by signally through the IL-4Rα and STAT6 pathway, not only regulates the development of AHR but also induces asthma-like features including tissue inflammation, mucus hypersecretion and goblet cell hyperplasia and eosinophilia. Indeed, IL-13 regulates eotaxin-1 production (Li et al., 1999). Thus it would appear that signals elicited by IL-5, eotaxins, eosinophils and IL-13 may be integrated to regulate aspects of allergic disease. Investigations in this thesis are designed to examine the collective roles of these factors in the induction of AHR and eosinophil recruitment.

Thus, these studies aimed to:

1. determine the interplay between IL-5 and eotaxins (eotaxin-1 and eotaxin-2) for the induction of pulmonary eosinophilia and AHR;
2. characterize the role of IL-5 and eotaxin-1 in the mechanism of IL-13 induced pulmonary eosinophilia and AHR;
3. investigate the spatial and temporal aspects underlying IL-13 induced AHR and inflammation, and
4. examine the role of STAT6 in eosinophil accumulation and AHR.
CHAPTER 2

Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model.
2.1 Introduction

Eosinophilic inflammation is hallmark feature of allergic disease and is thought to play a critical role in the development of asthma by inducing epithelial damage and AHR (Haczku 1998; Hall et al., 1998; Foster et al., 2001). Eosinophil differentiation, which is an important process in eosinophilic inflammation, is driven non-specifically by IL-3 and GM-CSF and specifically by IL-5 (Sanderson 1991). IL-5 is a key factor for the differentiation, activation and survival of both eosinophils and their progenitors (Kay et al., 1989; Gleich et al., 1990; Ohashi et al., 1990; Fukuda 1993; Foster et al., 1996; Robinson et al., 1999; Koh et al., 2002). IL-5 also plays a pivotal role in the expansion of the eosinophil pool in both blood and tissue (Katoh et al., 1992; Kopf et al., 1996; Sehmi et al., 1997). The central role that IL-5 plays in eosinophil function has identified this cytokine as key therapeutic target for the treatment of allergic disease. In particular the link between eosinophil accumulation in the lung and exacerbation of disease (induction of AHR) has identified this cytokine as an important mediator of enhanced airways reactivity. Indeed studies in animal models have shown an important role for IL-5 in the induction of inflammation and AHR (Foster et al., 1996; Hogan et al., 1997; Hamelmann et al., 1999). However, not all studies in animal models have linked IL-5 to the induction of AHR (Mould et al., 2000). Furthermore, eosinophilia and allergic inflammation in the lung do not always correlate with the development of AHR (Djukanovic et al., 1990; McFadden 1994) and a recent clinical trial with humanized anti-IL-5 monoclonal antibody did not inhibit the development of late phase AHR while significantly decreasing eosinophil numbers in the lungs and in the circulation (Leckie et al., 2000).

Differences in the ability of IL-5 to inhibit AHR have been highlighted in studies with C57BL/6 and BALB/c mice deficient in IL-5. Allergic IL-5-deficient mice of the C57BL/6 strain do not develop antigen-induced AHR (Foster et al., 1996), while BALB/c mice develop enhanced airways reactivity independently of this factor (Corry et al., 1996; Hogan et al., 1998). Notably, in IL-5-deficient BALB/c mice a greater tissue eosinophilia persists (>10 fold) by comparison to C57BL/6 mice deficient in this cytokine. This suggests that pathways which regulate tissue eosinophilia independently of IL-5 may regulate AHR in BALB/c mice, and that the degree of tissue eosinophilia in the absence of IL-5 is critically important for the development of AHR.
One possibility is that local chemokine systems can operate independently of IL-5 to regulate the accumulation of eosinophils into the inflamed lung. In particular, eotaxin-1 may be important because of its demonstrated potency and selectivity for eosinophil recruitment and its strong clinical correlation with disease (Yawalkar et al., 1999; Ying et al., 1999). Furthermore, although IL-5 and eotaxin-1 cooperate to regulate eosinophil migration, this chemokine can also regulate eosinophil migration independently of IL-5 (Mould et al., 1997). Notably, eotaxin-1 plays an important role by contrast to IL-5 in regulating eosinophil migration to sites of allergic inflammation of the gastrointestinal tract (Hogan et al., 2001).

Interestingly, overexpression of IL-5 or eotaxin-1 in the lungs of naïve mice does not result in the induction of AHR although there is a pronounced pulmonary eosinophilia (Mould et al., 2000). This observation suggests that other factors in association with IL-5, eotaxin-1 and eosinophils contribute to the development of AHR in the allergic lung. CD4⁺ Th2 cells and their cytokines (such as IL-4, IL-5 and IL-13) play key roles in the pathogenesis of asthma (Mazzarella et al., 2000; Venkayya et al., 2002). Administration of anti-CD4⁺ mAb abolishes the development of AHR in animal models of asthma supporting a critical role for this cell in the induction and expression of disease (Haczku et al., 1995; Foster et al., 2002). Recently, Ag-loaded eosinophils have been shown to activate CD4⁺ Th2-cells to promote cytokine production (MacKenzie et al., 2001). These studies are indicative that IL-5 and eotaxin-1 may contribute to the development of AHR by regulating CD4⁺ Th2 cell function through the recruitment of eosinophils. The secretion of IL-13 from activated Th2 cells may promote eotaxin-1 production and eosinophil accumulation and this pathway may also contribute to disease processes independently of IL-5.

In this Chapter we employ IL-5 and/or eotaxin-1 deficient BALB/c mice to determine the role of pathways operated by these factors in the induction of allergic disease of the lung. Our data show that eotaxin-1 plays a critical role in regulating eosinophil accumulation in the allergic lung independently of IL-5. Eotaxin-1 and IL-5 deficiency together not only abolished tissue accumulation of eosinophils, but also impaired the ability of Th2 cells to produce IL-4 and IL-13 and precluded the development of AHR after antigen inhalation. Thus IL-5 and eotaxin-1, potentially through eosinophils, regulate Th2 driven immune responses in the allergic lung.
2.2 Materials and methods

2.2.1 Genetically manipulated mice.

BALB/c mice (6-12 weeks) were employed in all experiments and were obtained from the Special Pathogen Free Facility or the Gene Targeting Facility, John Curtin School of Medical Research, Australian National University. IL-5-, eotaxin-1- and IL-5/eotaxin-1-deficient mice were backcrossed for 12 generations with the BALB/c strain. BALB/c IL-5 Tg mice (with approximately 49 transgene copies, male, 6-8 week old and backcrossed to the 12th generation (Dent et al., 1990)) were obtained from the University of Adelaide. Mice were treated according to Australian National University Animal Welfare guidelines and were housed in an approved containment facility.

2.2.2 Ovalbumin [OVA] treatment of mice.

Mice were sensitized at 6-8 weeks of age by intraperitoneal (i.p.) injection with 50 µg of OVA/1 mg Alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline. Non-sensitized mice received 1 mg of Alhydrogel in 0.9% saline. On day 24, 26, 28, and 30, all groups of mice were aeroallergen challenged with OVA (10mg/ml in 0.9% saline) for 30 min three times as previous described (Foster et al., 1996; Hogan et al., 1998). On day 23, 25, 27, 29, 31, six to eight animals per group were sacrificed and bone marrow samples were taken. Twenty-four hours after the last challenge, AHR was measured and then the mice were sacrificed by cervical dislocation and inflammation and morphological changes to the airways characterized.

2.2.3 Measurement of airways reactivity to methacholine

Airways reactivity to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function.
Before taking readings, the box was calibrated with a rapid injection of 1 ml of air into the main chamber. The pressure differences were measured between the main chamber containing the animal (lower chamber), and a reference chamber (upper chamber). This box pressure signal is caused by changes in volume and the resultant pressure changes in the main chamber during the respiratory cycle of the animal. A pneumotachograph with defined resistance in the wall of the main chamber acts as a low-pass filter and allows thermal compensation. The time constant of the box was determined to be approximately 0.02s. Inspiration and expiration are recorded by establishing start-inspiration and end-inspiration as the box pressure/time curve crosses, the change in the waveform can be quantified comparing the mean expiratory box pressure during early expiration (MP1) with the mean expiratory box pressure during late expiration (MP2) by measurement of pause where MP1 = mean expiratory box pressure 1; MP2 = mean expiratory box pressure 2; P = expiratory box pressure.

During bronchoconstriction, the changes in box pressure during expiration are more pronounced than during inspiration. Penh reflects changes in the waveform of the box pressure signal from both inspiration and expiration and combines it with the timing comparison of early and late expiration (Pause). Penh is a junction of the proportion of the pressure signal from inspiration and expiration and of the timing of expiration rather than a function of the absolute box pressure amplitude or the respiratory rate.

\[
\text{Penh} = \left[\frac{(T_e/0.3T_r)-1}{2}\right] \times \frac{2P_{ef}}{3P_{if}}
\]

* Penh: enhanced pause
  
  Te  expiratory time  
  Tr  relaxation time  
  Pef peak expiratory flow  
  Pif peak inspiratory flow

For the quantification of the dose-response to β-methacholine, mice were placed in the chamber and baseline reading taken and averaged for 3 min. Aerosolized methacholine (doubling concentrations in solution ranging from 3.125 to 50mg/ml) was then delivered through an inlet into the main chamber for 2 min and readings averaged over a period of 3 min after each dose was administered. Airways reactivity was expressed as a fold increase of Penh values for each concentration of methacholine compared to Penh.
values after H$_2$O challenge. The data are reported as the mean with the lower and upper limit of 95% confidence interval.

2.2.4 Characterization of lung morphology

Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed in 10% phosphate-buffered formalin, sectioned, and stained with alcian blue/periodic acid-Schiff or Carbol's Chromotrope-Hematoxylin for the identification of mucus-secreting cells and eosinophils respectively. The number of mucus staining cells and eosinophils in the central bronchi-bronchiole area were identified by morphological criteria and quantified as described by previously (Foster et al., 1996).

2.2.5 Collection and analysis of peripheral blood, bone marrow and BALF

Tracheas were cannulated and airway lumina were washed with 1ml of PBS (pH 7.2). Approximately 0.8ml of the instilled fluid was recovered per wash. The obtained BALF was pooled and centrifuged at 500× g for 5 min. BALF cells were resuspended in lymphocyte culture medium (100µl) and cell numbers were determined with a standard hemocytometer. BALF cells were cytocentrifuged at 100× g for 7 min to prepare cytospin slides.

The left thigh of each animal was excised and the bone lumina washed with $2 \times 1$ ml of PBS. Red blood cells were lysed by red-blood-cell lysis solution (14mM NH$_4$Cl, 1.7mM Tris-base, pH 7.2) for 7 min at 37°C. The bone marrow cells were centrifuged at 500× g for 5 min and then resuspended in 1 ml of PBS. The cell numbers were counted with a standard hemocytometer. Bone marrow cells were cytocentrifuged at 100× g for 7 min to prepare cytospin slides.

Peripheral blood were drawn from the tail vein and gently smeared onto slides. BALF cells, bone marrow cells and peripheral blood samples were differentially stained with May-Grunwald-Giemsma solution. Cell types were identified using standard morphological criteria and 200-300 leukocytes were counted per slide.
2.2.6 Isolation of CD4+ T-cells from naive spleens

Spleens were excised and gently pushed through sieves (70µm) in PBS/10% FCS. The resulting cell suspension was filter through nylon mesh (70µm) and centrifuged at 500× g for 5 min. The cell pellet was then resuspended in red blood cell lysis solution for 7 min at 37°C and centrifuged at 500× g for 5 min. The resulting pellet was resuspended in PBS/2% FCS. Cells were quantified with a cytometer and then centrifuged at 500× g for 5 min and the supernatant was removed. The cell pellet was then washed twice and resuspended in 200µl of PBS/2%FCS. CD4 positive cells were isolated with two midiMACS separation columns (Miltenyi Biotec GmbH, Germany), 10⁵ cells were washed in PBS/2%FCS and then incubated with phycoerythrin (PE)-conjugated anti-mouse CD4 mAbs (clone L3T4) for 30 min on ice. The purity of the CD4+ population was determined with a Becton and Dickinson FACScan flow cytometer.

2.2.6 Generation of Th2 cells from purified naive CD4+ T-cells

CD4+ T-cells (>98%) after two midiMACS columns separation were cultured at 5×10⁵ cells/ml in complete medium in the presence of anti-CD3 (3µg/ml)(clone 2C11), anti-CD28 (3µg/ml)(clone 37.51), recombinant murine IL-4 (500pg/ml) and anti-murine IFN-γ (10µg/ml; clone R46A2) for 4 days to generate CD4+ Th2 cells (Mattes et al., 2001). Cell-free culture supernatants were collected and stored in aliquots at -70° C until analysis by ELISA.

2.2.7 Analysis of cytokines by ELISA

IL-13 (R&D Systems, Minneapolis, MN), IL-4, IL-5, and IFN-γ (all from BD PharMingen) concentrations were determined in the culture medium of CD4+ Th2 cells by ELISA using paired antibodies and known standard.

Maxisorp 96-well assay plates (Nunc) were coated with the respective purified cytokine-antibodies in coating buffer (0.1M carbonate buffer, PH 9.5) and then incubated overnight at 4°C. After washing twice in 0.05%Tween/PBS (washing solution), plates were blocked with 2% skim powder in 0.05%Tween/PBS for 1 h at 37°C. Plates were washed 5 times in washing solution and incubated with serial
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

dilutions of respective cytokine standard (50µl) or cell-free medium (50µl) for 2 h at 37°C, then washed in washing solution 5 times. Plates were then incubated with respective biotinylated cytokine antibodies for 2 h at 37°C. Plates were washed 5 times in washing solution and then streptavidin alkaline phosphatase conjugate (Amersham Cat: RPN 1234) in 0.05%Tween/PBS was added and plates were incubated for 1 h at 37°C. Alkaline-phosphatase substrate solution was added after plates were washed and then colour was developed overnight at room temperature. After development, plates were read at 405nm with reference to 490nm on a microplate reader (BIO-TECH instruments Inc. Winooski, VT, USA).

2.2.8 Statistical analysis

The significance of differences between the means of experimental groups was analyzed using Student’s unpaired *t* test. Values were reported as the mean standard error of the mean (SEM). Differences in mean values were considered significant if *P*<0.05.
2.3 Results

2.3.1 Cooperation between IL-5 and eotaxin-1 for the regulation AHR

To investigate the role of IL-5 and/or eotaxin-1 on the development of AHR, airways responses were measured at day 31 in factor deficient mice. In SAL/OVA treated WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice, baseline levels of airways reactivity were not found to be significantly different in response to methacholine challenge (Figure 2.3.1). The levels of airways reactivity in OVA/OVA treated WT mice and IL-5- and eotaxin-1- deficient mice were significantly higher than that of the respective SAL/OVA treated mice. However, in the absence of both IL-5 and eotaxin-1, AHR in allergic mice was completely abolished (Figure 2.3.1). This data suggests that IL-5 and eotaxin-1 act in concert to regulate enhanced airways reactivity.

2.3.2 Effect of IL-5 and eotaxin-1 on peripheral blood eosinophilia

To examine the role of IL-5 and eotaxin-1 on the development of blood eosinophilia, blood samples were taken on day 23, 25, 27, 29 and 31 respectively. OVA-inhalation induced a slightly but insignificant increase in blood numbers of eosinophils in non-sensitized WT mice, whilst a pronounced blood eosinophilia was induced in OVA-sensitized WT mice (Figure 2.3.2). The basal levels of circulating eosinophils in non-sensitized or sensitized eotaxin-1-deficient mice were the highest amongst the non-sensitized or sensitized groups on day 23 (P<0.05). OVA sensitized eotaxin-1-deficient mice exhibited similar levels of circulating eosinophils as that of OVA sensitized WT mice, except on day 29 (Figure 2.3.2). On day 29, the level of eosinophils in OVA-sensitized eotaxin-1-deficient mice was 2-3 fold higher than that in OVA sensitized WT mice, suggesting that eosinophil movement into tissues was attenuated (P<0.05).

By contrast, the percentage of blood eosinophils in both IL-5- and IL-5/eotaxin-1-deficient mice were similar in both sensitized and non-sensitized groups. This data suggests that IL-5 plays a pivotal role in the development of blood eosinophilia in response to allergen challenge, whilst eotaxin-1 predominantly regulates the recruitment of eosinophils from the blood into tissues.
Figure 2.3.1 The development of AHR in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

WT mice and IL-5-, eotaxin-1-, and IL-5/eotaxin-1- deficient mice were injected i.p. with saline or OVA and challenged with OVA aerosol. Airways reactivity was measured the day after the last OVA aerosol by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. OVA/OVA treated WT mice and IL-5- and eotaxin-1-deficient mice exhibited heightened airways reactivity to methacholine in comparison to SAL/OVA treated mice. There was no significant difference between SAL/OVA and OVA/OVA treatment in IL-5/eotaxin-1-deficient mice. Data are the means ± SEM from n = 8-12 mice per group.

*P<0.05 compared to respective SAL/OVA treated mice.
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

---

**a. WT**

![Graph showing the effect of methacholine on WT mice.](image)

**b. IL-5**

![Graph showing the effect of methacholine on IL-5 mice.](image)

**c. eotaxin-1**

![Graph showing the effect of methacholine on eotaxin-1 mice.](image)

**d. IL-5/eotaxin-1**

![Graph showing the effect of methacholine on IL-5/eotaxin-1 mice.](image)

---

*Note: The graphs depict the effect of methacholine on different mouse models, showing a significant increase in methacholine sensitivity.*
Figure 2.3.2 Eosinophil levels in the peripheral blood of WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

Peripheral blood (PB) was examined on alternate days to challenge during the OVA aerosol regime. Eosinophils in the peripheral blood were identified using morphological criteria with May-Grunwald-Giemsa staining. OVA/OVA treated WT and eotaxin-1-deficient mice showed a higher level of peripheral blood eosinophils compared to the respective SAL/OVA treated group (a and c). OVA/OVA treated IL-5- and IL-5/eotaxin-1- deficient mice showed no difference in the peripheral blood eosinophil number by comparison to the respective SAL/OVA treated group (b and d). OVA/OVA treated WT and eotaxin-1-deficient group exhibited higher peripheral blood eosinophil levels compared to OVA/OVA treated IL-5- and IL-5/eotaxin-1- deficient groups. Data are the means ± SEM from n = 8 mice per group.

*P<0.05 compared to respective SAL/OVA treated mice. #P<0.05 compared to other OVA/OVA treated groups on day 23 or 29. ●P<0.05 compare to other SAL/OVA groups on day 23.
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

- **SAL/OVA**
- **OVA/OVA**

**a. WT**

**b. IL-5⁻/⁻**

**c. eotaxin-1⁻/⁻**

**d. IL-5/eotaxin-1⁻/⁻**
2.3.3 BALF eosinophilia is dependent on IL-5 and eotaxin-1

OVA/OVA treatment of mice resulted in a marked infiltration of eosinophils into the BALF compared to the respective SAL/OVA treatment of mice among the four groups (Figure 2.3.3). Total cell numbers in the BALF were similar in OVA/OVA treated WT and eotaxin-1-deficient mice. The number of eosinophils in total BALF cells were also similar in OVA/OVA treated eotaxin-1-deficient mice compared to OVA/OVA treated WT mice. Although the level of eosinophils in OVA/OVA treated IL-5- and IL-5/eotaxin-1- deficient mice were significantly higher than that in SAL/OVA treated respective group, they were significantly lower than that in OVA/OVA treated WT or eotaxin-1-deficient mice (P<0.05). The levels of alveolar macrophages in all OVA/OVA treated groups were similar and significantly higher than that in respective SAL/OVA treated groups. OVA/OVA treatment did not result in a significant difference of neutrophil infiltration as compared to SAL/OVA treatment among all groups. Lymphocyte infiltration was significantly increased compared OVA/OVA treatment to SAL/OVA treatment in the WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

2.3.4 Lung tissue eosinophilia is predominantly dependent on IL-5 and eotaxin-1

To investigate eosinophil accumulation in the lung tissue, histology samples were taken on day 31. OVA/OVA treatment of WT mice resulted in a marked accumulation of eosinophils in the lung tissues as compared not only to SAL/OVA treated WT mice but also to OVA/OVA treated IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice (P<0.05) (Figure 2.3.4). SAL/OVA treatment in WT mice induced a significant (very low level) recruitment of eosinophils into the lung tissue in comparison to SAL/OVA treatment in IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice (P<0.05) (or naïve WT mice which were not treated: data not shown). No significant difference in eosinophil numbers was found between OVA/OVA treated IL-5- and eotaxin-1- deficient mice. In the absence of both IL-5 and eotaxin-1, eosinophil accumulation in lung tissue in response to OVA/OVA treatment was dramatically suppressed in comparison to OVA/OVA treated WT mice and IL-5- and eotaxin-1- deficient mice. Although eosinophil numbers in the lung tissue of OVA/OVA treated IL-5/eotaxin-1-deficient mice were rare they were significantly elevated as compared to the respective SAL/OVA treated mice (P<0.05). These data suggests that IL-5 and eotaxin-1 play a crucial role in eosinophil
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

Figure 2.3.3 BALF leukocytes in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

The numbers of eosinophils and other inflammatory cells in BALF on day 31 are shown for WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice. Cell types were identified using morphological criteria with May-Grunwald-Giemsa staining. The number of eosinophils were significantly decreased in the absence of IL-5. Data are the means ± SEM from n = 8 mice per group.

*P<0.05 compared to respective SAL/OVA treated mice; #P<0.05 compared OVA/OVA treated IL-5- and IL-5/eotaxin-1- deficient mice.
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

Figure 2.3.4 Eosinophil numbers in lung tissue from OVA/OVA treated WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

The number of eosinophils in tissue are shown on day 31. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophils. The number of eosinophils in the lung tissue were enumerated in 7 - 10 similar high-powered fields (HPF) (x40 magnification) per mouse. Data are the means ± SEM from n = 4-6 mice per group.

*P<0.05 compared to respective SAL/OVA treated mice; #P<0.05 compared to OVA/OVA treated IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice; •P<0.05 compared to OVA/OVA treated IL-5/eotaxin-1-deficient mice; †P<0.05 compared to other SAL/OVA treated mice.
accumulation in lung tissue during allergic inflammation. Furthermore, other eosinophil recruitment factors such as eotaxin-2 may also be involved in eosinophil trafficking.

### 2.3.5 Role of IL-5 and eotaxin-1 in the regulation eosinophilia in the bone marrow

To investigate the effects of IL-5 and/or eotaxin-1 on basal levels of eosinophil numbers in the bone marrow, samples were taken from naïve WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice. Although the mean levels of bone marrow eosinophil number in IL-5- and IL-5/eotaxin-1- deficient mice were found to be lower than that in WT mice, the differences were not significant (Figure 2.3.5 a). In the absence of eotaxin-1, levels of bone marrow eosinophils were markedly elevated compared to that in WT mice.

To investigate the changes in bone marrow eosinophil numbers under allergic conditions, OVA/OVA treatment was administrated to WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice. On day 23 before aeroallergen challenge with OVA, eotaxin-1-deficient mice had a significantly elevated level of bone marrow eosinophils in comparison to WT mice and IL-5- and IL-5/eotaxin-1- deficient mice (P<0.05), whilst WT mice had higher levels of bone marrow eosinophils compared to IL-5- and IL-5/eotaxin-1- deficient mice (P<0.05) (Figure 2.3.5 b). However, no significant difference in bone marrow eosinophil numbers was observed between WT and eotaxin-1-deficient mice over the OVA challenge period. No significant difference was found between IL-5- and IL-5/eotaxin-1- deficient mice before and after the OVA challenge period in bone marrow eosinophil numbers.

### 2.3.6 Role of IL-5 and/or eotaxin-1 in the regulation of CD4 T-cell numbers in the spleen

To examine the impact of IL-5 on the CD4 T lymphocyte population, spleens were excised and the percentage of CD4 T-cells determined by FACS in naïve WT and IL-5 Tg mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice. IL-5- and IL-5/eotaxin-1-deficient mice had significantly lower levels of CD4 T-cells in spleens as compared to WT and IL-5 Tg mice and eotaxin-1-deficient mice (P<0.05) (Figure 2.3.6 a). No difference in the percentage of CD4 T cells in spleens was observed in eotaxin-1-deficient mice compared to that in WT mice. Over-expression of IL-5 resulted in a
Figure 2.2.5 Eosinophil levels in the bone marrow of WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1-deficient mice.

Bone marrow samples were taken on alternate days to challenge during the OVA aerosol regime. Cell types were identified using morphological criteria with May-Grunwald-Giemsa staining. (a). Naïve eotaxin-1-deficient mice showed a significant increase in the number of bone marrow eosinophils compared to WT mice and IL-5- and IL-5/eotaxin-1-deficient mice. (b). OVA/OVA treatment of WT and eotaxin-1-deficient mice induced an increase in the number of eosinophils in the bone marrow, whilst there was no change of eosinophil levels in the bone marrow of IL-5- and IL-5/eotaxin-1-deficient mice. Data are the means ± SEM from n = 8 mice per group.

*P<0.05 compared to OVA/OVA treated IL-5- and IL-5/eotaxin-1-deficient mice; 
#P<0.05 compared to OVA/OVA treated WT mice and IL-5- and IL-5/eotaxin-1-deficient mice.
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

a. Bone marrow leukocytes (naive)

![Graph showing BM leukocyte number x 10^6/ml]  
- WT
- eotaxin-1^−/−
- IL-5^−/−
- IL-5/eotaxin-1^−/−

P<0.003

b. Bone marrow eosinophils (OVA/OVA)

![Graph showing BM eosinophil number x 10^6/ml]  
- WT
- IL-5
- eotaxin-1^−/−
- IL-5/eotaxin-1^−/−
Figure 2.3.6 CD4$^+$ T-cells in spleen of WT and IL-5 Tg mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

Spleens from OVA/OVA treated WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1-deficient mice were excised on day 31, 24 hours after the last OVA challenge. Spleens were also taken from naïve WT mice and IL-5-, eotaxin-1-, and IL-5/eotaxin-1-deficient mice. Total cells were counted with a cytometer. CD4$^+$ T-cell percentages were identified using FACSscan with PE-conjugated anti-mouse CD4 (L3T4). (a) Naïve IL-5- and IL-5/eotaxin-1- deficient mice showed a low level of splenic CD4$^+$ T-cell number compared to WT and IL-5 Tg mice and eotaxin-1-deficient mice. Overexpression of IL-5 induced a significant increase in the splenic CD4$^+$ T-cell numbers compared to other groups. Eotaxin-1 deficiency did not alter CD4$^+$ T-cell numbers compared to WT. (b) OVA/OVA treated IL-5/eotaxin-1-deficient mice showed a significantly lower level in splenic CD4$^+$ T-cell numbers compared to other groups. No significant difference in splenic CD4$^+$ T-cell numbers was found between OVA/OVA treated WT mice and IL-5- and eotaxin-1- deficient mice. Data are the means ± SEM from n = 4 mice per group.

Figure 2.3.6 a, *P<0.05 compared IL-5- and IL-5/eotaxin-1- deficient mice; #P<0.05 compared to WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice; Figure 2.3.6 b, *P<0.05 compared to OVA/OVA treated WT mice and IL-5- and eotaxin-1-deficient mice.
Chapter 2: Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model.

(a) Splenic CD4+ cell number \( \times 10^6/\text{ml} \)

- WT
- IL-5 Tg
- IL-5\(^{-}\)
- eotaxin-1\(^{-}\)
- IL-5/eotaxin-1\(^{-}\)

(b) Splenic CD4+ cell number \( \times 10^6/\text{ml} \)

- WT
- IL-5\(^{-}\)
- eotaxin-1\(^{-}\)
- IL-5/eotaxin-1\(^{-}\)
marked increased in CD4+ T-cell number in the spleens in comparison to that of WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice (P<0.05).

Examination of spleens from OVA/OVA treated mice showed a significantly impaired capacity of CD4+ T-cells to proliferate when derived from IL-5/eotaxin-1-deficient mice compared to similarly treated WT mice and IL-5- and eotaxin-1- deficient mice (P<0.05) (Figure 2.3.6 b). No significant difference of splenic CD4+ T-cell numbers was observed between OVA/OVA treated WT mice and IL-5- and eotaxin-1- deficient mice. These data suggest that IL-5 has an important impact on the development of the naïve CD4+ T lymphocytes. Furthermore, IL-5 cooperatively with eotaxin-1 plays an important role in the proliferation of CD4+ T-cells derived from the spleen under allergic conditions.

2.3.7 Role IL-5 and eotaxin-1 in cytokine-secretion by CD4+ Th2 cells

To investigate the impact of IL-5 and/or eotaxin-1 on the Th2 cytokine-secreting capacity of CD4+ T lymphocytes, naïve CD4+ T lymphocyte from the spleens of WT and IL-5 Tg mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice were polarized to the Th2 type cells in vitro and their capacity to secrete IL-4, IL-5 and IL-13 was analyzed. Stimulation of CD4+ T-cells from the spleens of IL-5-, eotaxin-1-, IL-5/eotaxin-1- deficient, IL-5 Tg and WT naïve mice under identical conditions promoted the production of different levels of Th2 cell cytokines including IL-4, IL-5 and IL-13 (Figure 2.3.7). No IFN-γ was detected in any groups (data not shown). Notably, the levels of IL-4 in IL-5/eotaxin-1-deficient CD4+ T-cell cultures were significantly lower than that in WT CD4+ T-cell cultures (P<0.05) (Figure 2.3.7). The level of IL-13 produced by IL-5-deficient CD4+ Th2 cells was significantly reduced in comparison to those derived from WT mice and significantly higher than that derived from IL-5/eotaxin-1-deficient mice (P<0.05). The level of IL-13 produced by eotaxin-1-deficient CD4+ Th2 cells was reduced although not significant in comparison to those derived from WT mice, but significantly higher than the level of IL-13 produced by IL-5/eotaxin-1-deficient mice (P<0.05). The reduced levels of IL-13 did not correlate with an overall suppression of Th2-type cytokine production. IL-4 and IL-5 levels produced by eotaxin-1-deficient CD4+ Th2 cells were similar to that observed in WT CD4+ T-cell cultures. Surprisingly, overexpression of IL-5 resulted in a significantly elevated expression of all Th2 type cytokines including of IL-4 and IL-13 (P<0.05).
Figure 2.3.7 Intrinsic defect in IL-4 and IL-13 production by naïve splenic CD4+ Th2 cells in the absence of IL-5 and eotaxin-1.

Production of IL-4, IL-5 and IL-13 by purified CD4+ Th2 cells generated from the spleens of WT and IL-5 Tg mice and IL-5-, eotaxin-1- or IL-5/eotaxin-1- deficient mice. No IFN-γ was detected in any group (data not shown). CD4+ T-cells were derived from splenocytes as these T-cell cytokines profiles were directly reflective of those observed after stimulation of allergic PBLN cultures. Data represent the mean ± SEM from n = 3-4 cultures per group.

*P<0.05 compared to WT and IL-5 Tg mice and IL-5- and eotaxin-1- deficient mice; #P<0.05 compared to WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice; •P<0.05 compared to WT and IL-5 Tg mice.
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

(a) IL-4

(b) IL-5

(c) IL-13
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

2.4 Discussion

In this Chapter the roles of IL-5 and eotaxin-1 in the regulation of eosinophil expansion and migration and in the induction of AHR in experimental asthma were investigated. Both IL-5 and eotaxin-1 play key roles in the recruitment of eosinophils to lung, while IL-5 primarily regulates eosinophil expansion in the bone marrow and blood in response to antigen challenge in the lung of sensitized mice. Notably, both molecules act in concert to not only regulate eosinophil function but also modulate CD4⁺ Th2 cell proliferation and cytokine production. Therefore, deficiency in IL-5 and eotaxin-1 attenuates eosinophil accumulation in the lung and Th2 cell function, which correlates with abrogation of AHR.

IL-5 is an important regulator of eosinophil function in allergic diseases (Carlson et al., 1993; Sarmiento et al., 1995; Miyajima et al., 1997). IL-5 regulates eosinophilia and is able to enhance eosinophil responsiveness to chemokines (Shahabuddin et al., 2000). Notably, there is synergy between IL-5 and eotaxin-1 for the trafficking of eosinophils through a unique the interaction between the IL-5- and CCR3 receptors (Mould et al., 1997; Shahabuddin et al., 2000). Previous studies with both IL-5 deficient mice and anti-IL-5 mAb treated WT mice have demonstrated that blood and airway eosinophilia is markedly attenuated in the absence of IL-5 (Foster et al., 1996; Hamelmann et al., 1999). Lack of IL-5 in both IL-5- and IL-5/eotaxin-1- deficient mice resulted in the abolition of peripheral blood eosinophilia (Figure 2.3.2) and eosinophil expansion in the bone marrow compartment (Figure 2.3.5) in response to sensitization and antigen inhalation. These data confirm that IL-5 is essential for expansion of the eosinophil pool not only in the peripheral blood but also bone marrow in response to antigen challenge.

OVA sensitized eotaxin-1-deficient mice had greater eosinophil numbers, not only in the bone marrow, but also in the peripheral blood when compared to OVA sensitized WT mice before OVA challenge (Figure 2.3.5). In the healthy state, eotaxin-1 is responsible for the normal movement of eosinophils into tissues (Matthews et al., 1998). Eotaxin-1 has also been shown to induce the rapid release of eosinophils from the bone marrow (Mould et al., 1997; Palframan et al., 1998). These data are supported in this Chapter by the observation that naïve eotaxin-1-deficient mice had a significantly elevated level of eosinophils in the bone marrow compared to naïve WT mice. The
deficiency in eotaxin-1 appears to block, in part, basal eosinophil migration from the bone marrow resulting in accumulation in this compartment.

In contrast, no significant difference was seen in the level of eosinophils in the bone marrow of eotaxin-1-deficient mice compared to WT mice during the OVA challenge period. Eosinophil levels in the peripheral blood of OVA/OVA treated eotaxin-1-deficient mice were observed to increase rapidly during challenge and were significantly higher than that in the SAL/OVA treated mice. OVA/OVA treatment of eotaxin-1-deficient mice resulted in increased numbers of eosinophils in both airway tissue (Figure 2.3.4) and BALF (Figure 2.3.3). In the absence of both IL-5 and eotaxin-1, however, tissue and BALF levels of eosinophils were almost completely diminished. These data show that although IL-5 and eotaxin-1 can regulate eosinophil accumulation independently of each other, collectively they provide the central mechanism for the regulation of eosinophil migration and homing during allergic inflammation of the lung.

In addition to eosinophils, human T lymphocytes have been found to express the CCR3 receptor (Gerber et al., 1997). Although CCR3 has been identified on human T lymphocytes, murine Th2 cells were found not to express this receptor or respond to eotaxin-1 (Grimaldi et al., 1999). Notably, IL-5, but not eotaxin-1 deficiency predisposed to a significant impairment in the numbers of CD4+ T-cells in the spleen compared to WT mice. Moreover, overexpression IL-5 (IL-5 Tg mice) dramatically amplified the number of CD4+ T-cells in the spleen (Figure 2.3.6). These data strongly indicate that IL-5, but not eotaxin-1 has a marked impact on CD4+ T-cell proliferation. Eosinophils in the allergic lung can present antigen and traffic to local lymph nodes where they co-localize with T-cells and this granulocyte can induce proliferation and cytokine secretion from Th2 cells. Eosinophils can also secrete a wide range of T-cell growth and chemotactic factors and directly engage this lymphocyte through costimulatory molecule (Rothenberg 1998; Shi et al., 2000; MacKenzie et al., 2001). Therefore, the impaired T-cell proliferation may be achieved by the interaction between eosinophils and CD4+ T-cells either directly or indirectly. Thus, evidence is emerging that eosinophils may not only act as terminal effector cells, but can also actively modulate allergic inflammation by amplifying type 2 cytokine responses.

Our data are indicative that eosinophil deficiency caused by the absence of IL-5 and eotaxin-1 predisposes to an intrinsic defect in T-cells. Although the IL-5Rα chain
Chapter 2 Interplay between IL-5 and eotaxin-1 regulates the induction of allergic airways disease in a mouse model

(e) essential for IL-5 signal transduction) is expressed in B-lymphocytes and in limited studies has been implicated in the generation of cytotoxic T-lymphocytes, its expression in CD4+ T-helper cells has not been observed (Harada et al., 1987; Takatsu et al., 1987). A direct effect of IL-5 on this subclass of lymphocyte is therefore unlikely. Furthermore, IL-13, but not IL-5 production was reduced in CD4+ Th2 cells of eotaxin-1-deficient mice (Figure 2.3.7), suggesting that the T-cell defect was not due solely to the absence of IL-5 but involved processes downstream of these molecules. However, both IL-4 and IL-13 production were impaired in CD4+ Th2 cells from IL-5/eotaxin-1-deficient mice. IL-13 derived from CD4+ Th2 cells plays a central role in the onset of AHR and pathophysiology. The specific role for IL-13 in the mechanism of AHR was demonstrated by the delivery of recombinant murine IL-13 into the airways of naïve mice (Wills-Karp et al., 1998). However, depletion of IL-4 does not inhibit the development of AHR during allergen inhalation in a mouse model of asthma (Cohn et al., 1998; Grunig et al., 1998). Thus, collectively, the data suggests that eosinophils can provide factors that either directly or indirectly regulate IL-13 and IL-4 production from CD4+ Th2-cells. Importantly, limitation in IL-13 derived from CD4+ Th2 cell production occurred concomitantly with abrogation of AHR in IL-5/eotaxin-1 deficient mice.

The synonymous association between IL-5 and eosinophilia in conjunction with clinical trial data (with anti-IL-5) has led to the suggestion that eosinophils are not central mediators of asthma but are bystander cells recruited to the airways in response to aberrant Th2 cell activation (Leckie et al., 2000). However, data in this Chapter suggest that eosinophils may be recruited to the allergic lung independently of IL-5 by eotaxin-1-dependent mechanisms, and that this eosinophilia is directly linked to the development of disease. Eosinophilia (albeit reduced) is also observed in the lung of IL-5-deficient mice infected with Toxocara canis (Takamoto et al., 1997). Thus, although eotaxin-1 and IL-5 co-operate to regulate eosinophil recruitment to the allergic lung, they can also operate independently of one another to induce eosinophil accumulation. However, in the absence of both of these molecules, eosinophil accumulation in the allergic lung was almost ablated (sparse accumulation of eosinophilia in parenchyma) and AHR did not develop. These data indicate that although IL-5 can amplify eosinophil recruitment to the allergic lung, the primary role of this cytokine appears to be in the promotion of eosinophilia in the blood and bone marrow compartments in response to antigen provocation. In particular, in the absence of IL-5, eotaxin-1 induced
the recruitment of eosinophils to the lung without a significant blood eosinophilia or the expansion of the eosinophil pool in the bone marrow. Apparently, eosinophils produced by steady state hematopoiesis and/or residing in tissues can be efficiently recruited by eotaxin-1 to the allergic lung in the absence of IL-5.

Investigations have shown that eotaxin-1 not only promotes eosinophil accumulation in tissues, but also induces the release of this cell and its progenitors from the bone marrow (Palframan et al., 1998; Robinson et al., 1999). The lower abundance of eosinophils in the blood of allergic IL-5-deficient mice may mask the development of an eosinophilia in this compartment in response to eotaxin-1. Furthermore, it is likely that once eosinophils and/or their progenitors enter the circulation in IL-5-deficient mice in response to eotaxin-1, they are rapidly sequestered into the allergic lung. Indeed, evidence is accumulating for a local pulmonary role for eosinophil progenitors in the pathogenesis of allergic disease (Gibson et al., 1990; Inman et al., 1999). Potentially, in the absence of IL-5, eotaxin-1 may regulate the recruitment of eosinophils and their progenitors to the allergic lung that then undergo maturation in the Th2 immune environment.

In summary, this Chapter demonstrates that eosinophils are able to accumulate in the allergic lungs of BALB/c mice in the absence of IL-5 and promote disease. Eotaxin-1 is a prominent factor for maintaining normal migration of eosinophils in both bone marrow and peripheral blood compartment at baseline. IL-5 critically regulates the expansion of eosinophils in the blood and bone marrow in response to allergic stimuli and thus promotes tissue eosinophil accumulation. Importantly, IL-5 and eotaxin-1 cooperate to regulate not only eosinophilia but also Th2 cell production of IL-4 and IL-13 and thus Th2 immunity. It is also tempting to speculate that within the allergic lung, eosinophils may sequester antigen and localize to regional lymph nodes where they modulate IL-13 production from T-cells. This mechanism may help promote the development of AHR. The observation that eosinophils may regulate disease processes in the absence of IL-5 has important implications for therapeutic approaches to allergic disorders.
Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

3.1 Introduction

Eotaxin-1 and eotaxin-2 are members of the CC chemokine family (Forssmann et al., 1997), which selectively bind to the CCR3 receptor. Eotaxin-2 was named because of similar function to eotaxin-1, however these chemokines share only 39% identity at the amino acid level (Forssmann et al., 1997). Eotaxin-1 was first identified in BALF in an experimental model of allergic airways disease in guinea pigs (Jose et al., 1994). By using genomic analysis techniques, investigators identified a gene that encoded for an eosinophil selective chemokine and designated the gene product as eotaxin-2 (Kitaura et al., 1996; Forssmann et al., 1997; Patel et al., 1997; Shinkai et al., 1999). Sequence comparison analysis and chromosomal positioning showed that eotaxin-1 and eotaxin-2 are distantly related (Forssmann et al., 1997; Luster et al., 1997; Shinkai et al., 1999). Eotaxin-2 is located on human chromosome 7q11.23, whilst eotaxin-1 is positioned at 17q21.1 (Kitaura et al., 1996; Nomiyama et al., 1998).

Characterization of eotaxin-1 and eotaxin-2 expression, distribution and biological activity suggests that these chemokines possess common biological functions. They are eosinophil and basophil chemoattractants, and while efficacy levels are very similar, eotaxin-1 is more potent than eotaxin-2 in inducing eosinophil chemotaxis in vitro (Forssmann et al., 1997; Elsner et al., 1998; Shinkai et al., 1999; Zimmermann et al., 2000). Eotaxin-1 and eotaxin-2 also induce rapid and transient actin polymerization and modulate respiratory burst in eosinophils (Elsner et al., 1998).

The similar activities of the eotaxins are explained by their ability to signal exclusively through CCR3, which is primarily found on eosinophils (Rothenberg et al., 1999). Blockade of CCR3 with neutralizing mAb inhibits both eotaxin-1 and eotaxin-2 induce Ca²⁺ flux in human eosinophils (Elsner et al., 1998). Furthermore, eotaxin-1 and eotaxin-2 have been shown to induce CCR3 receptor internalization (Loetscher et al., 2001). Eotaxin-1 also induces complete cross desensitization of eotaxin-2 signalling confirming selective activation of CCR3 (Forssmann et al., 1997; Elsner et al., 1998; Zimmermann et al., 1999; Elsner et al., 2000; Dulkys et al., 2001).

Recent studies suggest that eotaxin-1 and eotaxin-2 may play important roles in regulating different components of eosinophil recruitment during allergic inflammation. By employing murine models, investigators have shown that inhibition of eotaxin-1
activity by neutralizing mAb or by gene disruption attenuates but does not abolish eosinophil accumulation in the lungs of allergic mice (Rothenberg et al., 1997; Campbell et al., 1998; Gonzalo et al., 1998; Matthews et al., 1998). This suggests that other eosinophil-specific chemokines can contribute to eosinophil trafficking during allergic inflammation (Rothenberg et al., 1997) and this has been supported by studies demonstrating IL-13 mediated pulmonary eosinophilia in IL-5/eotaxin-1 double deficient mice (Chapter 4). The data from clinical studies also suggest differential roles for eotaxin-1 and eotaxin-2 in eosinophil accumulation during allergic inflammatory responses (Ying et al., 1999). During the early phase response (6-hour) in allergen-induced late-phase cutaneous responses in atopic subjects, eosinophil recruitment correlated with increased levels of eotaxin-2, whereas elevated levels of eotaxin-1 correlated with the later 24-hour tissue eosinophilia (Ying et al., 1999). Whilst eotaxin-1 and eotaxin-2 have been shown to possess similar functional activity, they have differential patterns of expression, which may underpin defined individual roles. Eotaxin-1 is constitutively expressed in the thymus, lung and throughout the length of the gastrointestinal tract (esophagus, stomach and small intestine), whereas eotaxin-2 is expressed in the spleen and jejunum (Rothenberg et al., 1995; Zimmermann et al., 2000). Interestingly, the expression of eotaxin-1 and eotaxin-2 is upregulated in transgenic mice overexpressing IL-4 in the lung and also following intranasal administration of recombinant IL-4 (Zimmermann et al., 2000). Furthermore, IL-4-induced expression of eotaxin-1 and eotaxin-2 in the lung is dependent on STAT6 expression (Zimmermann et al., 2000). Collectively, these studies suggest differential roles for the eotaxins in regulating various components of eosinophil trafficking and potentially eosinophil function during allergic inflammatory responses. However, unlike eotaxin-1, the effect of eotaxin-2 on eosinophil trafficking into its lung and interactions with IL-5 and its potential role in the induction of AHR are poorly defined.

In this Chapter the potential contribution of eotaxin-2 to the induction of phenotypic characteristics of allergic airways disease was investigated by instilling this chemokine into airways of naïve WT and IL-5 Tg mice. The results demonstrate that eotaxin-2 promotes airway eosinophilia that was dependent on IL-5. Eotaxin-2, co-operatively with IL-5, also promoted mucus hypersecretion and the induction of AHR, which directly correlated with elevated levels of IL-13 in respiratory secretions. The induction of these pathological features of allergic airways disease by eotaxin-2 was critically dependent on signalling through the IL-4Rα chain and STAT6. These investigations, as
in Chapter 2, demonstrate co-operative interplay between eosinophil regulatory pathways and IL-13 signalling systems for the development of hallmark features of allergic disease of the lung.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

3.2 Materials and methods

3.2.1 Mice

See 2.2.1

Seven- to nine-week naïve WT and IL-5 Tg mice and IL-13-, IL-4Rα-, STAT6-, IL-5/eotaxin-1- and IL-5Tg/STAT6- deficient mice were bred in specific pathogen-free facilities, JCSMR. All animals were housed and treated according to the Australian National University guidelines for animal experimentation.

3.2.2 Induction of allergic phenotype by eotaxin-2

Mice were anesthetized by intravenous injection (i.v.) of 100µl Saffan solution (1:4 diluted in saline, v/v) and then intubated with a 22 gauge catheter needle, through which murine recombinant eotaxin-2 (1, 3 or 5µg dissolved in 20µl vehicle: 0.1% BSA/PBS) or vehicle control (0.1% BSA/PBS) were instilled into the airways of naïve WT or IL-5 Tg mice. AHR, mucus production, the number of airways eosinophils and levels of IL-13 in BALF were measured 24 h after instillation. In some experiments murine recombinant IL-5 (50ng dissolved in 200µl PBS) was i.v. administered to WT naïve mice daily for 4 consecutive days, followed by intratracheal instillation (i.t.) of IL-5 (1µg) and/or eotaxin-2 (3µg) (dissolved in a final volume of 20µl vehicle) on day 4, and lungs analyzed (as above) 24 h after instillation. Eotaxin-2 was produced by recombinant technology in bacteria from cDNA as previously reported (Peprotech Inc. NJ, USA) (Zimmermann et al., 2000). Mouse recombinant IL-5 was expressed and purified from the baculovirus expression system (Ingley et al., 1991). IL-5 (gift from: Professor Ian G. Young, Australian National University, Canberra, Australia) was purified to homogeneity by gel filtration (Superdex 75 Pharmacia LKB Biotechnology Inc. Piscataway, NJ) and ion exchange chromatography (Mono Q Pharmacia LKB Biotechnology, Inc.) and protein levels estimated by optical density at 280nm and Biorad protein assay using gamma globulin as the standard as described by the manufacturers.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary cosinophilia and IL-13 regulated AHR

3.2.3 mAb depletion of CD4+ cells

3.2.3.1 Generation of neutralizing antibodies

Hybridoma cell lines specific for anti-mouse CD4 (GK1.5) (ATCC, Manassas, VA, USA) and anti-rat CCR3 (6S2-19-4) (DNAX, Palo Alto, CA, USA) were grown in complete lymphocyte culture media (MLC: a base of Dulbecco’s modified eagle medium [DMEM; Gibco BRL, Grand Island, NY, USA]) supplemented with 10% fetal calf serum (FCS) and 1 mM Na pyruvate, 10 mM HEPES (pH 7.4), 2 mM glutamine, 50 µM penicillin, 50 µg/ml streptomycin, 100 µg/ml neomycin and 5 x 10^{-5} M mercaptoethanol) at 37°C, 5% CO₂. Swiss nude mice were injected i.p. with 500 µl pristine (2, 6, 10, 14-tetra-methyl-pentadecane; Sigma Chemical Co, St Louis, MO) one week before injection of hybridoma cells. On Day 7, hybridoma cells were washed and resuspended with PBS and then mice were injected i.p. with 5 million hybridoma cells in a volume of 200 µl PBS.

When ascites fluid had developed in the peritoneal cavity, the mice were sacrificed by cervical dislocation and sprayed with 75% ethanol. An 18-gauge needle was used to collect ascites fluid. The ascites fluid was centrifuged at 500 x g for 7 min at 4°C to remove cells and the supernatant was transferred to a sterile tube.

3.2.3.2 Quantitation of ascites fluid

Round-bottom ELISA plates (Dynatech, Chantill, VA, USA) were coated with goat anti-rat total IgG (Southern Biotechnology Associates Inc. Birmingham, AL, USA) in NaHCO₃ buffer (pH 9.2) and incubated at 4°C overnight. After washing twice in 0.05% Tween/PBS (washing solution), plates were blocked with 2% skim milk powder in 0.05% Tween/PBS for 1 h at 37°C. Plates were washed 5 times in washing solution and incubated with serial dilutions of ascites fluid and standard amount rat total IgG (Sigma Chemical Co, St Louis, MO) for 2 h at 37°C, then washed 5 times in washing solution. Plates were then incubated with biotinylated goat anti-rat total IgG (Southern Biotechnology Associates Inc. Birmingham, AL, USA) for 2 h at 37°C. Plates were washed 5 times in washing solution and then streptavidin alkaline phosphatase conjugate (Amersham Cat: RPN 1234) in 0.05% Tween/PBS was added and incubated for 1 h at 37°C. Alkaline-phosphatase substrate solution was added before plates were
washed twice and then colour was developed for 15 min at room temperature. After development, plates were read at 405nm with reference to 490nm on a microplate reader (BIO-TECH instruments Inc. Winooski, VT, USA).

3.2.3.3 Depletion of CD4 and CCR3 positive cells

Mice were injected i.p. with anti-CD4 or anti-CCR3 (200µl of 1mg mAb in saline) or control mAbs (200µl of 1mg J1.2 mAb isotype control [gift from Dr. Simon P Hogan, Australian National University, Canberra, Australia] in saline) 1 day prior to the first i.v. administration of IL-5 in naïve WT mice. IL-5 (50ng dissolved in 200µl PBS) was then administered i.v. daily for 4 consecutive days to naïve WT mice followed by instillation i.t. of IL-5 (1µg) and eotaxin-2 (3µg) (dissolved in a final volume of 20µl vehicle) on days 4 and airways were analyzed 24 h later.

3.2.4 Measurement of airways reactivity to methacholine

See 2.2.3

3.2.5 EPO activity assay

EPO activity in the supernatant of cell-free BALF was measured as previously described (Schneider et al., 1996). This assay is based on the oxidation of o-phenylenediamine (OPD) (Sigma Chemical Co, St Louis, MO) by EPO in the presence of hydrogen peroxide (H₂O₂). The substrate solution consisted of 12mM OPD, 0.005% H₂O₂, 10 mM HEPES (pH 7.4) and 0.22% cetyltrimethylammonium bromide (CTAB) (Aldrich Chemical Company, Inc. Milwaukee, WI, USA). Substrate solution (75µl) was added to cell-free BALF supernatants (75µl) in a 96-well microplate and incubated at room temperature for 15 min before stopping the reaction with 50µl of 4M cold sulfuric acid. Absorbence was measured at 490 nm using a spectrophotometer.

3.2.6 Measurement of IL-13 in BALF

See 2.2.7
Briefly, the concentration of IL-13 from cell-free BALF supernatant was determined by ELISA using the Abs AB-413-NA and AF-413-NA (R&D Systems, Minneapolis, MN).

3.2.7 Characterization of eosinophils and mucus staining cells in lung tissue

See 2.2.4

3.2.8 Comparison between eotaxin-1 and eotaxin-2 induced allergic phenotype

3.2.8.1 Induction of peripheral blood eosinophilia

Mice were injected i.v. with eotaxin-1 or eotaxin-2 [both 0.6μg/100μl/mouse] or control vehicle [100μl of 0.1%BSA/PBS]. Blood samples were taken before, at 30 min and 1 h after the i.v. injection. Blood eosinophilia was determined by diluting blood 1:10 in Discombe’s solution [Discombe, 1946] and counting positively staining cells after 10 min by light field microscopy using a haemocytometer.

3.2.8.2 Induction of AHR and pulmonary eosinophilia

BALB/c IL-5 Tg mice were anesthetized by i.v. injection of 100μl Saffan solution (1:4 dilution in saline, v/v) and then intubated with a 22-gauge catheter needle, through which murine recombinant eotaxin-1, eotaxin-2 (3μg dissolved in 20μl vehicle: 0.1% BSA/PBS) or vehicle control (0.1% BSA/PBS) were instilled into the airways. AHR, mucus production, peripheral blood and airways eosinophilia and levels of IL-13 in BALF were measured 24 h after instillations.

3.2.9 Statistical analysis

See 2.2.8
3.3 Results

3.3.1 Characterization of eotaxin-2 induced peripheral blood and pulmonary eosinophilia in WT and IL-5 Tg mice

Intratracheal instillation of 3µg of eotaxin-2 did not induce a peripheral blood, BALF or tissue eosinophilia in naïve WT mice (Figure 3.3.1 a, b and c). Interestingly, a decrease in peripheral blood eosinophil numbers was observed following i.t. instillation of 3 and 5µg of eotaxin-2 to IL-5 Tg mice (Figure 3.3.1 a), which correlated with a significant increase in BALF and tissue eosinophil numbers (Figure 3.3.1 b and c). Eosinophil recruitment induced by eotaxin-2 was dose dependent. Vehicle treatment did not significantly promote eosinophil accumulation in BALF or lung tissue (Figure 3.3.1 b and c). Differential analysis of leukocyte populations in BALF indicated that eotaxin-2 (3µg) selectively recruited eosinophils to the airways (data not shown). Tissue eosinophil numbers in IL-5 Tg mice were significantly higher than those observed in WT mice and/or WT mice receiving 3µg of eotaxin-2 (Figure 3.3.1 c). Increased tissue eosinophil levels in IL-5 Tg mice was associated with elevated levels of extracellular EPO, which increased after i.t. of 3 and 5µg of eotaxin-2 (Figure 3.3.1 d; P<0.05). No significant difference in the level of EPO activity was observed between naïve WT and IL-5 Tg mice. Furthermore, administration of eotaxin-2 (3µg) to WT mice did not significantly increase levels of extracellular EPO compared to naïve WT or IL-5 Tg mice (Figure 3.3.1 d).

3.3.2 Characterization of the effects of eotaxin-2 on airways reactivity in WT and IL-5 Tg mice

Baseline airways reactivity to increasing doses of methacholine was not significantly different between vehicle treated WT and IL-5 Tg mice (Figure 3.3.2). Administration of eotaxin-2 (3µg) to WT mice did not significantly enhance airways reactivity to methacholine compared to that treated with vehicle. No significant difference of airways reactivity was observed between naïve WT and IL-5 Tg mice. However, administration of 1, 3 and 5µg of eotaxin-2 to IL-5 Tg mice induced AHR. The increased airways responsiveness to methacholine peaked at treatment with 3µg eotaxin-2 (P<0.05).
Figure 3.3.1 Effect of eotaxin-2 on the levels of eosinophils in the peripheral blood, BALF and pulmonary tissue and on lung EPO activity in WT and IL-5 Tg mice.

Peripheral blood, BALF and pulmonary tissue eosinophilia and lung EPO activity were determined in WT and IL-5 Tg mice 24 h following i.t. administration of eotaxin-2 (1, 3 or 5µg/20µl 0.1%BSA/PBS). Responses to control vehicle are also shown. Eosinophils in BALF and peripheral blood were identified using morphological criteria with a May-Grunwald-Giemsa staining. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophils. The numbers of eosinophils in lung tissues were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. EPO levels in BALF were measured as described (Materials and Methods). Data are the means ± SEM from n = 4-6 mice per group.

Figure 3.3.1 a, *P<0.05 compared to WT mice either treated with eotaxin-2 (3µg) or vehicle; # P<0.05 compared to naïve IL-5 Tg or vehicle treated IL-5 Tg mice; Figure 3.3.1 b, *P<0.05 compared to WT mice treated with eotaxin-2 (3µg) or vehicle, and naïve IL-5 Tg mice and IL-5 Tg mice treated with vehicle or eotaxin-2 (1µg); Figure 3.3.1 c, *P<0.05 compared to WT mice treated with either eotaxin-2 (3µg) or vehicle; # P<0.05 compared to naïve IL-5 Tg mice or IL-5 Tg mice treated with eotaxin-2 (1µg) or vehicle; Figure 3.3.1 d, *P<0.05 compared to WT mice treated with eotaxin-2 (3µg) or vehicle, and naïve IL-5 Tg mice and IL-5 Tg mice treated with vehicle or eotaxin-2 (1µg).
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

a. Blood

b. BALF

c. Tissue

d. EPO
Figure 3.3.2 Airways reactivity to methacholine in eotaxin-2 treated WT and IL-5 Tg mice.

Airways reactivity to methacholine in WT and IL-5 Tg mice was determined 24 h following i.t. administration of eotaxin-2 (1, 3, or 5µg/20µl 0.1%BSA/PBS). Responses to control vehicle are also shown. Airways reactivity to methacholine challenge was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at 25mg/ml methacholine (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induced maximal responsiveness. Data are the means ± SEM from n = 4-6 mice per group.

*P<0.05 compared to WT mice treated with eotaxin-2 (3µg) or vehicle, and naïve IL-5 Tg or IL-5 Tg mice treated with vehicle; #P<0.05 compared to IL-5 Tg mice treated with eotaxin-2 (1µg).
3.3.3 Role of peripheral blood eosinophilia in the onset of eotaxin-2 induced pulmonary eosinophilia and AHR

The observation that pulmonary eosinophilia (Figure 3.3.3 d) and AHR (Figure 3.3.3 b) could only be induced by i.t. instillation of eotaxin-2 to IL-5 Tg mice suggested that the response was dependent on IL-5 and the induction of peripheral blood eosinophilia. To further investigate the interplay between IL-5, eosinophils and eotaxin-2 in the induction of AHR, an experimental model of eotaxin-2 induced AHR was developed in WT mice (Figure 3.3.3 a). Initially, WT mice were administered IL-5 (50ng in 200µl saline) i.v. over 4 days to promote a peripheral blood eosinophilia that was comparable to that observed during aeroallergen induced allergic airways disease (Webb et al., 2000). In the presence of the peripheral blood eosinophilia, i.t. instillation of eotaxin-2 (3µg) promoted a pulmonary eosinophilia (Figure 3.3.3 c), interestingly, the increased level of eosinophils in the lung was not associated with a significant alteration in airways reactivity to methacholine (Figure 3.3.3 a). However, i.t. administration of both IL-5 and eotaxin-1 in the presence of a peripheral blood eosinophilia induced by i.v. IL-5 promoted AHR concurrently with a pulmonary eosinophilia (Figure 3.3.3 a, P<0.05). Intratracheal administration of both IL-5 and eotaxin-2 in the absence of IL-5 i.v. treatment was also sufficient to promote pulmonary eosinophilia and AHR in WT mice (Figure 3.3.3 c). Collectively, these findings suggest that eotaxin-2 induced pulmonary eosinophilia and AHR is dependent on the presence of a peripheral blood eosinophilia and elevated pulmonary IL-5 levels. Furthermore, the observation that i.t. instillation of IL-5 following i.v. IL-5 treatment did not induce a pulmonary eosinophilia or AHR suggests that IL-5 in the pulmonary compartment alone is not sufficient for the induction of AHR.

These findings were supported by i.t. instillation of eotaxin-2 to IL-5 Tg mice (Figure 3.3.3 b and d). At baseline, the level of pulmonary eosinophilia in IL-5 Tg mice is approximately 10-fold higher than that observed in eotaxin-2/IL-5 i.t. challenged IL-5 i.v. injected WT mice (Figure 3.3.3 a and c). However, there was no significant difference in the level of airways reactivity at baseline when compared to vehicle treated WT or IL-5 Tg mice (Figure 3.3.3 a and b). Intratracheal administration of eotaxin-2 (3µg) increased the pulmonary eosinophil level by 2-fold in IL-5 Tg mice as compared to vehicle treated IL-5 Tg mice (Figure 3.3.3 c) and this increase was intimately associated with the development of AHR (Figure 3.3.3 b and d).
Figure 3.3.3 Role of IL-5 in eotaxin-2 mediated pulmonary eosinophilia and AHR in WT and IL-5 Tg mice.

Airways responses and pulmonary eosinophilia in WT (a and c) and IL-5 Tg mice (b and d). In WT mice responses were assessed 24 h following i.t. instillation of eotaxin-2 and/or IL-5 (3 and 1µg respectively/20µl 0.1%BSA/PBS) after 4 i.v. injections of IL-5 (50ng/200µl PBS) daily for 4 consecutive days. In IL-5 Tg mice responses were determined 24 h following i.t. instillation of eotaxin-2 (3µg/20µl 0.1%BSA/PBS). Airways reactivity to methacholine challenge was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at 25mg/ml of methacholine (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induced maximal responsiveness. Number of peribronchial/perivascular eosinophils per 7-10 similar high-powered fields (HPF) (x40 magnification) per mouse. Data are the means ± SEM from n = 4-6 mice per group.

Figure 3.3.3 a, *P<0.05 compared to vehicle group, IL-5 i.v. and i.t. group or IL-5 i.v. and eotaxin-2 i.t. group; Figure 3.3.3 b, *P<0.05 compared to vehicle or non treatment group; Figure 3.3.3 c, *P<0.05 compared to vehicle or IL-5 i.v. and i.t. group; Figure 3.3.3 d, *P<0.05 compared to vehicle or non treatment group.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

a. Penh % increase over baseline

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>IL-5 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (4x50ng) i.v.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (1ug) i.t.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eotaxin-2 (3ug) i.t.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

b. Penh % increase over baseline

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>IL-5 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (4x50ng) i.v.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (1ug) i.t.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eotaxin-2 (3ug) i.t.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

c. Eosinophil number/HPF

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>IL-5 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (4x50ng) i.v.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (1ug) i.t.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eotaxin-2 (3ug) i.t.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

d. Eosinophil number/HPF

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>IL-5 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (4x50ng) i.v.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-5 (1ug) i.t.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eotaxin-2 (3ug) i.t.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

3.3.4 Eotaxin-2 promotes the upregulation of pulmonary IL-13 levels and mucus secretion in IL-5 Tg mice

Intratracheal administration of eotaxin-2 to IL-5 Tg mice promoted a pulmonary eosinophilia and AHR response (Figure 3.3.1 and 3.3.2). AHR has been shown to be regulated in BALB/c mice by multiple pathways, which include IL-5/eosinophil dependent and IL-13 dependent pathways (Webb et al., 2000). To further clarify the mechanism of eotaxin-2 induced AHR, we determined IL-13 levels in BALF and measured mucus secretion following eotaxin-2 (i.t.) and IL-5 (i.t. and i.v.) treatment of WT and IL-5 Tg mice (Figure 3.3.4. a, b and c). Since the effects of eotaxin-2 were exaggerated in IL-5 Tg mice we initially investigated the effects of eotaxin-2 on IL-13 production and mucus secretion in IL-5 Tg mice (Figure 3.3.4 a and b). Intratracheal administration of eotaxin-2 (3µg) to IL-5 Tg mice induced a significant increase in BALF IL-13 levels compared to vehicle treated mice (Figure 3.3.4 a; P<0.05). Notably, the increase in IL-13 levels was directly associated with increased mucus secretion (a process that is highly dependent on IL-13) in these mice (Figure 3.3.4 b; P<0.05).

To identify whether eotaxin-2 induced upregulation of IL-13 is dependent on a peripheral- or pulmonary- eosinophilia or both, WT mice were i.t. challenged with eotaxin-2 in the presence and absence of i.t. IL-5. No significant increase in IL-13 levels was observed following i.v. and/or i.t. administration of IL-5 in the absence of eotaxin-2 (Figure 3.3.4 c). These data suggest that elevated pulmonary levels of IL-5 alone are insufficient to promote IL-13 production in respiratory secretions. Furthermore, i.v. or i.t. administration of IL-5 and i.t. instillation of eotaxin-2 did not enhance IL-13 levels in the BALF of WT mice (Figure 3.3.4 c). Only following both i.v. and i.t. administration of IL-5 and i.t. instillation of eotaxin-2 was a significant increase in BALF IL-13 levels observed (Figure 3.3.4 c).

3.3.5 Role of IL-4Rα, STAT6, IL-13 and eosinophil regulatory molecules in the induction of eotaxin-2 mediated pulmonary eosinophilia, AHR and increased IL-13 levels.

To identify the contribution of IL-4Rα, IL-13, STAT6 and IL-5/eotaxin-1 signalling pathways in the induction of eotaxin-2 mediated eosinophil infiltration, IL-5 (50ng) was administered i.v. to mice deficient in these factors, followed by i.t. challenge with IL-5
Figure 3.3.4 Effect of eotaxin-2 on IL-13 levels in BALF and on airway epithelial mucus secretion in WT and IL-5 Tg mice.

IL-13 levels in BALF (a) and numbers of mucus-secreting cell (b) were determined 24 h following i.t. instillation of eotaxin-2 (3µg/20µl 0.1%BSA/PBS) in IL-5 Tg mice. IL-13 levels in BALF (c) were determined 24 h following i.t. instillation of eotaxin-2 and/or IL-5 (3 and 1µg, respectively/20µl 0.1%BSA/PBS) in WT mice after i.v. injections of IL-5 (50ng/200µl PBS) daily for 4 consecutive days. Lung sections were stained with alcian blue/PAS for mucus-secreting cells. The numbers of mucus-secreting cells in the central bronchial epithelium regions were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. There was no mucus-staining cell observed in WT mice after any of the treatments (data not shown). Data represent the means ± SEM from n = 4-6 mice per group.

Figure 3.3.4 a and b, *P<0.05 compared to vehicle or non treatment group; Figure 3.3.4 c, *P<0.05 compared to all other groups.
a. BALF IL-13 (ng/ml)

- Vehicle
  - Eotaxin-2 (3ug) i.t.

b. Mucus-staining cell number/HPF

- Vehicle
  - Eotaxin-2 (3ug) i.t.

C. BALF IL-13 (ng/ml)

- Vehicle
  - IL-5 (4x50ng) i.v.
  - IL-5 (1ug) i.t.
  - Eotaxin-2 (3ug) i.t.

IL-5 Tg

WT
and eotaxin-1 (1µg and 3µg/20µl vehicle, respectively). AHR, eosinophil infiltration and IL-13 levels in BALF were determined. Eotaxin-2 mediated AHR was critically dependent on the IL-4Rα, IL-13, STAT6 and IL-5/eotaxin-1 (Figure 3.3.5 a-e). Abolition of AHR in IL-4Rα-, IL-13-, STAT6- and IL-5/eotaxin-1- deficient mice (figure 3.3.5) directly correlated with the inability of these mice to produce IL-13 in the lung in response to eotaxin-2 administration (Figure 3.3.6 b). Eotaxin-2 induced pulmonary eosinophilia was not dependent on IL-4Rα, IL-13 or STAT6 expression (Figure 3.3.6 b). However, pulmonary eosinophilia was abolished in IL-5/eotaxin-1-deficient mice suggesting that endogenous IL-5 and/or eotaxin-1- are, in part, critical for eotaxin-2 mediated pulmonary eosinophilia, possibly by maintaining circulating and bone marrow numbers of eosinophils (Figure 3.3.6 b; P<0.05). Furthermore, the abolishment of pulmonary eosinophilia in eotaxin-2 treated IL-5/eotaxin-1-deficient mice correlated with a significant reduction in IL-13 levels in BALF (Figure 3.3.5 b and c).

3.3.6 Role of CD4⁺ T-cells and CCR3 receptor in the onset of eotaxin-2 induced pulmonary eosinophilia and AHR

3.3.6.1 Role of CD4⁺ T-cells

Intratracheal administration of eotaxin-2 to WT mice in the presence of a peripheral blood eosinophilia induced the up-regulation of IL-13, pulmonary eosinophilia and AHR. Notably, in murine models of experimental asthma, CD4⁺ Th2 cells play a key role in IL-13 production, pulmonary eosinophilia and AHR. To elucidate whether CD4⁺ T-cells play a role in eotaxin-2 induced IL-13 production, pulmonary eosinophilia and AHR, mice were i.p. injected with anti-CD4 mAb followed by i.t. and i.v. administration of IL-5, and i.t. instillation of eotaxin-2. Eotaxin-2 instillation to mice pretreated with control mAb followed by i.v. and i.t. administration of IL-5 developed AHR, pulmonary eosinophilia and up-regulation of BALF IL-13 levels (Figure 3.3.7 and 3.3.8 a, c, d). Interestingly, depletion of CD4⁺ T-cells by anti-CD4 mAb abolished AHR, and suppressed pulmonary eosinophilia and IL-13 production induced by eotaxin-2 (Figure 3.3.7 and 3.3.8 a, c, d). Notably, the abolishment of AHR in eotaxin-2 treated mice depleted of CD4⁺ T-cells correlated with a significant reduction in IL-13 levels in BALF. Depletion of CD4⁺ T-cells exhibited no effect on numbers of circulating eosinophils (Figure 3.3.8 b).
Figure 3.3.5 Effect of eotaxin-2 on the development of AHR in WT mice and IL-4Rα-, IL-13-, STAT6- and IL-5/eotaxin-1- deficient mice.

Airways reactivity in WT mice and IL-5/eotaxin-1-, IL-13-, IL-4Rα- and STAT6-deficient mice was measured 24 h following i.t. instillation of eotaxin-2 and/or IL-5 (3 and 1µg, respectively/20µl 0.1%BSA/PBS) after i.v. injections of IL-5 (50ng/200µl PBS) daily for 4 consecutive days. Airways reactivity to methacholine challenge was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to respective group treated with IL-5 only (i.t. and i.v.); #P<0.05 as compared to IL-4Rα-, STAT6-, and IL-5/eotaxin-1- deficient mice treated with IL-5 (i.t and i.v.) and eotaxin-2 (3µg) i.t. at the same concentration of methacholine. There was no significant difference found in IL-4Rα-, STAT6-, and IL-5/eotaxin-1- deficient mice between IL-5 (i.t. and i.v.)+ eotaxin-2 and IL-5 (i.t. and i.v.) treatments.
a. WT

b. IL-5/eotaxin-1/−

---

IL-5
IL-5+eotaxin-2

---

c. IL-13/−

d. IL-4R/−

e. STAT6/−
Figure 3.3.6 Effect of eotaxin-2 on eosinophil accumulation and IL-13 levels in BALF of WT mice and IL-4Rα-, IL-13-, STAT6- and IL-5/eotaxin-1-deficient mice.

Eosinophil numbers in lung tissue (a) and BALF IL-13 levels (b) of WT mice and IL-4Rα-, IL-13-, STAT6- and IL-5/eotaxin-1-deficient mice were measured 24 h following i.t. instillation of eotaxin-2 and/or IL-5 (3 and 1μg, respectively/20μl 0.1%BSA/PBS) after i.v. injections of IL-5 (50ng/200μl PBS) daily for 4 consecutive days. The levels of IL-13 in BALF were measured by ELISA. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophils. The numbers of eosinophils in lung tissues were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to respective groups treated with IL-5 only (i.t. and i.v.).
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

**a.**

![Graph showing eosinophil number/HPF](image)

**b.**

![Graph showing BALF IL-13 (ng/ml)](image)
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

Figure 3.3.7 Role of CD4 and CCR3 positive cells in airways reactivity induced by eotaxin-2.

Airways reactivity was measured in WT mice 24 h following i.t. instillation of eotaxin-2 and IL-5 (3 and 1µg, respectively/20µl 0.1%BSA/PBS) after i.v. injections of IL-5 (50ng/200µl PBS) daily for 4 consecutive days. Mice were administrated with isotype control mAb or anti-mouse CD4 (GK1.5) or anti-mouse CCR3 mAb (6S2-19-4). Airways reactivity to methacholine was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to anti-CD4 or anti-CCR3 mAb treatments. There was no significant difference observed between anti-CD4 and anti-CCR3 mAb treatments.
Figure 3.3.8 Role of CD4 and CCR3 positive cells in the production of BALF IL-13 and induction of pulmonary and peripheral blood eosinophilia by eotaxin-2.

Eosinophils in the lung (a and c) and blood (b) and BALF IL-13 levels (d) were examined in WT mice 24 h following i.t. instillation of eotaxin-2 and IL-5 (3 and 1µg, respectively/20µl 0.1%BSA/PBS) after i.v. injections of IL-5 (50ng/200µl PBS) daily for 4 consecutive days. Mice were treated with isotype control or anti-mouse CD4 (GK1.5) or anti-mouse CCR3 mAbs (6S2-19-4). Eosinophils in BALF and peripheral blood were identified using morphological criteria by May-Grunwald-Giemsa staining. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophils. The number of eosinophils in lung tissues were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. IL-13 levels in BALF were measured by ELISA. Data are the means ± SEM from n = 4-6 mice per group.

Figure 3.3.8 a, *P<0.05 compared to anti-CD4 or anti-CCR3 mAbs treatments; #P<0.05 compared to anti-CCR3 mAb treatment; Figure 3.3.8 b, #P<0.05 compared to anti-CCR3 mAb treatments; Figure 3.3.8 c, *P<0.05 as compared to anti-CD4 or anti-CCR3 mAbs treatments; #P<0.05 compared to anti-CCR3 mAb treatment; Figure 3.3.8 d, *P<0.05 as compared to anti-CD4 or anti-CCR3 mAbs treatments.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

(a) BALF eosinophil number $\times 10^4$/ml

(b) PB eosinophils %

(c) Eosinophil number/HPF

(d) BALF IL-13 (ng/ml)
3.3.6.2 Role of CCR3 receptor

The CCR3 receptor, which is crucial for eosinophil homing and activation, is predominantly expressed on the surface of eosinophils and basophils: two cells which play an important role in the development of allergic disease (Kaplan 2001). To investigate whether the CCR3 receptor plays a role in eotaxin-2 induced pulmonary eosinophilia and AHR, mice were pretreated with anti-CCR3 mAb followed by i.t. and i.v. administration of IL-5 and i.t. instillation of eotaxin-2. Depletion of eosinophils by anti-CCR3 mAb totally abolished eotaxin-2 induced pulmonary eosinophilia and AHR (Figure 3.3.7 and Figure 3.3.8 a, c). Moreover, circulating eosinophils were totally eliminated by treatment with the anti-CCR3 mAb (Figure 3.3.8 b). Notably, the loss of eosinophils significantly suppressed IL-13 production induced in combination with IL-5 (Figure 3.3.8 d).

3.3.7 Eotaxin-2 induces pulmonary eosinophilia and IL-13 production but not mucus hypersecretion or AHR in IL-5Tg/STAT6-deficient mice

To further elucidate the mechanism and the contribution of IL-5 and eosinophils to the induction of eotaxin-2 induced AHR and mucus hypersecretion in the lung, IL-5 Tg mice in the BALB/c background were crossed to BALB/c STAT6-deficient mice. By comparison to IL-5 Tg mice, delivery of eotaxin-2 to the airways did not induce AHR or mucus hypersecretion in IL-5 Tg/STAT6-deficient mice (Figure 3.3.9 a and b). However, eotaxin-2 induced the recruitment of eosinophils to airway tissue and promoted the secretion of IL-13 into the BALF (Figure 3.3.9 c and d) in both IL-5 Tg and IL-5 Tg/STAT6-deficient mice. This further supports the previous results in WT mice (Figure 3.3.6) that eosinophils recruited to the airways play an important role in the production of IL-13 in the lung, which subsequently promotes AHR and mucus hypersecretion by STAT6 dependent pathways. Administration of eotaxin-2 significantly increased the number of mucus-staining positive cells in the epithelial region in IL-5 Tg mice (Figure 3.3.9 b), accompanied by the up-regulation of IL-13 in BALF (Figure 3.3.9 d). Although the levels of IL-13 were higher in BALF, mucus hypersecretion was abolished in IL-5 Tg/STAT6-deficient mice (Figure 3.3.9 b and d).
Figure 3.3.9 Eotaxin-2 induces pulmonary eosinophilia and IL-13 production but not mucus hypersecretion or AHR in IL-5 Tg/STAT6-deficient mice.

Airways reactivity (a), numbers of mucus-secreting cells (b) and eosinophils (c) in lung tissues and BALF IL-13 levels (d) in IL-5 Tg/STAT6-deficient and IL-5 Tg mice were determined 24 h following i.t. instillation of eotaxin-2 (3µg/20µl 0.1%BSA/PBS) or the same volume of vehicle. Airways reactivity to methacholine challenge was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at 25mg/ml of methacholine (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induced maximal responsiveness. Lung sections were stained with Carbol's Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissues and mucus-secreting cells in the central bronchial-epithelium regions were enumerated in 7-10 similar high-powered fields (HPF) (x40 magnification) per mouse. BALF IL-13 levels were measured by ELISA. Data are the means ± SEM from n = 4-6 mice per group.

Figure 3.3.9 a, *P<0.05 compared to all other groups; Figure 3.3.9 b, *P<0.05 compared to all other groups; Figure 3.3.9 c, *P<0.05 compared to respective vehicle group; Figure 3.3.9 d, *P<0.05 compared to respective vehicle group.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

![Bar charts showing the cooperative interplay between eotaxin-2 and IL-5](image)

- **a.** Penh % increase over baseline
  - Vehicle
  - Eotaxin-2
  - Methacholine [25mg/ml]

- **b.** Mucus-staining cell number/HPF
  - IL-5Tg/STAT6-/-
  - IL-5Tg

- **c.** Eosinophil number/HPF
  - IL-5Tg/STAT6-/-
  - IL-5Tg

- **d.** BALF IL-13 (ng/ml)
  - IL-5Tg/STAT6-/-
  - IL-5Tg
3.3.8 Comparison of the potency eotaxin-1 and eotaxin-2 for the induction of AHR, pulmonary eosinophilia and IL-13 production in the BALF

3.3.8.1 Eosinophilia induced by eotaxin-1 and eotaxin-2 in the peripheral blood

To investigate eosinophil release into the peripheral blood, WT mice were injected i.v. with eotaxin-1, eotaxin-2 (0.6µg/mouse/200µl PBS) or vehicle (200µl PBS). The effect of i.v. administration of eotaxin-2 on circulating levels of eosinophils has not been previously reported. In this study, eotaxin-1 induced a rapid blood eosinophilia at 30 minutes (Figure 3.3.10.1 P<0.05) that was similar to that reported previously (Mould et al., 1997). This eosinophilia returned to that observed in the vehicle treated group at 1 h. In contrast, eotaxin-2 had no effect on eosinophil release into the peripheral blood. This may indicate that eotaxin-1 and eotaxin-2 play different roles in the induction of eosinophil release into the peripheral blood, although both chemokines function through CCR3.

3.3.8.2 AHR induced by eotaxin-1 and eotaxin-2

To compare eotaxin-1 and eotaxin-2 induced AHR, naïve IL-5 Tg mice were administered i.t. with eotaxin-1 or eotaxin-2, and airways responses to methacholine were assessed after 24 h. The IL-5 Tg mice treated with eotaxin-1 or eotaxin-2 exhibited similar AHR that was significantly elevated compared to vehicle treated mice (Figure 3.3.10.2 P<0.05).

3.3.8.3 Comparison of the effect of eotaxin-1 and eotaxin-2 on pulmonary and BALF eosinophilia, mucus production and BALF IL-13 production

To compare the role of eotaxin-1 and eotaxin-2 in the induction of the allergic phenotypic in the lung, 3µg eotaxin-1 or eotaxin-2 were instilled i.t. into IL-5 Tg mice. Twenty-four h after instillation, both eotaxin-1 and eotaxin-2 induced BALF eosinophilia that was significantly higher than that in the vehicle treated group (Figure 3.3.10.4 a). Histological examination of lung tissue showed morphological changes to the lung of eotaxin-1 and eotaxin-2 treated mice, which included large number infiltrating eosinophils and mucus hypersecretion in epithelium (Figure 3.3.10.3 and .4). Both eotaxin-1 and eotaxin-2 induced significant eosinophil trafficking into BALF and
WT mice were injected i.v. with eotaxin-1 or eotaxin-2 (0.6µg/200µl PBS) or the same volume of PBS. Blood samples were taken before and at 30 and 60 min after i.v. injection of the chemokines or control vehicle for quantification of eosinophil number with Discombes' solution. Results represent mean ± SEM from n = 4-8 mice per group.

* P<0.05 compared to eotaxin-2 or vehicle treated mice at 30 min after i.v. injection. There was no significant difference observed between eotaxin-2 and vehicle treated mice.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR

![Graph showing Penh % increase over baseline against methacholine concentration with lines for vehicle, eotaxin-1, and eotaxin-2](image)

Figure 3.3.10.2 Comparison of the effect between eotaxin-1 and eotaxin-2 on airways reactivity.

Airways reactivity was measured 24 h following i.t. instillation of eotaxin-1 or eotaxin-2 (3µg respectively/20µl 0.1%BSA/PBS) in IL-5 Tg mice or the same volume of vehicle. Airways reactivity to methacholine was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 8-16 mice per group.

*P<0.05 as compared to vehicle treated mice. There was no significant difference between eotaxin-1 and eotaxin-2 treated mice.
Figure 3.3.10.3 Histological features of lung from eotaxin-1, eotaxin-2 and vehicle treated IL-5 Tg mice.

IL-5 Tg mice were treated with eotaxin-1 or eotaxin-2 (3µg/20µl 0.1%BSA/PBS) or the same volume of vehicle. Lungs were removed and fixed in 10% NBF 24 h following treatment and then stained with Carbol’s Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The 10× magnification is for i, iii and v (mucus-staining cell: stained dark red in epithelium) while 40× magnification is for ii, iv and vi (eosinophils: pointed with arrow). Eotaxin-1 (i and ii) and eotaxin-2 (iii and iv) treated mice exhibited large number of eosinophils in the peribronchial and perivascular regions compared to vehicle (v and vi) treated mice. Also, eotaxin-1 (i) and eotaxin-2 (iii) treated mice had a greater number of mucus-staining positive cells in epithelium compare to vehicle (v) treated mice.
Figure 3.3.10.4 Comparison of the effect between eotaxin-1 and eotaxin-2 on BALF IL-13 levels and lung tissue and BALF eosinophil and mucus secretary cell numbers.

BALF and pulmonary tissue eosinophil numbers (a and b), level of mucus production (c) and IL-13 levels in the BALF (d) of IL-5 Tg mice were examined 24 h after i.t. delivery of eotaxin-1 or eotaxin-2 (3µg/20µl 0.1%BSA/PBS) or the same volume of vehicle. Eosinophils in BALF were identified using morphological criteria with May-Grunwald-Giemsa solution. Lung sections were stained with Carbol's Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissues and mucus-secreting cells in the central bronchial epithelium regions were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. IL-13 levels in BALF were measured by ELISA. Data are the means ± SEM from n = 4-8 mice per group.

Figure 3.3.10.4 a, *P<0.05 compared to vehicle treated mice; Figure 3.3.10.4 b, *P<0.05 compared to eotaxin-2 and vehicle treated mice; #P<0.05 compared to vehicle treated mice; Figure 3.3.10.4 c, *P<0.05 as compared to vehicle treated mice; Figure 3.3.10.4 d, *P<0.05 as compared to vehicle treated mice.
Chapter 3 Cooperative interplay between eotaxin-2 and IL-5 regulates pulmonary eosinophilia and IL-13 regulated AHR.
peribronchial regions by comparison to vehicle treated animals (Figure 3.3.10.4 a and b). However, eotaxin-1 exhibited a much stronger capability for inducing eosinophil migration into the peribronchial region than eotaxin-2 (Figure 3.3.10.4; P<0.05). The mucus hypersecretion in response to either eotaxin-1 or eotaxin-2 treatment was significantly higher than in the vehicle treated group. No significant difference in the number of mucus-staining cells in epithelium was observed between eotaxin-1 and eotaxin-2 treated group (Figure 3.3.10.4 c). IL-13 levels in BALF from mice treated with either eotaxin-1 or eotaxin-2 were significantly higher than that in vehicle treated group. Similarly, no difference in IL-13 levels in BALF was found between eotaxin-1 and eotaxin-2 treated groups (Figure 3.3.10.4 d).
3.4 Discussion

The CC chemokines eotaxin-1 and eotaxin-2 have been identified as ligands that exclusively signal through CCR3 and selectively modulate eosinophil migration (Forssmann et al., 1997; Elsner et al., 1998). Clinical and experimental studies have demonstrated that these molecules regulate eosinophil trafficking at baseline and during allergic inflammatory responses (Ying et al., 1999; Zimmermann et al., 2000). However, the contribution of these molecules to eosinophil migration and induction of the pathological features of allergic disease is not fully understood. In this Chapter it was shown that eotaxin-2 in concert with IL-5 promotes selective eosinophil recruitment to the lung that predisposes to the development of AHR, mucus hypersecretion, and increased levels of EPO and IL-13 in respiratory secretions. Furthermore, both molecules can induce AHR.

Intratracheal administration of eotaxin-2 induced a pulmonary eosinophilia and AHR in IL-5 Tg mice but not in WT mice. However, following administration of IL-5 to the lung and blood of WT mice, eotaxin-2 induced both eosinophil accumulation in pulmonary tissue and AHR. These data suggest that eotaxin-2 mediated eosinophil accumulation in the lung was critically dependent on the presence of IL-5 and/or a peripheral blood eosinophilia. These findings were further supported by the demonstration that eotaxin-2 mediated eosinophil accumulation was abolished in IL-5/eotaxin-1-deficient mice. Furthermore, following administration of exogenous IL-5 i.t. and i.v., delivery of eotaxin-2 to the lung induced a pulmonary eosinophilia, confirming an important role for IL-5 and a peripheral blood eosinophilia in eotaxin-2 induced accumulation of eosinophilia in the lung. Thus like eotaxin-1, eotaxin-2 employs IL-5 as an important co-factor for the recruitment of eosinophil into the lung and the subsequent induction of pulmonary processes.

Eotaxin-2 has been reported to modulate a number of eosinophil effector functions in in vivo assays including actin polymerization, modulating respiratory burst and CCR3 internalization (Elsner et al., 1998; Elsner et al., 2000). Interestingly, the efficacy of eotaxin-2-induced respiratory burst in eosinophils is comparable to that of eotaxin-1 and C5a (Elsner et al., 2000). Intratracheal instillation of eotaxin-2 induced a pulmonary eosinophilia that was associated with elevated levels of extracellular EPO activity, suggesting that eotaxin-2 is also an effective activator of eosinophils in vivo. Eotaxin-2
was also shown to stimulate the upregulation of IL-13 levels in respiratory secretions. In the Chapter 2, IL-5/eotaxin-1-deficient mouse exhibited a very low level of bone marrow, circulating and tissue eosinophils and the failure to develop eosinophilia and AHR under allergen challenge. The failure to induce AHR and pulmonary eosinophilia by eotaxin-2 in IL-5/eotaxin-1-deficient mice also provided supporting evidence that eosinophils may be involved in initiating these responses. These findings, in IL-5/eotaxin-1-deficient mice and those in mice pretreated with anti-CCR3 mAb, where maximal levels of IL-13 in respiratory secretions were only observed in the presence of a significant pulmonary eosinophilia, indicates that this granulocyte directly or indirectly modulates IL-13 production. Recently, investigators have identified the presence of IL-13 in human eosinophils (Woerly et al., 1999). Thus, these finding are suggesting that activated eosinophils may either directly or indirectly be involved in IL-13 production.

Notably, eotaxin-2 induced upregulation of IL-13 production was shown to involve IL-4Rα and STAT6 pathways. In addition, it was shown that eotaxin-2 mediated IL-13 production is dependent on CD4+ T-cells or on cells expressing CCR3. Pretreatment with anti-CD4+ or anti-CCR3 mAbs abolished pulmonary eosinophilia and IL-13 up-regulation induced by eotaxin-2 and IL-5 administration. These observations suggest that both CD4+ T-cells and CCR3+ cells are critically involved in eotaxin-2 induced responses. Experimental investigations have demonstrated that IL-13 is predominantly produced by CD4+ T-cells (Levy et al., 1997; Bourreau et al., 2001). Notably, IL-13 levels, albeit reduced 3-4 fold, were detected in respiratory secretions following eotaxin-2 administration after the depletion of CD4+ T-cells. However, murine CD4+ T-cells have been shown not to express the eotaxin receptor, CCR3 (Grimaldi et al., 1999). Eosinophils may modulate CD4+ T-cell function and IL-13 production through direct or indirect mechanism. In this Chapter, it was shown that the maximal level of IL-13 in respiratory secretions was observed in the presence of IL-5 and a pulmonary eosinophilia. Collectively, these data suggest that IL-13 may be derived from CD4+ T-cells and that eotaxin-2 up-regulation of IL-13 may be through an indirect mechanism possibly via primed eosinophils modulating CD4+ T-cell activation.

The molecular mechanisms regulating the development of AHR are not yet understood. It is currently thought that two independent pathways, intimately associated with IL-13 and IL-5/eosinophils contribute to the induction of AHR. However, both pathways are
critically linked to the activation of CD4+ T-cells. IL-13 may contribute to AHR in a multifunctional manner such as directly priming ASM for enhanced responsiveness to spasmogenetic stimuli, promoting the release of spasmogenetic mediators, by inducing smooth muscle proliferation and/or by altering matrix protein deposition (Wills-Karp 2001). Eosinophils are also thought to contribute to AHR through the release of cytotoxic granule proteins that directly modulate ASM function and promote tissue damage (Rothenberg 1998). In this Chapter, we show that eotaxin-2 mediated AHR was dependent on CD4+ T-cells, IL-4Rα and STAT6 expression, and correlated with the up-regulation of IL-13. Furthermore, eosinophil accumulation could occur independently of AHR as demonstrated in the IL-4Rα-, IL-13- and STAT6- deficient mice, whereas, the presence of IL-13 was critically linked to eotaxin-2 induced AHR. These findings suggest a critical role for IL-13 in the induction of AHR and show that eotaxin-2 mediated IL-13-dependent AHR involves IL-4Rα/STAT6 signalling pathways and CD4+ T-cells. More importantly, it also suggests that eosinophils may modulate downstream pathways which regulate the development of AHR. Eosinophils secrete IL-4 to modulate cytokine production from CD4+ T-cells (Brunet et al., 1999; MacKenzie et al., 2001). Studies have also demonstrated that IL-4 enhances IL-13 production in CD45RA+ T-cells and in the absence of IL-4 signalling, IL-13 derived from CD4+ T-cells is significantly suppressed (Jung et al., 1996; Webb et al., 2000). Thus, IL-4 derived from activated eosinophils may possibly modulate IL-13 production from resident CD4+ T-cells in the lung. Investigators also demonstrated that eosinophils regulate cytokine production from cultured CD4+ Th2 cells through the secretion of IL-18 (Mattes et al., 2002). Interestingly, in IL-5/eotaxin-1 deficient mice (and anti-CCR3 mAb treated mice), eotaxin-2 mediated eosinophil accumulation and elevated IL-13 levels were ablated, as was the associated AHR. This again suggests that eosinophils are intimately involved in the eotaxin-2 mediated IL-13 production and also the subsequent development of AHR.

In contrast to the striking differences in overall sequence homology of eotaxin-1 and eotaxin-2, experimental investigations suggest that these molecules have very similar functional activities such as eosinophil chemoattraction, eosinophil activation and degranulation (Forssmann et al., 1997; Elsner et al., 1998; Ying et al., 1999). However, the evolutionary conservation of eotaxin-1 and eotaxin-2 may suggest that these molecules would have divergent non-redundant biological functions (Mayer et al., 2000). While, these molecules may have similar functionality, it is possible that in vivo,
in the presence of regulatory pathways of CCR3 effector function, eotaxin-1 and eotaxin-2 may possess divergent functions. For example, sequence divergence between eotaxin-1 and eotaxin-2 allows one to postulate that eotaxins may be susceptible to differential rates of degradation by proteolytic enzymes such as membrane-associated serine protease dipeptidyl peptidase IV (CD26) (Struyf et al., 1999; Mayer et al., 2000; Ye et al., 2000). Furthermore, eotaxin-1 has been shown to act as an endogenous antagonist of various other chemokine receptors including CXCR3 and CCR2 (Weng et al., 1998; Ogilvie et al., 2001). It is not yet known whether eotaxin-2 also antagonizes CXCR3, CCR2 or other chemokine receptors. Eotaxin-1, like eotaxin-2, was shown to induce AHR, eosinophil migration into the BALF and upregulate IL-13 and mucus production. However, eotaxin-1 exhibited a stronger capacity to induce pulmonary eosinophilia and also promoted a blood eosinophilia when administered i.v. by contrast to eotaxin-2. The capacity of eotaxin-1 and eotaxin-2 to promote lung IL-13 production and mucus secretion was similar (Figure 3.3.10.3). Recently, a study in atopic individuals demonstrated that eotaxin-1, but not eotaxin-2, was expressed during the rapid early phase response and this was associated with eosinophil accumulation (Ying et al., 1999). Whereas, the late phase eosinophil response was associated with eotaxin-2 but not eotaxin-1 expression. This may imply that eotaxin-1 and eotaxin-2 function differently in the temporal stages of inflammation during allergic responses.

In conclusion, the results in this Chapter demonstrate that eotaxin-2 in conjunction with IL-5 can promote eosinophil accumulation and activation and of elevated levels of IL-13 in the lung and induction of AHR. The induction of a pulmonary eosinophilia is dependent on the presence of a peripheral blood eosinophilia, and the presence of IL-5 and/or eotaxin-1 in the lung. The upregulation of IL-13 production is mediated through the IL-4Rα/STAT6 signalling pathways and is associated with eosinophil accumulation. CCR3+ cells regulate for eotaxin-2 induced AHR, pulmonary eosinophilia and IL-13 up-regulation. Furthermore, eotaxin-2 induced AHR is mediated by the IL-4Rα/STAT6 pathway, probably through the upregulation of IL-13 by CD4+ T-cells. Interestingly, while IL-13 was central for the induction of AHR, the role of eosinophils in the AHR response was possibly via either the upregulation of IL-13 production by CD4+ T-cells or modulation of ASM contraction by release granular proteins. These findings establish the potential importance of eotaxin-2 in the induction of key phenotypic characteristics of experimental asthma and suggest a possible link between eosinophil- and IL-13-mediated AHR responses.
CHAPTER 4:

Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR
4.1 Introduction

IL-13, an important CD4\(^+\) Th2 cell cytokine, has been identified a potential key regulator of the pathogenic mechanisms underlying allergic disease (Cohn et al., 1999; Zhu et al., 1999; Venkayya et al., 2002). IL-13 is expressed at elevated levels in atopic and nonatopic asthma (Huang et al., 1995; Humbert et al., 1997), atopic dermatitis (Akdis et al., 1997; Katagiri et al., 1997), and allergic rhinitis (Pawankar et al., 1995). Furthermore, IL-13 is produced in high quantities by Th2 cells, and this cytokine regulates an array of immunopathogenic effects that are hallmark features of asthma (IgE production, mucus hypersecretion, eosinophil recruitment, AHR, and the expression of CD23, leukocyte adhesion systems, and chemokines) (Zurawski et al., 1994; Bochner et al., 1995; Horie et al., 1997; Emson et al., 1998; Grunig et al., 1998; Wills-Karp et al., 1998). Investigations have also demonstrated a central role for IL-13 in the regulation of experimental asthma in mice (Grunig et al., 1998; Wills-Karp et al., 1998). Administration of IL-13 to naïve mice or chronic overexpression of this cytokine in the lung promotes mucus cell metaplasia, eosinophil accumulation, fibrosis and AHR (Wills-Karp et al., 1998). Furthermore, neutralization of IL-13 activity by treatment with the soluble form (fusion protein) of the IL-13Rα2 chain reverses AHR and pulmonary mucus cell hyperplasia in the allergic lung of allergen-challenged mice (Grunig et al., 1998).

Investigations have shown that IL-13 plays an important role in the induction of pulmonary eosinophilia by the upregulation of chemokines such as eotaxin-1, eotaxin-2, MCP-1 and MIP-1α in the lung (Goebeler et al., 1997; de Vries 1998; Li et al., 1999; Pype et al., 1999). Furthermore, CCR3-binding chemokines such as eotaxin-1 and eotaxin-2 play a critical role in the regulation of eosinophil infiltration into inflammatory tissues as shown and reviewed in previous Chapters. However, the role of IL-5 and eotaxin-1 in the mechanism of IL-13 induced eosinophil trafficking is not clear.

Airways remodeling contributes to the pathogenesis of asthma (Bento et al., 1998; Fish et al., 1999; Witczak et al., 2002). Abnormal regulation of matrix proteins in the asthmatic lung may contribute to airways remodeling (Huang et al., 1999; Lechapt-Zalcman et al., 2000). In particular, decreased levels of MMP and increased levels of tissue specific inhibitors of metalloproteinases (TIMP) (Lechapt-Zalcman et al., 2000;
Shaida et al., 2001) have been linked to remodeling events. Cysteine protease enzymes such as cathepsin -B and -S play crucial roles in the digestion of extracellular matrix proteins (Buhling et al., 1999). Mucin genes (e.g. Muc-2, -4, -5AC and -5B) are particularly important for the production of mucus in airways and overexpression may promote airways occlusion in asthmatics (Yoon et al., 1998; Bernacki et al., 1999; Rose et al., 2001). Although IL-13 expression has been shown to correlate with airways remodeling, it is not clear how this cytokine mediates these proteinases or mucin genes.

Thus, to further elucidate spatially and temporally the mechanisms involved in IL-13 induced AHR, pulmonary eosinophilia and mucus hypersecretion, these pulmonary responses were characterized in WT mice, which received recombinant murine IL-13 by i.t. administration. IL-4/IL-13-deficient mice were employed to identify the role of endogenous IL-4 and IL-13 in recombinant IL-13 induced pulmonary responses. Similarly IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice were also employed to investigate the role of these eosinophil regulatory molecules in IL-13 mediated processes. In this Chapter, RT-PCR was also employed to elucidate the underlying molecular changes in response to i.t. IL-13 challenge. In particular, transcription profiling was performed for factors implicated in regulating inflammation, AHR and airways remodeling. In this Chapter we show that IL-13 induces AHR independently of eosinophilia and mucus hypersecretion. Furthermore, IL-13 induces the expression of a wide range of inflammatory mediators indicating that this molecule is a key trigger for the induction of allergic inflammatory cascades.
4.2 Materials and methods

4.2.1 Mice

See 2.2.1 and 3.2.1

4.2.2 Intratracheal instillation of recombinant IL-13

Mice were anesthetized with an i.v. injection of 100µl Saffan solution (1:4 diluted in saline; v/v). Mice were then intubated with a 22-gauge catheter needle, through which murine recombinant IL-13 (gift from Genetics Institute, Cambridge, MA) (10µg dissolved in 20µl PBS) or vehicle control (PBS) was instilled. AHR, mucus production, and eosinophilia were measured at 6, 12, 24, 48, 96 h and 8 d after i.t. delivery of IL-13 to determine temporal responses.

4.2.3 Measurement of airways reactivity to methacholine

See 2.2.3

4.2.4 Characterization of eosinophils and mucus-staining cells in lung tissue

See 2.2.4

4.2.5 Collection and analysis of peripheral blood and BALF

See 2.2.5

Briefly, 24 and 48 h (peak responses for the pre-inflammatory phase [PIP] and inflammatory phase [IP], respectively) after i.t. delivery of IL-13 to the lung, tracheas were cannulated and airway lumina were washed with 2ml PBS. Approximately 1.7ml of the instilled fluid was recovered per wash.
4.2.6 Reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from IL-13 or PBS treated lung at 12, 48 h and 8 d by a standard method with RNAzol B (Biotech Laboratories, Houston, TX, USA). A RT-PCR procedure was performed as described previously (Svetic et al., 1991) and with some modification (Oswald et al., 1992). Briefly, RT-RNA was performed in 25μl (final volume) containing: 2.5mM of each of four deoxynucleotide triphosphates (dNTP); 1× reverse transcriptase buffer (50mM Tris-HCL, pH 8.3; 75mM KCL, 3mM MgCl); 8mM DTT; 0.5U random hexamers; 1μg total RNA; 200U reverse transcriptase (GIBCO, BRL, Gaithersburg, MD). The reaction mixture was incubated at 37°C for 60 min, then heated to 90°C for 5 minutes to denature the MMLV-reverse transcriptase and cooled on ice for 3 min. The final RT-PCR product was then diluted 1:8 by the addition of 175μl distilled H2O and stored at -20°C. After the appropriate number of PCR cycles, the amplified DNA was analyzed by gel electrophoresis.

The primers for all genes were purchased from GIBCO (GIBCO BRL products, New Zealand). The primer sequences for different factors are as below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>sense primer 5’-3’</th>
<th>antisense primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>gttggtacagggcagacatgttg</td>
<td>gattcaacttgccctcatcttaggc</td>
</tr>
<tr>
<td>MCP-1</td>
<td>accagccaacttcactgaagc</td>
<td>cagaattgctgaggtgttgtg</td>
</tr>
<tr>
<td>MCP-2</td>
<td>aagctcctgctgtctgcttag</td>
<td>atgagaaacagcagccaccgacc</td>
</tr>
<tr>
<td>MCP-3</td>
<td>aclactgctgctgctgcttag</td>
<td>gtaaaaatgggagaaggaggaat</td>
</tr>
<tr>
<td>MCP-5</td>
<td>ctaaccctcctgcttagc</td>
<td>ctaacccacttctccttg</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>tagtctagctggctggtgtag</td>
<td>cagttctcaacccagggctat</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>cccacctctgctgtttctc</td>
<td>gagggacccctcctggaat</td>
</tr>
<tr>
<td>MIP-2</td>
<td>aagttctgactgtctgtaatg</td>
<td>cttggggctctccggtgag</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>cacgactgtgctctcttagc</td>
<td>agagggtcacagccttttt</td>
</tr>
<tr>
<td>MDC</td>
<td>cctgggctctgctgcttctc</td>
<td>cagggggatggagggtagtaaa</td>
</tr>
<tr>
<td>RANTES</td>
<td>cccctacacatcactcttagc</td>
<td>gggagcgtataccaggggta</td>
</tr>
<tr>
<td>eotaxin-1</td>
<td>tccaccactgcagactccacag</td>
<td>cccacatcctctccatgcce</td>
</tr>
<tr>
<td>eotaxin-2</td>
<td>cgtgccagtccctttatctcc</td>
<td>cccctttgagggctgtgtt</td>
</tr>
<tr>
<td>TECK</td>
<td>ctgggttaccagcagagag</td>
<td>cctctgatccacacact</td>
</tr>
<tr>
<td>TARC</td>
<td>agtttctggtgccctag</td>
<td>ttgtggtgctctagtgc</td>
</tr>
</tbody>
</table>

108
### 4.2.7 Statistical analysis

See 2.2.8
Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

4.3 Results

4.3.1 Temporal characterization of IL-13 induced AHR, eosinophil accumulation and mucus secretion

Intratracheal instillation of 10µg of IL-13 into naïve WT mice induced a rapid and potent AHR. An early-onset PIP-associated AHR was observed between 6-24 h and was followed by an amplified and sustained IP-associated AHR that peaked at 48–96 h (Figure 4.3.1). The maximal IP AHR response (48 h) was significantly greater than the maximal PIP (12-24 h) response (Figure 4.3.1; P< 0.05). AHR returned to baseline levels at 8 d (Figure 4.3.1 f). No significant difference was found between the PBS treated groups at the different timepoints. This suggested that there were two temporally distinct responses relating to the induction of AHR.

Histological examination of lung tissue demonstrated morphological changes to the lung of WT mice treated with IL-13 from 48 h to 8 d which included eosinophil recruitment and mucus hypersecretion (Figure 4.3.2 a and Figure 4.3.3 a). Although some eosinophils were observed in the parenchyma of IL-13 treated mice, the majority of eosinophils were localized around peribronchial and perivascular regions. These morphological changes were not evident in the PBS treated groups at all timepoints. Large numbers of mucus-secreting cells were evident in the larger airways of IL-13 treated WT mice but not in the PBS treated groups 48 h and 8 d after IL-13 administrations.

Quantitative analysis of eosinophil infiltration and mucus-hypersecretion demonstrated that the PIP IL-13-induced AHR was not associated with eosinophil infiltration or mucus secretion in the lung (6-24 h) (Figure 4.3.1; Figure 4.3.2 b; Figure 4.3.3 b). However, 48 h following instillations, eosinophil numbers were significantly elevated in IL-13 treated mice when compared to PBS treated mice (P<0.05) (Figure 4.3.2 b). Similarly to eosinophil infiltration, mucus-staining cell levels were only significantly elevated 48 h after IL-13 administration (Figure 4.3.3 b) (P<0.05). Notably, mucus secretion and eosinophil numbers remained consistently elevated for up to 8 d following IL-13 challenge (Figure 4.3.2 b and Figure 4.3.3 b). These sustained responses to a single dose of IL-13 highlight the potency of this cytokine and its potential ability to generate allergic inflammatory cascades.
Figure 4.3.1 Characterization of the temporal development of IL-13 induced AHR.

Airways reactivity to methacholine in WT mice was determined at 6, 12, 24, 48, 96 h and 8 d following i.t. administration of IL-13 (10µg/20µl PBS). Responses to control vehicle (PBS, pH 7.4) are also shown. Airways reactivity to methacholine challenge was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 4-6 mice per group.

* P<0.05 compared to respective PBS treated groups at same dose of methacholine.
Figure 4.3.2a Histological features of WT lungs exposed to IL-13.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS). At 12, 48 h and 8 d following treatment, lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with Carbol’s Chromotrope-Hematoxylin for eosinophils. The magnification for i, iii and v is x10 and that for ii, iv and vi is x40. Eosinophil infiltration (arrow) was observed at 48 h (ii and iii) and 8 d (iv and v) following IL-13 treatment. Eosinophil infiltration was rare at 12 h (i and ii).
Figure 4.3.2b Kinetic characterization of IL-13 induced eosinophil infiltration.

Eosinophilia in lung tissue of WT mice was determined at 6, 12, 24, 48, 96 h and 8 d following i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS. Lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with Carbol’s Chromotrope-Hematoxylin for eosinophils. The numbers of eosinophils in lung tissues were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to the respective PBS treated groups.
Figure 4.3.3a Mucus hypersecretion in the lungs of IL-13 treated WT mice.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS). At 12, 48 h and 8 d following the treatment, lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with alcian blue/PAS for mucus-secreting cells. No significant increase in mucus staining cells around the bronchial epithelial region was observed at 12 h (i). Increased numbers of mucus-staining cells (stained with dark red in epithelium) were observed around bronchial epithelial regions at 48 h (ii) and 8 d (iii) following IL-13 treatment (×20 magnification).
(i) IL-13 12 h

(ii) IL-13 48 h

(iii) IL-13 8 d
Figure 4.3.3b Kinetic characterization of IL-13 induced mucus hypersecretion.

The numbers of mucus-staining cells in the lung tissues of WT mice were determined at 6, 12, 24, 48, 96 h and 8 d following i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS. Lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with alcian blue/PAS for mucus-secreting cells. The number of mucus-secreting cells in the bronchial epithelium regions was enumerated in 7-10 similar high-powered fields (HPF) (x40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to respective PBS treated groups.
4.3.2 BALF and peripheral blood leukocyte numbers in response to IL-13 administration in the PIP (12 h) and IP response (48 h)

Intratracheal administration of IL-13 induced macrophage accumulation in BALF in both PIP and IP responses. Macrophage numbers in BALF of IL-13 treated mice were significantly higher than that in the PBS treated mice at 12 h (Figure 4.3.4 b; P<0.05). However, no significant increase in lymphocytes, neutrophils and eosinophils in BALF were found in IL-13 treated mice in comparison to PBS treated mice at 12 h. At 48 h, IL-13 not only induced a significant increase in macrophages but also lymphocytes, eosinophils and neutrophils in BALF compared to PBS treated mice (Figure 4.3.4 d; P<0.05). No significant change of leukocytes in peripheral blood was observed in response to IL-13 or PBS instillation at both 12 and 48 h (Figure 4.3.4 a and b).

4.3.3 RT-PCR detection of messenger RNA [mRNA] expression in the lungs

IL-13 induced AHR but not pulmonary eosinophilia or mucus hypersecretion at 12 h. IL-13 induced AHR, pulmonary eosinophilia and mucus hypersecretion at 48 h, and pulmonary eosinophilia and mucus hypersecretion but not AHR at 8 d. These three timepoints were selected to investigate whether molecules related to IL-13 induced responses were differentially regulated and could be linked to the expression of a particular allergic inflammatory phenotype.

4.3.3.1 Expression of chemokine genes

In response to IL-13 stimulation at 12 h, expression of eotaxin-1, eotaxin-2, MDC, TARC, MCP-1, MCP-2 and MCP-3 in the lungs appeared to be strongly induced and these increases correlated with to the elevated levels of macrophages in BALF and the onset of PIP AHR; expression of MIP-1α, -1β, -2 and -3α appeared to be only slightly increased compared to PBS treatment group, and no difference was observed in the expression of TECK and RANTES. At 48 h, expression of eotaxin-1, eotaxin-2, MDC, TARC, TECK, MCP-1, and -2 was pronounced; expression of TARC, MIP-1α, -1β and -2 was also increased; there was no difference in the level of expression MCP-3, MIP-3α and RANTES in response to IL-13 administration as compared to PBS treated mice. At 8 d, increased expression of MCP-2 and MIP-1α was still detectable in response to IL-13 administration; other chemokines returned to levels of expression similar to that
Figure 4.3.4 Characterization of leukocyte numbers in the peripheral blood and BALF of IL-13 and PBS treated WT mice.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS) or the same volume of PBS. Peripheral blood (a and c) and BALF (b and d) were examined at 12 and 48 h following the administration of IL-13 or PBS. Cell types were stained with May-Grunwald-Giemsa. Data are the mean ± SEM from n = 4-10 mice per group.

* P<0.05 compared to respective PBS treated groups.
Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

**a. PB (12 h)**

**b. BALF (12 h)**

**c. PB (48 h)**

**d. BALF (48 h)**

<table>
<thead>
<tr>
<th>Leukocytes Type</th>
<th>PBS</th>
<th>IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference compared to PBS control.*
of PBS treated mice. Interestingly, expression of MCP-5 in lungs appeared to be inhibited at 12 and 48 h after IL-13 delivery and only at day 8 did this chemokine slightly return to normal expression levels (Figure 4.3.5).

4.3.3.2 Expression of genes related to airways remodeling

To investigate the role of IL-13 in lung tissue fibrosis, the level of mRNA of MMPs in IL-13 treated WT mice was compared to those expressed in PBS treated WT naïve mice at 12, 48 h and 8 d. MMPs and cathepsins are large groups of extracellular enzymes, which are associated with inflammation, tissue remodeling and degradation of connective tissue (Bank et al., 2000; Kekow et al., 2000). In response to IL-13, expression of MMP -2, -9, -14, TIMP-1, cathepsins -B and -S was not strongly induced. However, expression of MMP-12 and -13 appeared to be strongly enhanced in lung tissue 48 h after IL-13 administration compared to the PBS treated group, and increased expression of the mRNA encoding these two MMPs persisted to 8 days after IL-13 stimulation (Figure 4.3.6).

Airways mucus obstruction is a complex process encompassing mucin gene regulation, mucin protein secretion and goblet cell hyperplasia (Rose et al., 2001). To define the mechanism whereby IL-13 regulates mucus overproduction, the levels of Muc-2, -4, -5AC and -5B mRNA after IL-13 treatment were compared to PBS treatment in WT mice at 12, 48 h and 8 d (Figure 4.3.6). There was no increase in Muc-2 mRNA levels at 12 h, however, expression was enhanced at 48 h. At 8 d, IL-13 induced Muc-2 mRNA expression had returned to near baseline. For Muc-4, no difference was observed between IL-13 and the PBS treated group at 12 h and 8 d, and Muc-4 expression was detected in only one sample at 48 h. Increased expression of Muc-5AC mRNA was weakly induced at 12 h, although expression was significantly elevated at 48 h by IL-13. Some weak expression persisted at 8 d compared to PBS treated mice at the respective time point. Muc-5B mRNA level was not significantly different between IL-13 and PBS treated mice at any timepoints (Figure 4.3.6).
Figure 4.3.5 Effect of IL-13 on respiratory chemokine mRNA expression.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS) or the same volume of PBS. Lung samples were examined by RT-PCR at 12, 48 h and 8 d following the administration of IL-13 or vehicle. The effects of IL-13 treatment on the levels of mRNA encoding HPRT, MCP-1, MCP-2, MCP-3, MCP-5, MIP-1α, MIP-1β, MIP-2, MIP-3α, MDC, RANTES, eotaxin-1, eotaxin-2, TECK and TARC are shown. IL-13 differentially regulated the expression of all chemokines tested except RANTES.
### Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

<table>
<thead>
<tr>
<th>Control</th>
<th>PBS (12 h)</th>
<th>IL-13 (12 h)</th>
<th>PBS (48 h)</th>
<th>IL-13 (48 h)</th>
<th>PBS (8 d)</th>
<th>IL-13 (8 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **HPRT**
- **MCP-1**
- **MCP-2**
- **MCP-3**
- **MCP-5**
- **MIP-1α**
- **MIP-1β**
- **MIP-2**
- **MIP-3α**
- **MDC**
- **RANTES**
- **Eotaxin-1**
- **Eotaxin-2**
- **TECK**
- **TARC**
Figure 4.3.6 IL-13 induced expression of respiratory matrix proteinase and mucin genes.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS) or the same volume of PBS. Lung samples were examined by RT-PCR at 12, 48 h and 8 d following the administration of IL-13 or vehicle. The effects of IL-13 treatment on the levels of mRNA encoding HPRT, MMP-2, -9, -12, -13, -14, cathepsin-B, -S, TIMP-1, Muc-2, -4, -5AC and -5B are shown. IL-13 induced increased expression of respiratory MMP-12, -13, Muc-2 and -5AC.
Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

<table>
<thead>
<tr>
<th>Control</th>
<th>PBS (12 h)</th>
<th>IL-13 (12 h)</th>
<th>PBS (48 h)</th>
<th>IL-13 (48 h)</th>
<th>PBS (8 d)</th>
<th>IL-13 (8 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- HPRT
- MMP2
- MMP9
- MMP12
- MMP13
- MMP14
- Cathepsin B
- Cathepsin S
- TIMP-1
- Muc-2
- Muc-4
- Muc-5AC
- Muc-5B
4.3.4 Endogenous IL-4 and IL-13 are not necessary for recombinant murine IL-13 induced pulmonary responses

IL-4/IL-13-deficient mice were employed to investigate the interplay between exogenous IL-13 and endogenous IL-4 and IL-13 for the induction of AHR, pulmonary eosinophilia and mucus-hypersecretion. Intratracheal administration of IL-13 to IL-4/IL-13-deficient mice induced AHR at 24 and 48 h as compared to PBS treated IL-4/IL-13 deficient mice (Figure 4.3.7; P<0.05). This suggests that endogenous IL-13 and IL-4 were not required for the induction of AHR by exogenous IL-13. Eosinophil recruitment was insignificant at 24 h but significantly elevated at 48 h compared to PBS treated IL-4/IL-13-deficient mice (Figure 4.3.8 a and b; P<0.05). Although mucus-staining cells were not significantly enhanced in response to IL-13 at 24 h, mucus secretion levels were markedly increased at 48 h as compared to PBS treated IL-4/IL-13-deficient mice (Figure 4.3.8 c and d; P<0.05). Furthermore, the levels of eosinophils and mucus-staining cells in IL-13 treated IL-4/IL-13-deficient mice at 48 h were both significantly higher than that at the 24 h timepoint. Compared to IL-13 treated WT mice at 48 h, IL-4/IL-13-deficient mice exhibited similar levels of eosinophils in the lungs and AHR but a significantly decreased levels of mucus-secreting cells in the epithelium in response to IL-13 treatment. These data suggest that the exogenous IL-13 is sufficient to induce AHR, pulmonary eosinophilia and mucus-hypersecretion in the absence of endogenous IL-4 and IL-13.

4.3.5 Role of IL-5 and/or eotaxin-1 in IL-13 induced AHR, eosinophil recruitment and pulmonary mucus hypersecretion

Although eosinophils were not recruited to the airways in significant numbers during the PIP response, low numbers of resident cells at baseline were observed (Figure 4.3.2 a i and ii) (Figure 4.3.2 b). To determine if IL-13 induced AHR, eosinophil recruitment and mucus hypersecretion occurred via pathways that were dependent on basal levels of eosinophils, the pulmonary responses to IL-13 were characterized in IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice. IL-5-, eotaxin-1-, and IL-5/eotaxin-1- deficient mice were treated with IL-13 and responses were characterized 24 and 48 h. In IL-5-, eotaxin-1-, and IL-5/eotaxin-1- deficient mice, IL-13 induced AHR at both 24 and 48 h (Figure 4.3.9). However, compared to WT mice, AHR in these factor-deficient mice was attenuated at 48 h, although this failed to reach significance (Figure 4.3.9 b). IL-13
Figure 4.3.7 IL-13 induced AHR in IL-4/IL-13-deficient mice.

Airways reactivity was measured by barometric plethysmography 24 or 48 h after i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS in IL-4/IL-13-deficient mice. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to respective PBS treated groups.
Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

---

**a. 24 h**

- **PBS**
- **IL-13**

![Graph showing the percentage increase of methacholine with PBS and IL-13 at 24 hours.](image)

**b. 48 h**

![Graph showing the percentage increase of methacholine with PBS and IL-13 at 48 hours.](image)
Eosinophils and mucus-secreting cells in the lung tissues of IL-4/IL-13-deficient mice were determined at 24 or 48 h after i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS. Lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with Carbol’s Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissues and mucus-secreting cells in the central bronchial epithelium regions were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to respective PBS treated groups; # P<0.05 compared to IL-13 treated IL-4/IL-13-deficient mice at 24 h.
Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

**a. 24 h**

![Eosinophil number/HPF](chart)

**b. 48 h**

![Eosinophil number/HPF](chart)

**c. 24 h**

![Mucus-staining cell number/HPF](chart)

**d. 48 h**

![Mucus-staining cell number/HPF](chart)
Figure 4.3.9 IL-13 induced AHR in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice.

Airways reactivity to methacholine was measured by barometric plethysmography at 24 or 48 h after i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice. Data represent the percentage increase in enhanced pause (Penh) at 25mg/ml (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induces maximal responsiveness. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to respective PBS treated groups.
Chapter 4: Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR

a. 24 h

b. 48 h

methacholine [mg/ml]
treatment of WT mice and IL-5- and eotaxin-1- deficient mice resulted in infiltration of eosinophils by 48 h (Figure 4.3.10 a). IL-13-mediated accumulation of eosinophils in IL-5- and eotaxin-1- deficient mice was reduced compared to WT mice. Eosinophil recruitment in IL-5/eotaxin-1-deficient mice was significantly reduced compared to WT mice and IL-5-, and eotaxin-1- deficient mice. In the absence of IL-5, eotaxin-1, or IL-5/eotaxin-1, IL-13-induced mucus secretion was not significantly impaired (Figure 4.3.10 b).
Figure 4.3.10 IL-13 induced eosinophil accumulation and mucus secretion in WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice at 48 h.

Eosinophils and mucus-secreting cells in the lung tissue of WT mice and IL-5-, eotaxin-1- and IL-5/eotaxin-1- deficient mice were determined at 48 h after i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS. Lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with Carbol’s Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissues and mucus-secreting cells in the central bronchial epithelium regions were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to respective PBS treated groups; †P<0.05 compared to IL-13 treated eotaxin-1-deficient mice; •P<0.05 compared to IL-13 treated IL-5-deficient mice; # P<0.05 compared to IL-13 treated IL-5/eotaxin-1-deficient mice.
Chapter 4 Spatial and temporal characterization of IL-13 induced pulmonary inflammation and AHR.

a. 

![Graph showing eosinophil number/HPF](image)

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>!***</td>
</tr>
<tr>
<td>IL-5⁻/⁻</td>
<td></td>
<td>#**</td>
</tr>
<tr>
<td>eotaxin-1⁻/⁻</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>IL-5/eotaxin-1⁻/⁻</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

b. 

![Graph showing mucus-staining cell number/HPF](image)

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>IL-5⁻/⁻</td>
<td>eotaxin-1⁻/⁻</td>
<td>IL-5/eotaxin-1⁻/⁻</td>
</tr>
<tr>
<td>30</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* Statistically significant difference compared to WT group.

#* Significant difference compared to IL-5⁻/⁻ group.

#** Significant difference compared to eotaxin-1⁻/⁻ group.

** Significant difference compared to IL-5/eotaxin-1⁻/⁻ group.
4.4 Discussion

The potent spasmogenic and inflammatory actions of IL-13 have identified this molecule as a potential regulator of AHR and airways obstruction in asthma (Huang et al., 1995; Grunig et al., 1998; Wills-Karp et al., 1998). However, the subsequent cellular and molecular components employed by this cytokine to induce AHR and allergic disease in the lung have not been fully elucidated. In this Chapter, a mouse model system was employed to delineate the contribution of IL-13 to the temporal induction of AHR, eosinophilic inflammation and mucus secretion. It demonstrated that IL-13 induced a biphasic response that was characterized by a PIP and IP AHR. The PIP AHR response occurred independently of eosinophilic inflammation and mucus hypersecretion. By contrast, the IP AHR was associated with an amplified AHR that correlated with the recruitment of eosinophils to the airways and the production of mucus. IL-13 induced IP eosinophilia recruitment was partly dependent on IL-5 and/or eotaxin-1.

Intratracheal administration of IL-13 resulted in a pronounced AHR in naïve WT mice within 6 h. Peak AHR occurred at 48 h following IL-13 treatment, and was accompanied with a striking pulmonary tissue eosinophilia and mucus hypersecretion. Interestingly, AHR returned to near baseline 8 d after IL-13 administration, despite the persistence of pulmonary eosinophilia and mucus hypersecretion. In atopic asthma, antigen provocation of the airways induces a biphasic bronchoconstrictor response (Bochner et al., 1994). The early phase response is characterized by acute enhanced bronchospasm within 15-30 minutes after challenge (Pepys 1975), followed by a late phase response which can last for 48 to 72 h (Hargreave et al., 1974). The early phase AHR is believed to be mediated by IgE-mast cell degranulation and the release of preformed spasmogenic mediators (e.g. histamine) and occurs independently of cellular inflammation and mucus hypersecretion. The late phase response is characterized by T-lymphocyte and eosinophil accumulation, mucus hypersecretion, airway wall remodeling and AHR (Diaz et al., 1989). Both guinea pig and murine model systems have provided corroborative evidence demonstrating the presence of an early- and late-phase responses (Hutson et al., 1988; Frew et al., 1990; Cieslewicz et al., 1999).

Delivery of IL-13 to the airways of naïve WT mice promoted a biphasic AHR that is kinetically different from allergen induced early phase responses. However, the PIP
AHR occurred independently of the recruitment of eosinophils to the lung and the induction of mucus hypersecretion. These results suggest that IL-13 can rapidly prime for bronchoconstriction and identifies a potential role for this cytokine in early bronchospasm responses in asthmatics (potentially by being released from mast cells). The sustained effects (up to 8 days) of IL-13 also suggest that when released during the early phase response this molecule may not only induce early onset bronchoconstriction but also potently activate inflammatory cascades that induce hallmark features (mucus hypersecretion and eosinophilia) of the late phase response. Interestingly, by day 8, IL-13-induced AHR had dissipated, whilst mucus hypersecretion and eosinophilia were still pronounced in the lung. These observations suggest that the association or dissociation between inflammation and AHR that is often observed in clinical and experimental settings will be critically dependent on the time of analyses. The results further suggest that IL-13 may induce AHR by two pathways: One independent of inflammation and the other by promotion of inflammatory cascades.

Previous investigations have demonstrated that both IL-4 and IL-13 are critically involved in airways mucus secretion (Cohn et al., 1999). Moreover, chronic overexpression of IL-4 or IL-13 in the airways induces mucus hypersecretion, goblet cell hyperplasia, and airways epithelial cell hypertrophy (Rankin et al., 1996; Zhu et al., 1999). The observation that mucus hypersecretion can be induced in IL-4/IL-13-deficient mice by the administration of IL-13 definitively demonstrates that IL-13 is critical for the induction of this allergic response. The observed decline in mucus secretion in IL-13-treated IL-4/IL-13-deficient mice as compared to WT mice may be explained by the lack of baseline expression of IL-13 in the recipient animals or a reduction in residual Th2 cells (IL-13 secreting) in the pulmonary compartment because of the deficiency of IL-4.

Increased numbers of macrophages were observed in BALF in the PIP and IP induced by IL-13. In contrast, increased numbers of lymphocytes, neutrophils and eosinophils were not observed in BALF in the PIP, however, they were observed in the IP. This data strongly suggests that macrophages may be involved in IL-13 induced asthma-like responses. Furthermore, enhanced levels of mRNA encoding MDC, MCP-1, -2, -3, MIP-1β, MIP-2 and TARC were observed following IL-13 administration during the PIP response, and sustained expression of these chemokines remained during the IP response. Increased expression of TECK was only observed during the IP response.
Activated macrophages are the very important cellular sources of these chemokines (Van Coillie et al., 1999). Since macrophages have been identified as expressing the IL-4Ra/IL-13Rα1 complex (Andersson et al., 1997), it is tempting to speculate that IL-13 acts on macrophages to induce the production of these chemokines, which subsequently recruit inflammatory cells to sites of macrophage activation, and maintain and extend macrophage activation. Infiltrating and activated inflammatory cells may play a critical role in the development of the IP response. In particular, enhanced expression of eotaxin-1 and eotaxin-2 may account for the increased recruitment of eosinophils around peribronchial regions.

Among the hallmark features of asthma are mucus hypersecretion and goblet cell hyperplasia in airway epithelium (Hogaboam et al., 2000). Bronchial epithelial cells are important in host defence, by providing a barrier and through secreting mucus (Ordonez et al., 2001). Respiratory epithelial cells from allergic patients have been shown to express IL-13Rα1 (Akaiwa et al., 2001). Bronchial epithelial cells can also produce proinflammatory factor including cytokines (e.g. IL-6 and IL-8) and chemokines (e.g. eotaxin, RANTES and MCP-1) (Noah et al., 1993; Li et al., 1999; Momoi et al., 1999). It is therefore also possible that IL-13 directly stimulates bronchial epithelial cells and over stimulation in asthma contributes to mucus hypersecretion, leukocyte recruitment, epithelial cell shedding and airways occlusion and AHR in asthma. Of the 11 genes encoding mucin production, Muc-2, -4, -5AC and -5B are thought to play an important role in pulmonary mucin secretion (Dohrman et al., 1998; Hanaoka et al., 2001; Groneberg et al., 2002). Moreover, Muc-5AC mRNA and protein have been strongly associated with goblet cell metaplasia in murine airways (Zuhdi Alimam et al., 2000). Forty-eight hours after IL-13 administration, mRNA encoding Muc-2 and -5AC was significantly upregulated, which correlates with mucus hypersecretion during the IP response. This data suggests that Muc-2 and -5AC may account for the IL-13 induced mucus hypersecretion.

IL-5 and eotaxin-1 have previously been shown to be critical regulators of eosinophil trafficking and tissue accumulation at baseline and also during inflammatory responses (Mould et al., 1997; Mishra et al., 1999). IL-13 is thought to synergise with this pathway by regulating eotaxin-1 expression. Furthermore, chronic overexpression of IL-13 in the lung specifically promotes eotaxin-1 production (Zhu et al., 1999). Consistent with these findings, IL-13 mediated eosinophil accumulation was
significantly attenuated in IL-5-, eotaxin-1-, and IL-5/eotaxin-1-deficient mice. The level of eosinophil accumulation in eotaxin-1 deficient mice was significantly lower than that of IL-5-deficient mice. These data confirm the importance of the interaction between IL-5 and eotaxin-1 for localization of eosinophils in tissues and show that IL-13 is integrated into this eosinophil regulatory pathway (Chapter 2). Interestingly, the level of IL-13-mediated eosinophil accumulation was low when compared to an allergic inflammatory response (which is 6-8 fold greater) (Hogan et al., 1998; Webb et al., 2000). This may reflect the requirement for the upregulation of both IL-5 and eotaxin-1 and the induction of synergistic interactions to promote a maximal eosinophilic response. In particular, IL-5 promotes a blood eosinophilic pool in response to antigen provocation, which eotaxin-1 can subsequently sequester into tissues. Although eosinophilia induced by IL-13 is largely dependent on both IL-5 and eotaxin-1, significant numbers of eosinophils homed to lung tissue in comparison to vehicle control. Interestingly, IL-13 mediated a small but significant eosinophil accumulation independently of both eotaxin-1 and IL-5. This may be explained by IL-13 inducing the upregulation of other eosinophil recruitment chemokines including eotaxin-2, MCPs and MIPs in the lung.

In conclusion, IL-13 induced an initial AHR response (PIP) in WT mice that occurred within 6 h (peaked at 12-24 h) of administration and developed independently of eosinophilic inflammation and mucus hypersecretion in the lung. The PIP response was immediately followed by an inflammatory phase (IP) (48 h) that was characterized by an amplified AHR, which correlated with the development of mucus hypersecretion and eosinophilic infiltration of the lung. The IP response lasts for 8 days with sustained eosinophilic infiltration of the lung and mucus hypersecretion but AHR dissipates by this time. Activated macrophages and their related molecules may drive IL-13 induced responses. Expression of mRNA encoding Muc-2 and -5AC induced by IL-13 may account for mucus hypersecretion in the epithelium. Furthermore, IL-13 mediated eosinophil accumulation, is in part, through IL-5 and eotaxin-1. However, IL-13 can mediate eosinophil accumulation independently of these molecules, probably via the upregulation of other CCR3 activating chemokines such as eotaxin-2.
CHAPTER 5

Investigation of the mechanisms underlying IL-13 induced AHR
5.1 Introduction

In Chapter 4, it was shown that alveolar macrophage numbers significantly increased after IL-13 administration to the lung. Macrophages contribute to innate and adaptive immune responses (Gordon 1998; Stoy 2001). NO, which is one of important factors produced by activated macrophages, has an essential role not only in antimicrobial immunity but also in transmitting neuronal signals (Nijkamp et al., 1995). Numerous studies in humans and animal models have demonstrated that inhaled NO induces vascular dilation, and reverses vascular constriction (Frostell et al., 1993; Adatia et al., 1994; Booke et al., 1996; Shirai et al., 1996; Tan et al., 1997; Naoki et al., 1999). Thus, these data suggest that the production of NO in the pulmonary system may be important for both normal lung function and in regulating immune responses.

NO is also produced by endothelial cells, epithelial cells, smooth muscle cells, sensory nerve cells and inflammatory cells in airways tissue (Rosbe et al., 1996; Nagano 1999; Hess et al., 2000; Kohn et al., 2001). iNOS plays an important role in the production of NO which is synthesized from the semi-essential amino acid L-arginine in rodent models of allergic disease (Yan et al., 1995; Lee et al., 2001). NO appears to be an important factor for the bronchodilator response (Charan et al., 1997; Therminarias et al., 1998). Endogenous NO is thought to induce smooth muscle relaxation by activating guanylate cyclase, raising c-GMP levels and spasmogen induced contractile responses (Nijkamp et al., 1995). As the substrate of NO, L-arginine is also metabolized by arginase I and arginase II. Arginase I, is an important enzyme in the urea cycle (Ratner 1973). Arginase I is thought to be expressed almost exclusively in the cytosol of liver cells under normal conditions, but has also been shown to be upregulated in macrophages and smooth muscle cells under acute inflammatory conditions (Waddington et al., 1998). Arginase I is also induced by IL-4 and IL-13 in cultured vascular smooth muscle cells (Wei et al., 2000). By contrast, arginase II is found in the mitochondrial matrix and expressed at low levels in many tissues (Jenkinson et al., 1996; Mori et al., 1998; Wu et al., 1998). As a substrate of arginase, L-arginine itself may stimulate protein synthesis by forming arginyl-tRNA complexes (Rosenthal 1977; Sivaram et al., 1990). Ornithine aminotransferase [OAT] and ornithine decarboxylase [ODC] are also importantly involved in the metabolism of arginine (Manteuffel-Cymborowska et al., 1995; Durante 2001).
Although the results in Chapter 4 indicated that IL-13 can induce multifaceted asthmatic responses including AHR, pulmonary eosinophil recruitment and mucus hypersecretion and the expression of many inflammatory molecules, the mechanisms underlying IL-13 induced AHR remains unclear. To further elucidate the mechanisms involved in IL-13 induced AHR and the requirement of mucus hypersecretion and eosinophilia for this process, Swiss nude mice and IL-4Rα- and STAT6- deficient mice were employed to investigate IL-13 induced pulmonary responses. In this Chapter, the role of IL-13 in the expression of NO-associated enzymes and the role of NO and L-arginine in IL-13 induced AHR and pulmonary eosinophilia and mucus hypersecretion was also investigated. We also investigated the effect of disodium cromoglycate [DSCG] on IL-13 induced responses and whether IL-13 can enhance airways responsiveness to acetycholine and non-cholinergic agents (adenosine and histamine) which also induce abnormal constriction responses in asthmatics. In this Chapter we link IL-13 induced arginase activity with the development of AHR, furthermore we show central roles for IL-4Rα chain and STAT6 in the induction of IL-13 mediated responses.
5.2 Materials and methods

5.2.1 Mice

See 2.2.1.

Seven to nine week old naïve WT and Swiss nude mice and IL-4Rα-, STAT6- deficient mice were bred in specific pathogen-free facilities, JCSMR. All animals were housed and treated according to the Australian National University guidelines for animal experimentation.

5.2.2 Intratracheal instillation of murine recombinant IL-13

See 4.2.2

Briefly, mice were intubated with a 22-gauge needle through which mice received murine recombinant IL-13 (10µg, some 1 or 3µg dissolved in 20µl PBS) or vehicle control (20µl PBS) under anesthetisia. AHR, mucus production, and eosinophilia were measured at 24 and 48 h (some at 12 h) (peak responses for PIP and IP, respectively) after i.t. instillation of IL-13 to mice.

5.2.3 Measurement of airways reactivity to methacholine

See 2.2.3

5.2.4 Characterization of eosinophils and mucus-staining cells in lung tissue

See 2.2.4

Lung tissues representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with alcian blue/periodic acid-Schiff for enumeration of mucin-secreting cells or Charbol’s chromatrope hematoxylin for identification of eosinophils. The number of mucus-staining cells and eosinophils in the central bronchi-bronchiole area was identified by morphological criteria and quantified as previously described.
5.2.5 Drug treatment

WT mice were injected i.v. with DSCG (Sigma Chemical Co, St Louis, MO) or vehicle (PBS) at 2mg/kg in sterile PBS daily for 4 consecutive days. On day four, mice were instilled i.t. with IL-13 (10µg/20µl PBS) or 20µl PBS. 24 h after i.t. treatment, airways reactivity readings were taken.

5.2.6 Measurement of airways reactivity to different spasmogens

Airways reactivity to different spasmogens (acetylcholine, adenosine and histamine) was assessed in conscious, unrestrained mice by barometric plethysmography apparatus (BUXCO, Troy, NY). Twenty-four hours after IL-13 treatment of WT mice, airways responses were measured as described previously in section 2.2.3.

5.2.7 Treatment with NO-modulating agents

WT mice were administered with 10µg murine recombinant IL-13 in 20µl PBS or PBS alone. Forty-eight hours after IL-13 treatment, mice were placed in the plethysmograph chamber and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (at 12.5, 25 and 50mg/ml) was then delivered through an inlet into the main chamber for 2 min and readings were averaged over a period of 3 min after each dose was administered. Some IL-13 treated mice were administered i.p. with 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (HOBAT, 100mg/kg in saline, gift from Dr. Bill W Cowden, Canberra, Australia) 5 min before placing the mice in the plethysmograph chamber.

5.2.8 RT-PCR analysis

RT-PCR was performed as described in Section 4.2.6

The primers for all genes were purchased from GIBCO (GIBCO BRL products, New Zealand). The sequences for HPRT are described in section 4.2.6. The primer sequences for different factors are as below:
<table>
<thead>
<tr>
<th>gene</th>
<th>sense primer 5’-3’</th>
<th>antisense primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginase I</td>
<td>cagttggcagttggaagcatct</td>
<td>gttctcttcactcacttgccaat</td>
</tr>
<tr>
<td>Arginase II</td>
<td>ctggctgaagttgtagtaga</td>
<td>gggagaaggaaggcaaggtgtgat</td>
</tr>
<tr>
<td>ODC</td>
<td>tgattacctctgtcaggcagtgc</td>
<td>ctcaaacagcaatcactcaccaca</td>
</tr>
<tr>
<td>OAT</td>
<td>tgatacctctgtcaggcagtgc</td>
<td>ctcaaacagcaatcaccacccaca</td>
</tr>
<tr>
<td>iNOS</td>
<td>cggagcctttagacctcaacaga</td>
<td>gtctgaatgtcagggaagtaggt</td>
</tr>
<tr>
<td>STAT6</td>
<td>tcttgccaagacctgtcattgge</td>
<td>ctgagttaggcaactgtcctcttccat</td>
</tr>
</tbody>
</table>

### 5.2.9 Role of L-arginine in IL-13 induced AHR

WT mice were injected i.p. with L-arginine (200mg/kg diluted in saline) (Sigma Chemical Co, St Louis, MO) or the same volume of saline once every twelve hour for 4 consecutive days. On day three, mice were then instilled i.t. with IL-13 (1µg/20µl PBS) or the same volume of PBS. Twelve hour after IL-13 treatment, airways response to methacholine was measured by barometric plethysmography.

### 5.2.10 Arginase activity assay

Arginase activity assay was described previously (Corraliza et al., 1994). Briefly, lung tissues were excised and the cells lysed with 0.5ml 0.1% Triton X100. After 30 min, 0.5ml of buffer (25mM Tris-HCl and 5 mM MnCl₂, pH 7.4) was added. Arginase was then activated by heating for 10 min at 56°C. Arginine hydrolysis was carried out by incubating 25µl of the activated lysate with 25µl of 0.5 M arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 400µl of an acidic mixture (H₂SO₄, H₃PO₄ and H₂O, 1:3:7 v/v). The urea was measured at 540nm after addition of 25µl of 9% α-isonitrosopropiophenone (dissolved in 100% ethanol) and heating at 100°C for 45 min.

### 5.2.10 Statistical analysis

See 2.2.8
5.3 Results

5.3.1 Airways response to different spasmogens in IL-13 treated WT mice

To investigate the effect of IL-13 on different spasmogenic responses, airways reactivity was assessed in response to acetycholine, adenosine and histamine (Figure 5.3.1) twenty-four hour after IL-13 or PBS treatment. Airways reactivity to acetycholine in IL-13 treated groups exhibited a significant increase in comparison to PBS treated groups (P<0.05). However, no significant difference was observed between IL-13 and PBS treated groups in response to adenosine or histamine. This data suggests that IL-13 primes for cholinergic responses.

5.3.2 Characterization of responses to IL-13 challenge in Swiss nude mice

Swiss nude mice were employed to investigate the role of T-cells in IL-13 induced responses. T-cell function in these mice is severely impaired (Viac et al., 1976; Mesfin et al., 1979). Treatment of Swiss nude mice with IL-13 resulted in a significant increase in airways reactivity to methacholine compared to PBS treated mice at 24 and 48 h (Figure 5.3.2). Eosinophils in bronchial region were increased about 2 fold by IL-13 treatment compared to PBS treatment at 24 h, and by about 7 fold in the IL-13 treated group compared to PBS treated group at 48 h (Figure 5.3.3 a and b). Peribronchial eosinophilia induced by IL-13 was approximately 3 fold higher at 48 h compared to the 24 h time point. The numbers of mucus-staining cells in the epithelium in IL-13 treated nude mice was significantly higher (about 15 fold increase) than that in PBS treated group at 48 h (Figure 5.3.3 c and d). Significant increase in the numbers of mucus-staining cells was also seen in IL-13 treated group at 48 h compared to 24 h. No significant difference in mucus-staining cells was found between the IL-13 treated and PBS treated group at 24 h.

5.3.3 Effect of DSCG on IL-13 induced AHR

DSCG has been demonstrated as an anti-inflammatory agent, which acts mainly through stabilization of mast cells to inhibit degranulation (Advenier et al., 1984; Cockerham et al., 1986; Stenton et al., 1998). WT mice treated with IL-13 and DSCG demonstrated
Figure 5.3.1 IL-13 induced airways responsiveness to spasmogens.

Airways responsiveness to the spasmogens, acetylcholine, adenosine and histamine was measured by barometric plethysmography 24 h after i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS. Data represent the percentage increase in enhanced pause (Penh) at 25mg/ml (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induced maximal responsiveness. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to the respective PBS treated groups.
Figure 5.3.2 IL-13 induced AHR in Swiss nude mice.

Airways responses to methacholine were measured by barometric plethysmography 24 (a) or 48 h (b) after i.t. administration of IL-13 (10µg/20µl PBS) or the same volume of PBS in Swiss nude mice. Data represent the percentage increase in enhanced pause at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to the respective PBS treated groups.
Chapter 5 Investigation of the mechanisms underlying IL-13 induced AHR

---

**a. 24 h**

- **PBS**
- **IL-13**

**b. 48 h**

- **PBS**
- **IL-13**

*Graphs showing the percentage increase over baseline for methacholine concentrations of 0, 10, 20, 30, 40, and 50 mg/ml at 24 and 48 hours. The data is represented with error bars, indicating statistical significance.*

---
Figure 5.3.3 IL-13 induced eosinophil accumulation and mucus secretion in Swiss nude mice.

Eosinophil and mucus-secreting cells in the lung tissue of Swiss nude mice were determined 24 h (a and c) or 48 h (b and d) after i.t. administration of IL-13 (10µl/20µl PBS) or the same volume of PBS. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissues (a and b) and mucus-secreting cells in the central bronchial epithelium regions (c and d) were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to the respective PBS treated groups. # P<0.05 compared to IL-13 treated groups at 24 h time point.
Chapter 5 Investigation of the mechanisms underlying IL-13 induced AHR

a. 24 h

IL-13 | PBS
---|---
2.5 | *

b. 48 h

IL-13 | PBS
---|---
10.0 | * #

C. 24 h

IL-13 | PBS
---|---
2.5 | *

D. 48 h

IL-13 | PBS
---|---
20.0 | * #
no significant difference in airways responsiveness compared to mice treated with IL-13 and vehicle control (Figure 5.3.4).

5.3.4 Role of IL-4Rα and STAT6 in the onset of IL-13 induced AHR

The observation that AHR was induced rapidly but was also maintained for 48 h (which correlated with the induction of pulmonary eosinophilia and mucus production) suggested that IL-13 acts via multiple pathways (Chapter 4: Figure 4.3.1). The sustained effect (over 8 d) of IL-13 in the lung suggested that this cytokine activates endogenous pathways that subsequently liberate mediators that maintained AHR (48 h), eosinophilia and mucus production (up to 8 d). Initially, it was decided to investigate the requirements for the IL-4Rα subunit and STAT6 in the induction of AHR (Figure 5.3.5). IL-4Rα- and STAT6- deficient mice were employed to determine the role of this receptor and its downstream signalling processes in IL-13 mediated AHR. IL-13-mediated AHR was ablated in mice deficient in these molecules (Figure 5.3.5) both at 24 and 48 h.

5.3.5 Role of IL-4Rα and STAT6 in the induction of eosinophil recruitment and mucus hypersecretion induced by IL-13

IL-13 induced IP AHR was associated with eosinophilic infiltration and mucus hypersecretion (Chapter 4). To identify the contribution of the IL-4Rα chain and STAT6 signaling pathways in these processes, mice deficient in these molecules were treated with IL-13 at 24 and 48 h. AHR, eosinophilia and mucus production in the lung were abolished in IL-4Ra- and STAT6- deficient mice at both time points (Figure 5.3.6).

5.3.6 Treatment with 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene [HOBAT] completely abolishes IL-13 induced AHR but not pulmonary eosinophilia and mucus hypersecretion in WT mice

HOBAT is a compound to rapidly generate NO by automatically degradation once dissolved (Hrabie et al., 1993). Due to the short half-life (approximately 40 min) in vivo of HOBAT, this agent was administered i.p. to mice 5 min (100mg/kg in saline) before placing the mice in plethysmograph chambers. The procedure for measuring airways
Figure 5.3.4 Onset of IL-13 induced AHR in WT mice treated with disodium cromoglycate (DSCG).

WT mice were treated i.v. with 2 mg/kg DSCG in PBS or vehicle control (PBS) daily for 4 consecutive days. Additionally, these mice were instilled i.t. IL-13 (10µg/20µl PBS) or the same volume of PBS on day 4. Twenty-four hour after IL-13 treatment, airways responses to methacholine were measured by barometric plethysmography. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 6-8 mice per group.

* P<0.05 compared to PBS with or without DSCG treated groups; #P<0.05 compared to PBS with or without DSCG treated groups
Figure 5.3.5 IL-13 induced AHR in WT mice and IL-4Rα- and STAT6- deficient mice.

Airways reactivity to methacholine was measured by barometric plethysmography at 24 (a) or 48 h (b) after administration i.t. of IL-13 (10µg/20µl PBS) or the same volume of PBS in WT mice or IL-4Rα- and STAT6- deficient mice. Data represent the percentage increase in enhanced pause (Penh) at 25mg/ml (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induces maximal responsiveness. Data are the means ± SEM from n = 4-10 mice per group.

* P<0.05 compared to the respective PBS treated groups; # P<0.05 compared to IL-13 treated IL-4Rα- and STAT6- deficient mice.
Chapter 5 Investigation of the mechanisms underlying IL-13 induced AHR

a. 24 h

b. 48 h
Figure 5.3.6 IL-13 induced eosinophil accumulation and mucus secretion in WT mice and IL-4Rα- and STAT6- deficient mice.

Eosinophils and mucus-secreting cells in the lung tissue of WT mice and IL-4Rα- and STAT6- deficient mice were determined 24 (a and c) or 48 h (b and d) after administration i.t. of IL-13 (10µg/20µl PBS) or the same volume of PBS. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophil and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissues and mucus-secreting cells in the central bronchial epithelium regions were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-10 mice per group.

*P<0.05 compared to the respective PBS treated groups; #P<0.05 compared to IL-13 treated WT mice at 24 h time point; ♦P< 0.05, compared to IL-4Rα- and STAT6-deficient mice treated with IL-13 at 48 h time point;
Chapter 5 Investigation of the mechanisms underlying IL-13 induced AHR

- **PBS**
- **IL-13**

### a. 24 h
- WT
- IL-4R<sup>−/−</sup>
- STAT6<sup>−/−</sup>

### b. 48 h
- WT
- IL-4R<sup>−/−</sup>
- STAT6<sup>−/−</sup>

### c. 24 h
- WT
- IL-4R<sup>−/−</sup>
- STAT6<sup>−/−</sup>

### d. 48 h
- WT
- IL-4R<sup>−/−</sup>
- STAT6<sup>−/−</sup>
Figure 5.3.7 Supplementation of NO abolished IL-13 induced AHR but not pulmonary eosinophil accumulation and mucus secretion in WT mice.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS) or the same volume of PBS. Forty-eight h after the treatment, airways reactivity to methacholine (a) was measured by barometric plethysmography. In some experiments, 5 min before the measurement of airways reactivity, HOBAT or control were injected i.p. into IL-13 treated animals and then airways responses were measured. Data represent the percentage increase in enhanced pause at 25mg/ml (and is representative of responses obtained with a full dose response curve to methacholine 3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induced maximal responsiveness. Lung sections were stained with Carbol’s Chromotrope-Hematoxylin for eosinophil and alcian blue/PAS for mucus-secreting cells. The number of eosinophils in lung tissue (b) and mucus-secreting cells in the central bronchial epithelium regions (c) were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 8-10 mice per group.

* P<0.05 compared to the PBS treated mice. # P<0.05 compared to IL-13 plus HOBAT treated mice.
Chapter 5 Investigation of the mechanisms underlying IL-13 induced AHR

- **a.**
  - PBS
  - IL-13
  - IL-13+HOBAT
  - IL-13+control
  - Penh % increase over baseline

- **b.**
  - Eosinophil number/HPF

- **c.**
  - Mucus-staining cell number/HPF
reactivity was modified by using only three doses to complete measurements within 40 min. WT mice were treated with IL-13, and AHR and lung samples were taken after 48 h. Treatment with HOBAT abolished AHR induced by IL-13 (Figure 5.3.7 a). Neither HOBAT nor control treatment had any effect on pulmonary eosinophilia and mucus hypersecretion induced by IL-13 (Figure 5.3.7 b and c). These data suggest that NO plays an important role in the regulation of IL-13 induced AHR.

5.3.7 IL-13 induced expression of arginase I in lungs is dependent on STAT6

To define the contribution of IL-13 to the regulation of the expression of enzymes associated with NO biosynthesis, RT-PCR was employed to detect the mRNA expression levels in lung tissue of arginase I, arginase II, OAT, ODC and iNOS at 12, 48 h and 8 d after IL-13 treatment (Figure 5.3.8). No obvious increase or decrease in mRNA expression of ODC, OAT and iNOS was observed at 12, 48 h and 8 d after IL-13 instillation. Treatment with IL-13 strongly promoted arginase I expression at 12 and 48 h, which was slightly higher at 48 h than that at 12 h. The induced expression of arginase I in IL-13 treated mice was reduced to a similar level as seen in PBS treated mice on 8 d. No obvious difference appeared in arginase II expression on 48 h and 8 d between IL-13 and PBS treated mice. However, a slight increase in the expression of arginase II was observed 12 h after IL-13 treatment compared to PBS treated mice. These data demonstrate that IL-13 i.t. treatment strongly induces arginase I expression but does not influence the expression of other NO-associated enzymes in the lung.

To investigate the role of STAT6 in IL-13 induced arginase I expression, NO-associated enzymes were examined in WT and STAT6-deficient mice 12 h after IL-13 treatment (Figure 5.3.9). No expression of arginase I appeared in IL-13 treated STAT6-deficient mice, however, expression of arginase II, iNOS, OAT, ODC remained unchanged. This implied that administration of IL-13 specifically induced arginase I expression and this process was critically dependent on STAT6 activation.

5.3.8 L-arginine supplementation enhances IL-13 induced AHR

As the substrate of arginase I, L-arginine is important for the citrulline-arginine cycle that generates many products including NO, polyamines and glutamate (Wiesinger 2001). To elucidate the role of L-arginine in IL-13 (1μg/20μl PBS) induced AHR, this
Figure 5.3.8 Effect of IL-13 on mRNA expression of NO-associated respiratory enzyme.

WT mice were instilled i.t. with IL-13 (10µg/20µl PBS) or the same volume of PBS. Frozen lung samples were examined by RT-PCR at 12, 48 h and 8 d following the administration of IL-13 or PBS. The effects of IL-13 treatment on the levels of mRNA of HPRT, arginase I, arginase II, OAT, ODC and iNOS are illustrated. IL-13 increased expression of arginase I mRNA, but not the other NO-associated factors.
Figure 5.3.9 IL-13 induced arginase I expression is dependent on STAT6.

WT or STAT6-deficient mice were instilled with IL-13 (10µg/20µl vehicle) or the same volume of PBS. Frozen lung samples were examined by RT-PCR 12 h following administration of IL-13 or vehicle. The effects of IL-13 treatment on the levels of mRNA of HPRT, arginase I, arginase II, OAT, ODC and iNOS are shown. Depletion of STAT6 abolished enhanced pulmonary expression of arginase I mRNA that was induced by IL-13.
amino acid was injected i.p. into mice for four consecutive days and then AHR and lung arginase activity was determined. Airways reactivity of IL-13 treated mice, with or without L-arginine was significantly enhanced compared to the respective PBS treated mice (Figure 5.3.10 a). L-arginine treatment significantly promoted IL-13 induced AHR. IL-13 treatment markedly increase lung arginase activity, however, treatment with L-arginine did not promote this effect (Figure 5.3.10 b).
Figure 5.3.10 L-arginine supplementation promotes IL-13 induced AHR.

WT mice were injected i.p. with L-arginine (200mg/kg diluted in saline) or the same volume of saline every 12 h for 4 consecutive days. Mice were then treated with IL-13 (10µg/20µl PBS) or the same volume of PBS on day 4. Twelve hours after the treatment, airways response to methacholine (a) was measured by barometric plethysmography. Data represent the percentage increase in enhanced pause at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Lungs were excised and stored at −80°C for arginase assays (b). Data are the means ± SEM from n = 8-10 mice per group.

Figure 5.3.10 a, * P<0.05 compared to other groups. †P<0.05 compared to PBS treated mice; Figure 5.3.10 b, # P<0.05 compared to the respective PBS treated mice.
Chapter 5 Investigation of the mechanisms underlying IL-13 induced AHR

---

**Graph a.**

- **PBS**
- **PBS + L-arginine**
- **IL-13 + vehicle**
- **IL-13 + L-arginine**

**Graph b.**

- **PBS**
- **IL-13**
5.4 Discussion

The mechanisms regulating the induction of AHR in asthma are unknown. Mast cells through the release of histamine and adenosine (among other factors) may regulate early bronchoconstriction, while activated Th2 cells and eosinophils may promote the late-phase response. IL-13 is also released from activated mast cells and Th2 cells, suggesting that this cytokine may prime ASM for enhanced airways responsiveness to spasmogens in both the early and later-phases. Notably asthmatics are hyperresponsive to histamine, adenosine and cholinergic stimuli (acetylcholine and methacholine) (Miller et al., 1977; Anderton et al., 1979; Takano et al., 1998) In this Chapter we show that IL-13 was able to prime the lung for enhanced responsiveness to cholinergic spasmogen, but not histamine or adenosine (which are also used to determine clinical severity of disease). This data suggests that while IL-13 may prime the lung for enhanced responsiveness, it may not contribute to pathways employed by histamine and adenosine to induce AHR. DSCG also failed to inhibit IL-13 induced responses suggesting that IL-13 did not act via mast cell activation or by non-specific anti-inflammatory pathways.

T lymphocytes are the key regulators of the development of airways inflammation and AHR (Ray et al., 1999; Yssel et al., 2001). Swiss nude mice were employed to examine the role of T lymphocytes in IL-13 induced AHR, pulmonary eosinophilia and mucus hypersecretion. These mice lack a functional thymus, and T lymphocytes do not develop to maturity (Viac et al., 1976; Mesfin et al., 1979). Delivery of IL-13 to the airways of Swiss nude mice enhanced airways reactivity at both 24 and 48 h. Eosinophil recruitment and mucus hypersecretion in the lung were also observed at these two time points after IL-13 treatment. The data here suggest that IL-13 induces AHR by direct stimulation of resident airway cells (e.g. fibroblasts and epithelial cells) (Lordan et al., 2002; Venkayya et al., 2002). Indeed, bronchial epithelial cells have been shown to regulate T lymphocyte migration into the lung compartment by production of T-cell specific chemokines (e.g. MDC and TARC) (Sekiya et al., 2000; Berin et al., 2001). IL-13 activated lung cells may contribute to mucus hypersecretion and also recruit eosinophils by secreting eosinophil specific factors such as eotaxin-1 (Cook et al., 1998; Corry 1999; Li et al., 1999; Kawaguchi et al., 2000). Hence, even with the impairment in T lymphocytes, eosinophil recruitment and mucus hypersecretion were still induced after IL-13 treatment in Swiss nude mice.
Clinically, it has been demonstrated that the arginine/NO synthase pathway is highly relevant to the pathophysiology of many diseases (Sonaka et al., 1994; Carraway et al., 1998; Grasemann et al., 1999; Liu et al., 2001). Furthermore, NO regulates many physiological and pathological effects in vivo (Willenborg et al., 1999). Administration of the NO donor (HOBAT) in vivo totally abolished IL-13 induced AHR at 48 h, which is the time point of maximal AHR and inflammation. NO has been predominantly described as a signaling molecule, that effects its functions by simple diffusion through biological membranes rather than through any specific receptor system (Willenborg et al., 1999). NO regulates cyclic guanosine monophosphate (cGMP) activity, which plays a role in relaxation of vascular smooth muscle (Ignarro et al., 1987; Trigo-Rocha et al., 1993; Archer et al., 1994). However, while supplementation of NO with HOBAT completely inhibited IL-13 induced AHR, it did not alter eosinophil recruitment and mucus hypersecretion. However, inhibition of these features was not expected when one considers HOBAT was administrated just prior to methacholine inhalation.

L-arginine is a semi-essential amino acid that is associated with numerous physiological and pathological processes (Nguyen van 1965; Cunin et al., 1986; Davis et al., 1988; Morel et al., 1996). Moreover, L-arginine has been identified as the substrate for the endogenous production of NO (Grant et al., 1998; Gobert et al., 2000; Piacenza et al., 2001). However, administration of L-arginine in IL-13 treated WT mice did not suppress AHR, but significantly promoted hyperreactivity increasing the sensitivity of IL-13 treated mice to methacholine. Thus in the presence of IL-13 arginase activity is increased which depletes the substrate available for NO production by iNOS. This mechanism has been previously suggested to be operative (Rutschman et al., 2001).

Notably, the prominent feature of arginine/NO metabolism is the activation of a complex network of enzymes that include arginase I, arginase II, OAT, ODC and iNOS (Wu et al., 1998; Morris 2002). Arginase I is predominantly expressed in periporal hepatocytes and functions to convert arginine nitrogen into urea (Yu et al., 1995; Watanabe et al., 1997; Miyanaka et al., 1998). Arginase II expression is more pronounced in the mitochondria of cells located in extra-hepatic tissues (Gotoh et al., 1997). Arginase I is also upregulated in cultured macrophages and smooth muscle cells (Waddington et al., 1998; Wei et al., 2000). In this Chapter, only arginase I expression was upregulated in the lungs of mice treated with IL-13. The temporal expression of arginase I closely correlated with the development of AHR. Both AHR and arginase I
expression were enhanced at 12 and 48 h, but not at 8 d following IL-13 treatment. These observations suggested that processes regulated by arginase I underpin the development of IL-13 induced AHR.

Cytosolic arginase I is thought to play a pivotal role in the synthesis of polyamines (putrescein, spermine, spermidine) and with ODC are also thought to be important for cell growth and differentiation (Oredsson et al., 1985; Singh et al., 1992; Thomas et al., 2001). Polyamines modulate protein synthesis by stimulating the initiation of mRNA translation and mediating the sensitivity of RNA to endogenous RNases (Igarashi et al., 1997; Igarashi et al., 2000). Importantly, polyamines can interact with ion channels such as K+ channels and AMPA/kainate receptors (Williams 1997). Investigation of cardiac myocytes revealed that lowering polyamine levels tended to decrease the excitability by reducing rectification of K+ channels (Nichols et al., 1996). Polyamines, in particular spermine, also significantly inhibit the activity of iNOS and the subsequent production of NO (Hu et al., 1994; Blachier et al., 1997). In this Chapter, there was no dramatic change in the expression of arginine/NO related enzymes except arginase I. It is therefore tempting to speculate that IL-13 induced AHR was dependent on the increased pulmonary expression of arginase I. Arginase I may then stimulate the production of polyamines but more importantly redirect arginine away from NO production removing the protective bronchodilatory effect.

IL-13 is thought to primarily signal through the IL-13Rα1/IL-4Rα complex to initiate inflammatory responses (Zurawski et al., 1993; Tomkinson et al., 2001) and STAT6 is pivotal for IL-4 receptor complex mediating intracellular signaling events (Kaplan et al., 1996; Takeda et al., 1996). In the present Chapter using factor deficient mice, both the PIP and IP AHR responses were shown to be critically dependent on signaling through IL-4Rα and STAT6. Notably, IP mucus secretion and eosinophil recruitment were ablated in the absence of the IL-4Rα and STAT6. The abolition of IL-13-induced PIP AHR in IL-4Rα- and STAT6-deficient mice suggests that IL-13 mediated signaling is through the IL-4Rα-STAT6 signalling system. Furthermore, expression of arginase I induced by IL-13 was critically dependent on STAT6 activation in the lung. These observations are also consistent with the fact that STAT6 activation is downstream of IL-4Rα mediated signaling (Takeda et al., 1996). The IL-13 receptor complex has been identified on macrophages and is also found on other pulmonary cells such as bronchial epithelial cells and smooth muscle cells (Heinzmann et al., 2000; Akaiwa et al., 2001).
Thus, further understanding of the interaction of IL-13 and pulmonary cells will provide new insights into asthma pathogenesis.

In conclusion, the pathogenic effects of IL-13 are predominantly mediated through the IL-4Rα/STAT6 signalling pathways. IL-4Rα and STAT6 are critical for the development of AHR, pulmonary eosinophil accumulation and mucus overproduction during both PIP and IP responses. IL-13 induced AHR, eosinophil recruitment and mucus hypersecretion occurs independently of T lymphocytes. IL-13 upregulates lung expression and activity of arginase I and the temporal expression of arginase I in the lung strongly correlates with the development of IL-13 induced AHR. Importantly, IL-13 induced expression of arginase I, but not other NO-associated enzymes (e.g. arginase II, iNOS, ODC and OAT) is dependent on STAT6 activation. Supplementation of mice with L-arginine potentiates IL-13 induced AHR. In addition, supplementation of NO with HOBA completely abolished IL-13 induced AHR but not pulmonary eosinophil accumulation or mucus overproduction. These findings establish the potential importance of IL-13 in the induction of key phenotypic characteristics of experimental asthma and suggest that IL-13 signaling through IL-4Rα and STAT6, and the fine balance of NO metabolism in airways are important targets for therapeutic intervention of this disease.
CHAPTER 6

Peripheral eosinophilia but not arginase I or AHR

is induced in the absence of STAT6
6.1 Introduction

STAT6 is a transcription factor, which regulates the production of many proinflammatory molecules (Leonard et al., 1998; Hoey et al., 1999), and has been shown to play a pivotal role in IL-4 and IL-13 induced intracellular signaling events (Izuhara et al., 1996; Heim 1999). STAT6 is essential for IL-4 induced polarization of CD4⁺ T-cells to the Th2 type response (Takeda et al., 1996). Investigations on STAT6-deficient mice have demonstrated an impaired development of CD4⁺ Th2 cells (Kaplan et al., 1996) and increased Th1 immune responses (Stamm et al., 1998). Furthermore, STAT6 has been shown to be critical for the development of AHR, mucus hypersecretion and eosinophil accumulation in the allergic lung of mice (Kuperman et al., 1998; Miyata et al., 1999). However, the effect of STAT6 deficiency on eosinophil numbers in the blood and bone marrow during allergic inflammation remains unknown. Inhibition of allergic inflammation and disease processes in the lung of STAT6-deficient mice may be due to a generalized deficiency in Th2 responses and/or loss of STAT6 regulated processes in pulmonary tissues.

In previous Chapter, IL-13 was shown to require STAT6 for the induction of AHR, eosinophil recruitment and mucus hypersecretion in naive mice. IL-13 induced expression of arginase I in the lungs was also dependent on STAT6 (Figure 5.3.11). Previous studies have also shown that IL-4/IL-13 induced activation of STAT6 results in increased arginase I expression in cultured vascular smooth muscle cells (Wei et al., 2000). Currently, it is not clear how STAT6 influences the expression of NO-associated enzymes under allergic conditions. Nor has it been delineated as to how STAT6 modulates eosinophil accumulation in the lung during allergic inflammation.

To further understand the role of STAT6 in regulating eosinophil trafficking and the induction of AHR, STAT6-deficient mice were sensitized and challenged with OVA. The role of STAT6 in the induction of chemokine production including MCPs (MCP-1, -2, -3 and -5), MIPs (MIP-1α, -1β, -2 and -3α), eotaxin-1 and eotaxin-2 in the lung was identified. The contribution of IL-5 and of CD4⁺ and CD8⁺ T-cells to the regulation of eosinophil trafficking and the contribution of NO to induction of AHR, in OVA sensitized STAT6-deficient mice were investigated. Results from this Chapter show that IL-5 can regulate eosinophil expansion in the bone marrow and peripheral blood in response to sensitization independently of STAT6 and normal development of Th2
cells. However, the lack of chemokine signals in the inflamed lungs in the absence of STAT6 impairs eosinophil accumulation in the airways. STAT6 also modulated arginase I expression, NO production and AHR in the allergic lung and deficiency of this transcription factor predisposed to a significant decrease in baseline airways reactivity to methacholine.
6.2 Materials and methods

6.2.1 Mice

See 2.2.1 and 3.2.1

6.2.2 OVA treatment of mice

See 2.2.2

6.2.3 Measurement of airways reactivity to methacholine

See 2.2.3

6.2.4 Characterization of lung morphology

See 2.2.4

6.2.5 Collection and analysis of peripheral blood, bone marrow and BALF

See 2.2.5

6.2.6 Depletion of CD4⁺, CD8⁺- cells and IL-5

6.2.6.1 Generation of neutralizing antibodies

Hybridoma cell lines specific for anti-CD4 (GK1.5), anti-CD8 (YTS 169.4), and anti-IL-5 (TRFK5) mAbs were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). Generation and quantitation of mAbs was performed as described in 3.2.3.1 and 3.2.3.2.
6.2.6.2 Procedure for the depletion of CD4⁺- and CD8⁺- cells and IL-5

Mice were i.p. injected with anti-CD4, anti-CD8 or anti-IL-5 (200µl of 1mg of the respective mAbs in saline) or isotype control mAb (as in Chapter 3) at day 19 during the OVA treatment regime as described in 6.2.2, and again every four days thereafter.

6.2.7 RT-PCR analysis

See 4.2.6

6.2.8 Assay of serum reactive nitrogen intermediate (RNI; nitrite and nitrate)

Measurement of the levels of serum RNI was carried out as described (Rockett et al., 1994). Initially, 30µl of plasma was dispensed into 1.5ml plastic tubes (Eppendorf Inc. Hamburg, Germany) in duplicate. Sodium nitrite (Sigma Chemical Co, St Louis, MO) and sodium nitrate (Sigma Chemical Co, St Louis, MO) standards (concentration range: 1 µM to 1mM) were then prepared separately in 30µl volumes of pooled (50 mice) normal mouse plasma. Control tubes contained 30µl of PBS (pH 7.2) and acts as a blank. A standard curve for sodium nitrite was developed by the addition of 20 µl of distilled H₂O or 15µl of NADPH solution (stock 1.25mg/ml in distilled H₂O) with 5µl of nitrate reductase (stock 5U/ml in distilled H₂O)(Boehringer Mannheim, Sydney, Australia). Samples received the same volume of distilled H₂O or NADPH plus nitrate reductase solution. All samples were incubated at room temperature for 20 min after which Griess reagent (100µl) [1% sulphanilamide (Sigma Chemical Co, St Louis, MO) + 0.1% N-(1-naphthyl) ethylene diamine-2 HCl (Sigma Chemical Co, St Louis, MO) in two molar sulphuric acid] and 10% trichloroacetic acid (100µl) were added to all samples. This was mixed thoroughly before centrifuging for 15 min at 500× g in a microcentrifuge (Model 5414C, Eppendorf, Hambrug, Germany). Two 100µl samples of the supernatant from each tube was transferred to a 96-well, flat-bottom plate (Nunc, Roskilde, Denmark) and the absorbance was read on a microplate reader (Dynatech MR 600, Dynatech Scientific, Inc., Camberidge, MA, USA) using a test wavelength of 540nm and a reference wavelength of 630nm. Plasma nitrite and nitrate were calculated by reading the absorbance directly from the respective standard curve. The results were expressed as micromolar concentrations of nitrite and nitrate (RNI).
6.2.9 Treatment of with \(\text{N}^\text{G}\)-methyl-L-arginine [L-NMA]

The competitive inhibitor of NO synthesis, L-NMA, inhibits the increase in plasma nitrate (Rolph et al., 1996). STAT6-deficient mice were administrated with either L-NMA (200mg/kg/day in 200µl saline) (gift from Dr. Bill W Cowden, Canberra, Australia) or the same volume of vehicle control for four days by i.p. injection daily. On day 4, airways reactivity to methacholine was measured as described in section 2.2.3.

6.2.10 Statistical analysis

See 2.2.8
6.3 Results

6.3.1 Characterization of eosinophil expansion in the bone marrow, peripheral blood and BALF of STAT6-deficient mice

To investigate the role of STAT6 in eosinophil expansion and migration, cell numbers were determined in the bone marrow, peripheral blood and BALF of WT and STAT6-deficient mice that were sensitized and challenged with OVA/OVA or SAL/OVA. OVA/OVA treated WT mice demonstrated a pronounced infiltration of inflammatory cells into the BALF. In particular, the levels of eosinophils in the BALF were significantly higher, compared to SAL/OVA treated WT mice (Figure 6.3.1 a). Very few eosinophils were found to accumulate in BALF of OVA/OVA treated STAT6-deficient mice (0.2±0.2×10^4 eosinophils/ml) and numbers were significantly lower than in OVA/OVA treated WT mice (90.2±11.6×10^4 eosinophils/ml). However, macrophage accumulation was similar in OVA/OVA treated WT and STAT6-deficient mice. Neutrophils in BALF of OVA/OVA treated STAT6-deficient mice were also significantly decreased compared to that of OVA/OVA treated WT mice.

Eosinophils in the peripheral blood of OVA/OVA treated STAT6-deficient mice exhibited a more dramatic accumulation compared to OVA/OVA treated WT mice. OVA/OVA treatment of both WT and STAT6-deficient mice resulted in a significant increase of eosinophils in the peripheral blood compared to respective SAL/OVA treated group (Figure 6.3.1 b). No increase in eosinophil number in the peripheral blood was found in SAL/OVA treated mice during OVA challenge.

OVA/OVA treatment of WT mice generated a pronounced increase in eosinophil numbers in the bone marrow compared to SAL/OVA treated mice (Figure 6.3.1 c). The number of eosinophils in the bone marrow of STAT6-deficient mice after OVA/OVA treatment was significantly lower than that in OVA/OVA treated WT mice. The number of eosinophils in the bone marrow of SAL/OVA treated WT and STAT6-deficient mice were not significantly different.
Chapter 6: Eosinophil accumulation in the absence of STAT6 in a mouse model of allergic airways disease

a.

**BALF leukocyte number x 10^4/ml**

- lymphocyte
- neutrophil
- eosinophil
- macrophage

<table>
<thead>
<tr>
<th></th>
<th>WT OVA/OVA</th>
<th>WT SAL/OVA</th>
<th>STAT6^/- OVA/OVA</th>
<th>STAT6^/- SAL/OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF leukocyte</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number x 10^4/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b.

**PB eosinophil (%)**

<table>
<thead>
<tr>
<th></th>
<th>D23</th>
<th>D25</th>
<th>D27</th>
<th>D29</th>
<th>D31</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SAL/OVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT OVA/OVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT6^/- SAL/OVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT6^/- OVA/OVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* and # indicate significant differences.
Chapter 6 Peripheral eosinophilia but not arginase I and AHR is induced in the absence of STAT6

Figure 6.3.1 Eosinophilia in BALF, peripheral blood and bone marrow in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice.

WT and STAT6-deficient mice were exposed i.p. to SAL or OVA and challenged with an aerosol of OVA. BALF leukocyte number (a) and eosinophil number in the bone marrow (c) were determined on day 31. Eosinophils in peripheral blood (b) were examined on alternate days to antigen challenge during the aerosol regime. Data represent mean ± SEM for n = 8-12 mice per group.

Figure 6.3.1 a, *P<0.05 compared to respective SAL/OVA treated mice, #P<0.05 compared to OVA/OVA treated STAT6-deficient mice; Figure 6.3.1 b, *P<0.05 compared to other groups, #P<0.05 compared to SAL/OVA treated mice; Figure 6.3.1 c, *P<0.05 compared to respective SAL/OVA treated mice, #P<0.05 compared to OVA/OVA treated STAT6-deficient mice;
6.3.2 Eosinophil numbers and mucus production in the lungs of STAT6-deficient mice

Histological examination of lung tissue indicated that mucus hypersecretion in the epithelium and eosinophil accumulation around peribronchial and perivascular regions were absent in OVA sensitized STAT6-deficient mice (Figure 6.3.2). OVA/OVA treated WT mice exhibited significant infiltration of eosinophils into the bronchial regions and mucus hypersecretion in the epithelium in comparison to SAL/OVA treated WT mice.

Quantitation of tissue number of eosinophils and mucus-producing cells is shown in Figure 6.3.3. The number of eosinophils in peribronchial and perivascular regions and mucus-producing cells in the epithelium of OVA/OVA treated WT mice were significantly higher than those in SAL/OVA treated WT mice and OVA/OVA treated STAT6-deficient mice. Mucus-producing cells were not observed in the epithelial layer of OVA/OVA treated STAT6-deficient mice, which was in marked contrast to OVA/OVA treated WT mice.

6.3.3 mRNA expression of chemokines in the lungs of STAT6-deficient mice

To investigate the role of STAT6 in the expression of chemokines, the levels of mRNA for eotaxin-1, eotaxin-2, MDC, TARC, TECK, RANTES, MCP-1, -2, -3 and -5, MIP-1α, -1β, -2 and -3α were examined in the lungs of SAL/OVA and OVA/OVA-treated WT and -STAT6-deficient mice on day 31 (Figure 6.3.4). Pronounced mRNA expression for eotaxin-1 and eotaxin-2 was observed in the lungs of OVA/OVA treated WT mice and neither of these two chemokines was expressed in the lungs of OVA/OVA treated STAT6-deficient mice. SAL/OVA treatment of both WT and STAT6-deficient mice showed low mRNA expression of these two chemokines. Expression of MCP-1, -2, -3 and -5, MIP-1α and -1β appeared to be increased when OVA/OVA treatment was compared to SAL/OVA treatment of both WT and STAT6-deficient mice. Interestingly, the expression of MCPs and MIPs appeared to increase in the lungs of OVA/OVA treated STAT6-deficient mice compared to that of OVA/OVA treated WT mice. No significant difference in the expression of MDC, TECK or TARC was observed in SAL/OVA and OVA/OVA treated WT or STAT6-deficient mice.
WT and STAT6-deficient mice were exposed i.p. to SAL or OVA and challenged with an aerosol of OVA. On day 31, lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with Carbol’s Chromotrope-Hematoxylin for eosinophils. OVA/OVA treated WT mice demonstrated large numbers of inflammatory cells (primarily eosinophils [arrow]) in the peribronchial and perivascular regions (i) compared to OVA/OVA treated STAT6-deficient mice (iii) (40× magnification). No significant infiltration of eosinophils was observed in SAL/OVA treated WT (ii) or STAT6-deficient (iv) mice.
Figure 6.3.2b Mucus-staining-cell number in the lungs of SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice.

WT and STAT6-deficient mice were exposed i.p. to SAL or OVA and challenged with an aerosol of OVA. On day 31, lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with alcian blue/PAS for mucus-secreting cells. Increased numbers of mucus-staining cells (i: epithelial cells stained by dark red) were observed around bronchial epithelial regions in OVA/OVA treated WT mice (i) (10× magnification). No increase in the number of mucus-staining cells were observed in SAL/OVA treated WT (ii), OVA/OVA or SAL/OVA treated STAT6-deficient mice (iii and iv respectively).
Figure 6.3.3 Characterization of the numbers of eosinophils and mucus-staining cells in lung tissue in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice.

WT and STAT6-deficient mice were exposed i.p. to SAL or OVA and challenged with an aerosol of OVA. On day 31, lungs were perfused with saline, removed and fixed in 10% NBF before sectioning and staining with Carbol’s Chromotrope-Hematoxylin for eosinophils and alcian blue/PAS for mucus-secreting cells. The numbers of eosinophils in lung tissue (a) and mucus-secreting cells in the central bronchial epithelium regions (b) were enumerated in 7-10 similar high-powered fields (HPF) (×40 magnification) per mouse. Data are the means ± SEM from n = 4-6 mice per group.

*P<0.05 compared to other groups.
Chapter 6: Eosinophil accumulation in the absence of STAT6 in a mouse model of allergic airways disease

(a) Eosinophil number/HPF

(b) Mucus-staining cell number/HPF
Figure 6.3.4 Respiratory chemokine mRNA expression in lungs of SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice.

WT and STAT6-deficient mice were exposed (i.p.) to saline or OVA and challenged with an aerosol of OVA. On day 31, lungs were examined by RT-PCR. The levels of mRNA encoding HPRT, MCP-1, MCP-2, MCP-3, MCP-5, MIP-1α, MIP-1β, MIP-2, MIP-3α, MDC, RANTES, eotaxin-1, eotaxin-2, TECK and TARC are shown. Eotaxin-1 and eotaxin-2 were significantly inhibited in OVA/OVA treated STAT6-deficient mice.
<table>
<thead>
<tr>
<th>Control PCR</th>
<th>WT OVA/OVA</th>
<th>WT SAL/OVA</th>
<th>STAT6-/­ OVA/OVA</th>
<th>STAT6-/­ SAL/OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TECK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TARC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-3α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3.4 AHR and serum RNI levels in STAT6-deficient mice

OVA/OVA treated WT mice developed a significant AHR as compared to SAL/OVA treated WT mice (Figure 6.3.5). Both SAL/OVA and OVA/OVA treated STAT6-deficient mice demonstrated significantly lower airways reactivity by comparison to not only OVA/OVA treatment but also SAL/OVA treatment of WT mice. No difference was found between the two STAT6-deficient groups.

RNI is an index of endogenous NO production (MacMicking et al., 1992). The levels of serum RNI in SAL/OVA and OVA/OVA treated STAT6-deficient mice were significantly higher than that in SAL/OVA and OVA/OVA treated WT mice (Figure 6.3.5 b). No significant difference in serum RNI was found between SAL/OVA and OVA/OVA treated WT mice, or between SAL/OVA and OVA/OVA treated STAT6-deficient mice.

6.3.5 mRNA expression of NO-associated enzymes in the lungs of STAT6-deficient mice

To investigate the role of STAT6 in the regulation of NO-associated enzymes in the lung, mRNA expression of arginase I, arginase II, iNOS, OAT and ODC were examined in the lungs of SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice on day 31 (Figure 6.3.6). Arginase I expression was induced in the lungs of OVA/OVA treated WT mice, but not in the lungs of OVA/OVA treated STAT6-deficient mice. SAL/OVA treatment of both WT and STAT6-deficient mice did not increase the mRNA expression of arginase I. Arginase II, OAT and ODC were constitutively expressed and levels of activation were not increased in any group. Expression of iNOS in the lung of OVA/OVA treated WT mice appeared to be inhibited as compared to the other three groups.

6.3.6 Administration of L-NMA increased the basal airways reactivity of STAT6-deficient mice

To identify the role of NO in decreased basal airways reactivity in STAT6-deficient mice, naïve mice deficient in this factor were treated with L-NMA, an inhibitor of NO synthesis (Rolph et al., 1996). Administration of L-NMA to STAT6-deficient mice
Figure 6.3.5 Characterization of airways reactivity and serum RNI in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice.

WT and STAT6-deficient mice were exposed i.p. to SAL or OVA and challenged with an aerosol of OVA. On day 31, airways reactivity to methacholine (a) was determined by barometric plethysmography and the levels of serum RNI (b) were examined. Data represent the percentage increase in enhanced pause (Penh) at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 4-6 mice per group.

Figure 6.3.5 a, * P<0.05 compared to other groups, #P<0.05 compared to SAL/OVA and OVA/OVA treated STAT6-deficient mice; Figure 6.3.5 b, * P<0.05 compared to SAL/OVA and OVA/OVA treated WT mice.
Chapter 6: Eosinophil accumulation in the absence of STAT6 in a mouse model of allergic airways disease

(a) Eosinophil accumulation in response to increasing methacholine concentrations. The graph shows the percentage increase over baseline for each group. WT SAL/OVA (white squares), WT OVA/OVA (black squares), STAT6−/− SAL/OVA (white circles), STAT6−/− OVA/OVA (black circles).

(b) Nitrite concentration (RNI) in WT and STAT6−/− mice. The bars represent the mean ± SEM. * denotes significant difference compared to WT SAL/OVA, and # denotes significant difference compared to WT OVA/OVA.
Figure 6.3.6 NO-associated respiratory enzyme mRNA expression in SAL/OVA and OVA/OVA treated WT and STAT6-deficient mice.

WT and STAT6-deficient mice were exposed i.p. to SAL or OVA and challenged with an aerosol of OVA. On day 31, lungs were examined by RT-PCR. The expression of mRNA encoding HPRT, arginase I, arginase II, OAT, ODC and iNOS are shown. STAT6 regulates the expression of respiratory arginase I mRNA. Expression of iNOS was inhibited in OVA/OVA treated WT mice.
generated a marked increase of airways reactivity in comparison to vehicle control, and resulted in similar levels of airways reactivity to that observed in naïve WT mice (Figure 6.3.7).

6.3.7 Role of IL-5, CD4 and CD8 positive cells in eosinophil accumulation in peripheral blood and bone marrow of OVA/OVA treated STAT6-deficient mice

To investigate the mechanism that regulated eosinophilia in the absence of STAT6, OVA/OVA treated STAT6-deficient mice were administrated with 1mg to murine IL-5, CD4, CD8 or isotype control mAb at day 19 by i.p. injection and every four days thereafter during OVA/OVA treatment regime. Administration of mAb to IL-5, significantly suppressed bone marrow eosinophil expansion (2.6×10^5 cells/ml) as compared to that in control mAb treated group (6.9×10^5 cells/ml) (P<0.05) (Figure 6.3.8 a). Levels in the bone marrow were similar to levels observed in SAL/OVA treated WT mice (2.9×10^5 cells/ml) or STAT6-deficient mice (2.8×10^5 cells/ml) (Figure 6.3.4 a) without mAb treatment. Moreover, bone marrow eosinophil levels in OVA/OVA treated STAT6-deficient mice, that received anti-IL-5-mAb, were significantly lower than those observed in OVA/OVA treated STAT6-deficient mice treated with depleting mAbs to either CD4 (P<0.05) or CD8 (P<0.05). However, administration of depleting mAbs to either CD4 or CD8 in OVA/OVA treated STAT6-deficient mice had no significant effect on eosinophil numbers in the bone marrow as compared to control mAb treated mice.

The administration of anti-IL-5 mAb significantly suppressed eosinophilia in the peripheral blood. The administration of depleting mAb to CD4 or CD8 did not effect eosinophil accumulation in the blood. Thus, IL-5 may regulate eosinophil movement in the absence of STAT6 and normal Th2 immunity.
Figure 6.3.7 L-NMA treatment enhanced basal airways reactivity of STAT6-deficient mice.

Naïve STAT6-deficient mice received L-NMA or vehicle control. Airways reactivity to methacholine was determined by barometric plethysmography. Airways reactivity of naïve WT mice was also assessed. Data represent the percentage increase in enhanced pause at a range of concentrations of methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml) over baseline reactivity in the absence of cholinergic stimuli. Data are the means ± SEM from n = 8-12 mice per group.

* P<0.05 compared to naïve WT mice and L-NMA treated STAT6-deficient mice.
Figure 6.3.8 Role of CD4⁺-, CD8⁺- cells and IL-5 in the regulation of eosinophil numbers in the bone marrow and peripheral blood of OVA/OVA treated STAT6-deficient mice.

STAT6-deficient mice were exposed i.p. to OVA and challenged with an aerosol of OVA. Mice were i.p. treated with anti-CD4, anti-CD8, anti-IL-5 mAbs at day 19, and every four days thereafter. Eosinophil number in bone marrow (a) was determined on day 31. Peripheral blood (b) was examined on alternate days to OVA challenge during the aerosol regime. Cell types were identified using morphological criteria with May-Grunwald-Giemsa staining. Data are the means ± SEM from n = 8-12 mice per group. There was no significant difference in eosinophil number between anti-CD4, anti-CD8 and isotype mAbs treatment in bone marrow or peripheral blood.

* P<0.05 compared to IL-5 mAb treated group.
Chapter 6: Eosinophil accumulation in the absence of STAT6 in a mouse model of allergic airways disease

a.

![Bar chart showing BM eosinophil numbers](chart_a)

Control Ab  CD8 Ab  CD4 Ab  IL-5 Ab

P<0.0006  P<0.006  P<0.005

b.

![Line graph showing PB eosinophil percentage](chart_b)

Control Ab  CD8 Ab  CD4 Ab  IL-5 Ab

D23  D25  D27  D29  D31
6.4 Discussion

In previous Chapters, it was shown that the administration of IL-13 induced marked eosinophil recruitment in the lung by potential up-regulation of a wide range of chemokines, and the development of a pronounced AHR that was abolished by supplementation of mice with HOBAT, an NO donor. Importantly, these effects are critically dependent on STAT6. Thus, it was of interest to determine the relevance of these observations, in the absence of STAT6, under allergic inflammatory conditions. STAT6-deficient mice do not develop AHR or airway mucus cell hypertrophy and have a significantly suppressed pulmonary inflammatory (in particular eosinophilia) response when sensitized and challenged with OVA (Akimoto et al., 1998; Kuperman et al., 1998). However, these studies did not characterize eosinophilic responses in the blood and bone marrow compartments.

As previously shown (Akimoto et al., 1998; Kuperman et al., 1998), we also see that OVA/OVA treated STAT6-deficient mice did not develop eosinophilia in either the BALF or pulmonary tissue and mucus hypersecretion (Figure 6.3.1). However eosinophils accumulated in the blood and bone marrow of OVA/OVA treated STAT6-deficient mice. By contrast to OVA/OVA treated WT mice levels of eosinophils were greater in the blood but reduced in the bone marrow of STAT6-deficient mice. Characterization of chemokine mRNA levels in the lung revealed that only expressions of eotaxin-1 and eotaxin-2 were dependent on STAT6, other chemokines (such as MDC, TECK, TARC, RANTES, MCPs and MIPs) were expressed independently of STAT6 in response to OVA/OVA treatment. These results further highlight the importance of the eotaxin family (by contrast to other chemokine subfamilies) in the recruitment of eosinophils to the airways of OVA/OVA treated mice.

Notably, blockade of IL-5 action in OVA/OVA treated STAT6-deficient mice resulted in the abolition of eosinophil expansion in the bone marrow and blood compartments. STAT6 is critical for CD4\(^+\) Th2 cell development (Kaplan et al., 1996). CD4\(^+\) Th2 cells are the predominant cellular source of IL-5, which is an essential factor for the development of eosinophilia (Hogan et al., 1998; Lee et al., 1998; Lalani et al., 1999; Fort et al., 2001). However, CD8\(^+\) T-cells, NK cells and mast cells are also able to produce IL-5 (Le Gros et al., 1994; Bressler et al., 1997; Walker et al., 1998). These cellular sources of IL-5 may regulate eosinophil expansion during allergen challenge in
STAT6-deficient mice. However, depletion of CD4\(^+\) and CD8\(^+\) cells in OVA/OVA treated STAT6-deficient mice did not suppress the levels of eosinophils in the bone marrow and also had no effect in the development of peripheral blood eosinophilia. These results suggest that although CD4\(^+\) Th2 cells play a central role in the induction of eosinophilia, other cells (e.g. NK cells and mast cells) may also contribute to the expansion of eosinophils in bone marrow and blood by the production of IL-5 and this occurs independently of STAT6. Notably CD3 positive cells have also been directly linked with the induction of eosinophilia in the bone marrow in response to allergic stimuli (Wood et al., 2002). By contrast, activation of STAT6 dependent pathways exclusively regulates migration into the lung, potentially through the regulation of eotaxin-1 and eotaxin-2.

AHR is a hallmark feature of allergic asthma. OVA/OVA treated STAT6-deficient mice did not develop enhanced airways reactivity to methacholine. Furthermore, airways reactivity of both SAL/OVA and OVA/OVA treated STAT6-deficient mice was significantly lower than that of not only OVA/OVA treated but also SAL/OVA treated WT mice. These effects were accompanied by increased levels of serum RNI in the STAT6-deficient mice. RNI reflects the level of NO production (MacMicking et al., 1992). Examination of the expression of pulmonary NO-associated enzymes showed increased expression of arginase I and decreased expression of iNOS in the lungs of OVA/OVA treated WT mice. By contrast no expression of arginase I and no change in the expression of iNOS in the lungs of STAT6-deficient mice were observed. Other NO-associated enzymes remained unchanged during antigen challenge in both WT and STAT6-deficient mice. It is thus indicative that the induced expression of arginase I or iNOS is important for the induction of AHR, possibly through the regulation of the production of endogenous NO. In Chapter 5 and other studies, the production of NO has been linked to the development of AHR (Henriksen et al., 2000; Ricciardolo et al., 2001). Furthermore, IL-13 also induces the expression of arginase I in the pulmonary compartment through the STAT6 pathway (Chapter 5). IL-13 also increases arginase activity as shown in Chapter 5 and in cultured murine macrophages (Rutschman et al., 2001). The downstream products of arginase I (e.g. polyamines such as spermine) inhibit NO production by inhibiting iNOS activity (Southan et al., 1994; Szabo et al., 1994; Baydoun et al., 1998). Up-regulation of arginase I depletes the shared substrate, L-arginine, of iNOS and inhibits the production of NO (Rutschman et al., 2001). Furthermore, inhibiting NO production by L-NMA in naïve STAT6-deficient mice
restored basal airways reactivity (to levels similar to that of naïve WT mice). Collectively, the above observations suggest that STAT6 is involved in the regulation of iNOS and the subsequent production of NO which play a important role in the development of AHR.

In conclusion, investigations in this Chapter show that allergic inflammatory pathways are regulated by the activation of STAT6, in particular the accumulation of eosinophils in the lung, induction of mucus hypersecretion and the development of AHR. The development of pulmonary eosinophilia and AHR may be intimately dependent on eotaxins, and iNOS and arginase-I activity, respectively. The pulmonary expressions of eotaxin-1, eotaxin-2 and arginase I were critically dependent on STAT6. The expression of iNOS in lungs was also significantly inhibited in WT mice during allergen challenge. NO may also regulate basal levels of airways reactivity to methacholine. Eosinophil accumulation in the blood and bone marrow compartments of STAT6-deficient mice are regulated by IL-5 independently of normal Th2 immunity. Treatment with anti-CD4 or anti-CD8 mAbs did not suppress the development of eosinophilia in the blood or the bone marrow of OVA/OVA treated STAT6-deficient mice suggesting that IL-5 was derived from other cellular sources. Thus, investigations highlight the importance of STAT6 in the development of allergic inflammation, eosinophilia and AHR.
CHAPTER 7

General discussion and summary
7.1 Pathophysiology of asthma

Asthma is a chronic disease of the lung of which airways inflammation and obstruction, mucus hypersecretion, enhanced serum IgE, pulmonary eosinophilia, airways wall remodeling and AHR are hallmark features (Kay 1991; Bochner et al., 1994; Holgate 1997; Wills-Karp 1999). The infiltration of CD4⁺ Th2 cells and eosinophils into the pulmonary compartment is a predominant clinical feature of asthma and these cells are thought to play key roles in pathogenesis (Bochner et al., 1994; Foster 1999; Wills-Karp 1999). CD4⁺ Th2 cells are thought to regulate airways inflammation and disease by secreting a range of cytokines that promote the recruitment and activation of inflammatory cells in the lung and modulate the function of resident pulmonary cells (Wills-Karp 1999). The aims of experiments in this thesis were to examine the interplay between the Th2 cytokines IL-5 and IL-13, and the eosinophil regulatory molecules, eotaxin-1 and eotaxin-2, for the regulation of eosinophilia, mucus hypersecretion and AHR in experimental models of allergic airways disease. This chapter will summarize the major findings described in this thesis and further discuss their potential contribution to the pathogenesis of allergic airways disease.

7.2 Interplay between IL-5, eotaxin-1 and eotaxin-2 for the regulation of eosinophilia, mucus secretion and AHR

The mechanisms involved in the regulation of eosinophil trafficking under allergic inflammatory conditions are complex. CD4⁺ Th2 cells are thought to be intimately involved in the regulation of eosinophilia through the secretion of IL-5. Indeed, IL-5 provides the essential signal for the induction of eosinophilia in the bone marrow and blood in response to allergic inflammatory stimuli (Hogan et al., 1998; Mould et al., 2000). However, investigations have shown that CC chemokines, in particular those belonging to the eotaxin family (e.g. eotaxin-1 and eotaxin-2) are important cofactors with IL-5 for eosinophil recruitment into tissues.

Data in this thesis support numerous investigations that have identified IL-5 as a key regulator of the development of eosinophilia and that this cytokine acts cooperatively with eotaxin-1 to regulate the trafficking of eosinophils to the lung (Collins et al., 1995; Foster et al., 1996; Kopf et al., 1996; Mould et al., 1997). Eotaxin-2, like eotaxin-1, also binds to CCR3 to regulate eosinophil function (Forssmann et al., 1997). Eotaxin-2
is elevated in the airways of asthmatic patients and may regulate the function of eosinophils in a manner similar to that of eotaxin-1 (Ying et al., 1999).

To investigate the role of IL-5 and eotaxin-1 in the development of eosinophilia, IL-5 and/or eotaxin-1 deficient mice were employed in a mouse model of allergic airways disease induced by OVA sensitization. The development of eosinophilia in the blood and bone marrow compartments was critically dependent on IL-5 in response to allergen inhalation. Expansion of eosinophil numbers in the blood and lung were not dependent on eotaxin-1, however, tissue accumulation was significantly attenuated in the absence of this chemokine. By comparison to WT mice, IL-5- and eotaxin-1-deficient mice had reduced numbers of eosinophils in the lung, however, eosinophilic inflammation was marked and significantly greater than that observed in non-treated control mice. Eosinophils did not accumulate in the inflamed lungs of mice deficient in both IL-5 and eotaxin-1. Moreover, the absence of eosinophils in lung tissue of IL-5/eotaxin-1-deficient mice directly correlated with the inability of these mice to develop AHR, suggesting that a critical level of eosinophils in the pulmonary compartment underpins enhanced airways reactivity. Examination of CD4+ Th2 cell function in IL-5/eotaxin-1- deficient mice revealed an impairment of CD4+ T-cell proliferation and Th2 cytokine production (notably IL-13). These studies indicated that the eosinophil regulatory molecules IL-5 and eotaxin-1 (potentially through eosinophils) modulate Th2 function and the subsequent development of allergic airways disease. While the mechanism of modulation was not investigated, eosinophils have been shown to regulate Th2 cells cytokine production through antigen presentation and this granulocyte can secrete an array of immunomodulatory factors that may effect T cell function (Del Pozo et al., 1992; Shi et al., 2000; MacKenzie et al., 2001). Interestingly, other work from our laboratory suggests a direct role for eosinophils (rather that IL-5 or eotaxin-1) in the modulation of cytokine production from Th2 cells through the secretion of IL-18 (Mattes et al., 2002).

It is tempting to speculate that eosinophils may sequester antigen in the inflamed lung and subsequently localize to local lymph nodes where they modulate IL-13 production from T cells during expansion by the secretion of IL-18. This mechanism may have evolved to promote the expulsion of parasites from the intestinal mucosa. Eosinophils loaded with parasitic antigens may enhance production of IL-13 by T-cells in gut-associated lymphoid tissue, which subsequently promotes the expulsion of the pathogen
by increasing gastrointestinal motility by amplifying cholinergic responsiveness and enhancing mucus secretion. This mechanism may provide a fundamental link between the innate (eosinophils) and adaptive (T-cells) immune responses for the regulation of IL-13 production and host protection from pathogens. These observations also indicate that IL-5 and IL-13 signaling systems are not necessarily mutually exclusive effector mechanisms, but may also be integrated (through eosinophils) to regulate certain aspects of allergic disease or immunity against parasite. Further the observation that eosinophils may regulate disease processes in the absence of IL-5 has important implications for therapeutic approaches to allergic disorders.

Eotaxin-1 has been previously shown to regulate the release of eosinophils from the bone marrow, which is important for maintaining a basal level of eosinophil trafficking into tissues (Matthews et al., 1998; Mould et al., 2000). Administration of recombinant eotaxin-1 intravenously to naïve WT mice induced a rapid and transient increase in blood eosinophil numbers. By contrast, i.v. injection of eotaxin-2 showed no effect on the levels of blood eosinophils. However, eotaxin-2 and eotaxin-1 had similar effects in the lung, inducing a pronounced eosinophilia (eotaxin-1 was more potent) and AHR. Furthermore, eotaxin-2 binds to CCR3, induces actin polymerization, Ca\(^{2+}\) mobilization and the release of reactive oxygen species from eosinophils with similar efficacy as eotaxin-1 (Forssmann et al., 1997; Elsner et al., 1998). Both molecules also cooperate with IL-5 to promote tissue eosinophilia. The inability of eotaxin-2 to generate a blood eosinophilia is intriguing but may reflect discrete changes in structure by comparison to eotaxin-1, and a greater affinity to bind to plasma proteins. Eotaxin-1 and eotaxin-2 are used differentially in the recruitment of eosinophils into sites of allergic inflammation in the skin (Ying et al., 1999), and the inability of eotaxin-2 to promote a blood eosinophilia may be linked to defined phases of the allergic response providing tighter regulation of migration.

The administration of eotaxin-2 to the airways of IL-5 Tg mice induced AHR, a marked pulmonary eosinophilia, mucus hypersecretion and the production of IL-13 in BALF. The effects of eotaxin-2 was completely inhibited by the administration of anti-CCR3 mAb indicating the central role for eosinophil, and not a direct role for IL-5 or eotaxin-2, in the development of these allergic responses. Like eotaxin-1, eotaxin-2 cooperated with IL-5 to induce eosinophil accumulation in the lung and AHR, further highlighting the importance of IL-5 and molecules that act through the CCR3 receptor for the
specific regulation of eosinophil migration. Studies with eotaxin-2 also further supported the concept that eosinophil regulatory pathways modulate the production of IL-13 in the lung. Examination of BALF revealed increased levels of IL-13 in both IL-5/eotaxin-2 treated WT mice and eotaxin-2 treated IL-5 Tg mice.

Investigations in factor deficient mice indicated that the production of IL-13 in the lung underpinned the development of AHR through the IL-4Rα/STAT6 pathway. Notably, AHR and mucus hypersecretion was not induced by eotaxin-2 in IL-5 Tg/STAT6-deficient, although pulmonary infiltration of eosinophils and the production of IL-13 persisted. This data indicates the importance of STAT6, down stream of eosinophil recruitment and IL-13 production, for the development of these responses. Depletion of CD4+ cells in IL-5/eotaxin-2 treated WT mice inhibited the increase of IL-13 in the BALF and the induction of AHR, although significant numbers of eosinophils infiltrated into the lungs. This data suggests that CD4+ cells are IL-13 play a key role in the induction of AHR by IL-5 and eotaxin-2. IL-5/eotaxin-2 induced AHR and eosinophil infiltration was also abolished in IL-5/eotaxin-1-deficient mice or WT mice treated with anti-CCR3 mAb. Collectively, these data with eotaxin-2 and IL-5 again identify important links between eosinophils and T-cells for the regulation of defined aspects of allergic disease.

The production of IL-5 and eotaxin-1 and/or eotaxin-2 (temporally regulated during the phases of allergic inflammation) promotes the accumulation of eosinophils into tissues. Eosinophils may activate/amplify IL-13 production from T-cells that subsequently regulates the development of AHR and mucus production through the IL-4Rα/STAT6 pathway. Eotaxin-1 may also cooperate with IL-5 to regulate blood eosinophilia.

### 7.3 IL-13 induced pulmonary eosinophilia and two phases of AHR

IL-13 is necessary and sufficient to induce pulmonary eosinophilia and AHR (Wills-Karp et al., 1998). To further define the molecular mechanisms whereby IL-13 induces pulmonary eosinophilia, mucus hypersecretion and AHR, recombinant murine IL-13 was administrated to the lungs of WT mice. IL-13 induced AHR was characterized by two phases: PIP and IP. Pulmonary eosinophil accumulation and mucus hypersecretion were only associated with AHR during the IP. Pulmonary eosinophilia and mucus hypersecretion induced by IL-13 lasted up to 8 days, while levels of airways reactivity...
had returned to baseline levels by this time. Experiments on IL-4/IL-13 double deficient mice demonstrated that IL-13 was sufficient to induce pulmonary eosinophilia, mucus hypersecretion and AHR without the participation of endogenous IL-4 and IL-13, suggesting that the sustained effects of IL-13 were regulated through other pathways. IL-13 partially utilized IL-5 and eotaxin-1 to promote pulmonary eosinophilia, however, AHR and mucus hypersecretion were not attenuated in IL-5/eotaxin-1 deficient mice.

Examination of pulmonary mRNA expression of chemokines and transcripts related to airways remodeling showed that IL-13 regulated the production of a wide range of inflammatory molecules. In particular, eotaxin-1 and eotaxin-2 were significantly upregulated by IL-13 during both PIP and IP responses, suggesting an important role in the recruitment of eosinophils during the IP. Furthermore, administration of IL-13 into the lung differentially regulated the expression of TECK, TARC, MDC, MCPs (MCP-1, -2 and -3) and MIPs (MIP-1α, -1β, -2 and -3α). These chemokines mediate the activation and migration of a range of inflammatory cells including of T lymphocytes, neutrophils, eosinophils, NK cells and monocytes (Van Coillie et al., 1999; Kaplan 2001). These data were supported by increased numbers of lymphocytes, neutrophils, eosinophils and macrophages in BALF after IL-13 treatment. Notably, MDC and TARC play important roles in the interaction between APCs and memory T-cells and thus promote T-cell responses with a Th2 profile (Andrew et al., 1998; Vestergaard et al., 2000).

MMPs are a large group of extracellular enzymes, which play important roles in the turnover and degradation of connective tissue proteins (Nagase et al., 1999; Tierney et al., 1999). Moreover, TIMP-1, MMP-2, -9, -12, -13 and -14, cathepsin-B and -S are critically involved in the development of inflammation, repair and other tissue remodeling processes (Dahlen et al., 1999; Perez-Ramos et al., 1999; Tschesche et al., 2000; Cataldo et al., 2001; Taggart et al., 2001; Ohbayashi 2002). Interestingly, expression of mRNA of only MMP-12 and -13 in lungs appeared increased in response to IL-13 treatment. In rodents, MCP-12 and -13 have been identified as important proteinases associated with macrophages (Parks et al., 2001; Tetley 2002). Macrophages from MMP-12 deficient mice fail to degrade elastin and many other matrix substrates (Shipley et al., 1996). MMP-13 also plays an important role in the pathogenesis of tissue destruction (Lindy et al., 1997). Notably, macrophages in BALF of IL-13 treated WT mice were significantly increased compared to vehicle control.
during both PIP and IP responses. Taken together, these data indicate that IL-13 may regulate the production of MMP-12 and -13 to induce airways remodeling and it is likely that activated macrophages may participate in the expression of these two proteinases and potentially AHR.

Mucus hypersecretion and goblet cell metaplasia are important features of allergic asthma. Mucin glycoproteins are the predominantly found in the airway mucus layer that acts as a protective barrier against pathogens (Litt et al., 1974; Rose et al., 1979). Several mucin genes (e.g. Muc-2, -4, -5AC and -5B) are expressed in normal respiratory tract tissues (Rose et al., 2001) and are thought to be responsible for the production of mucin glycoproteins in the airways. The Muc-2 gene is also significantly induced in rat airways of experimental chronic bronchitis (Jany et al., 1991). The Muc-4 gene is involved in the integrity and renewal of airway epithelium (Moniaux et al., 2000), and possibly in the development of lung cancers (Nguyen et al., 1996; Hanaoka et al., 2001). Muc-5AC mRNA and protein accompanied the goblet cell metaplasia which was induced in a mouse model of asthma (Zuhdi Alimam et al., 2000). Muc-5B more likely plays a role in the production of lung mucus in obstructive lung disease (Sheehan et al., 1995; Thornton et al., 1997; Wickstrom et al., 1998). Both Muc-2 and Muc-5AC are upregulated in a guinea-pig model of allergic asthma (Li et al., 2001). IL-4 induces the expression of Muc-2 in cultured mouse airway epithelial cells (Dabbagh et al., 1999). In this thesis, administration of IL-13 significantly increased the expression of Muc-2 and -5AC in the lungs of mice during the IP response, which accompanied airway mucus hypersecretion and goblet cell metaplasia. This implies that Muc-2 and -5AC play an important role in IL-13 induced mucus hypersecretion and goblet cell metaplasia. Collectively, these data suggest that IL-13 is a potent activator of a number of important inflammatory cascades that have significant implications for asthma pathogenesis and the exacerbation of disease and identify this cytokine as a pivotal target for the treatment of disease.

7.4 Supplementation of NO completely inhibits IL-13 induced AHR

AHR is a hallmark feature of asthma and regulated by many factors (Wills-Karp 1999; Primhak et al., 2002). Many investigations have shown that a range of factors (such as CD4+ T-cells, B-cells, mast cells and eosinophils and their products) are involved in the development of AHR (Gleich et al., 1988; Garssen et al., 1990; Hogan et al., 1998). In
particular, CD4$^+$ T-cells are thought to be the central mediators of allergic airways responses (Umetsu et al., 1997; Wills-Karp 1999; Foster et al., 2002). Swiss nude mice are athymic animals that have severely impaired T-cell function (Gaillard et al., 1998). Thus, Swiss nude were employed to investigate the role of T lymphocytes in IL-13 induced AHR at baseline. IL-13 still induced significant AHR during the PIP response, however, AHR induced by IL-13 was markedly attenuated in Swiss nude mice during the IP response. Pulmonary eosinophilia and mucus hypersecretion occurred independently of T lymphocytes. This suggests that normal T lymphocyte function is important in the amplification of airways responses to methacholine during the IP response after IL-13 treatment.

Mast cells are important in the initiation of the early-phase of the asthmatic response. However, their role and those related molecules (such as histamine and IgE) in the development of AHR in experimental model of allergic asthma remain controversial (Lack et al., 1995; Geba et al., 1997; Hamelmann et al., 1999; Wilder et al., 1999; Wills-Karp 1999; Mayr et al., 2002). Treatment of mice with DSCG, inhibiting mast cell function, demonstrated that mast cells are not essential in the onset of IL-13 induced AHR in BALB/c mice at baseline.

IL-13 may induce AHR by directly regulating the function of airway resident cells such as macrophages, epithelial cells or ASM cells (Doherty et al., 1993; Jordan et al., 1997; Li et al., 1999). Notably, pulmonary expression of arginase I significantly correlated with the temporal development of IL-13 induced AHR. However, levels of arginase II and iNOS remained unchanged. Arginase I is normally a liver enzyme involved in the urea cycle (Jenkinson et al., 1996), and is also upregulated at sites of inflammation (Waddington et al., 1998). The downstream products of arginase I not only promote the synthesis of protein and interact with membrane ion channels (Balasundaram et al., 1991; Bowie et al., 1995; Liu et al., 2000; Wang et al., 2000; Camon et al., 2001), but also inhibit the synthesis of NO (Mossner et al., 2001). Administration of L-arginine, a substrate of both arginase I and iNOS, significantly promoted IL-13 induced AHR. Interestingly, supplementation of NO completely inhibited IL-13 induced AHR even at peak responses, but had no effect on the development of pulmonary eosinophilia and mucus hypersecretion. NO, a highly diffusible small molecule plays a crucial role in bronchial dilation and neuron transmission in vivo (Adatia et al., 1994; Archer et al., 1994; Byrick et al., 1999; Naoki et al., 1999). This implies that increased expression of
pulmonary arginase I participates in the onset of IL-13 induced AHR by multi-factorial pathways in particular by regulation of the endogenous production of NO.

IL-13 predominantly binds the IL-4Rα/IL-13Rα1 complex, which then activates STAT6 (Murata et al., 1996; Palmer-Crocker et al., 1996). IL-4Rα- and STAT6-deficient mice treated with IL-13 did not develop pulmonary eosinophilia, mucus hypersecretion and the two phases of AHR were abolished. This suggests that IL-13 exerts its biological effects by binding to the IL-4Rα/IL-13Rα1 complex and activating of STAT6. Importantly, IL-13 induced arginase I expression was also dependent on STAT6.

7.5 Role of STAT6 in a mouse model of allergic airways disease

CD4+ Th2 cells are thought to be the key regulators of allergic asthma (Robinson et al., 1993; Hogan et al., 1998; Wills-Karp 1999). STAT6 has been identified as a crucial mediator in the polarization of CD4+ Th2 cells in response to IL-4 stimuli (Kaplan et al., 1996). Increasing evidence in STAT6-deficient mice suggests that this transcription factor is a key regulator of the development of pulmonary eosinophilia and AHR (Takeda et al., 1996; Kuperman et al., 1998; Miyata et al., 1999). In our studies IL-13 appeared to regulate AHR by activating arginase I activity and altering NO metabolism in a STAT6 dependent manner but the relevance of this mechanism during allergic airways disease was not established. OVA/OVA treated STAT6-deficient mice did not develop AHR or eosinophilia in the lung as previously described (Takeda et al., 1996; Kuperman et al., 1998). This effect in OVA/OVA treated STAT6 deficient mice was accompanied by significantly increased levels of serum NO metabolites. Examination of pulmonary mRNA showed significantly increased expression of arginase I in OVA/OVA treated WT mice but not in OVA/OVA treated STAT6-deficient mice. Moreover, the expression of iNOS was markedly inhibited in OVA/OVA treated WT mice but not in OVA/OVA treated STAT6-deficient mice. The levels of AHR in both SAL/OVA and OVA/OVA treated STAT6-deficient mice were significantly lower that that observed in not only OVA/OVA but also SAL/OVA treated WT mice. Furthermore, by inhibiting NO production with L-NMA, the basal airways reactivity of naïve STAT6-deficient mice recovered to the similar levels to that observed in naïve WT mice. This indicates that the differential expression of arginase I and iNOS in the
l lung is crucially dependent on the activation of STAT6 and not only contributes to the development of AHR but also regulates baseline airways reactivity.

Notably, although tissue accumulation of eosinophils in OVA/OVA treated STAT6-deficient mice was abolished, this leukocyte accumulated in the bone marrow and peripheral blood compartments. Examination of pulmonary chemokine mRNA revealed that eotaxin-1 and eotaxin-2 were absent in OVA/OVA treated STAT6-deficient mice, although other chemokines were still expressed. In combination with Chapter 5, these data indicate that eotaxin-1 and eotaxin-2 are key regulators of pulmonary eosinophilia and their production may be regulated by IL-13 in a STAT6 depended manner. This is supported by extensive studies on these two molecules in both human and mouse models (Forssmann et al., 1997; Mould et al., 1997; Elsner et al., 1998; Ying et al., 1999). Depletion of IL-5 by anti-IL-5 mAb completely inhibited the onset of eosinophilia in both the bone marrow and the blood compartments in OVA/OVA treated STAT6-deficient mice. These data once again emphasize the critical role of IL-5 in the development of systemic eosinophilia (Foster et al., 1996; Kopf et al., 1996; Kotsimbos et al., 1997; Garlisi 1999; Lalani et al., 1999). Although extensive investigations show that CD4+ Th2 cells are the predominant cellular source of IL-5, other inflammatory cells (e.g. CD8+ T-cells and NK cells) are also capable of producing this cytokine (Ying et al., 1997; Hogan et al., 1998; Walker et al., 1998; Hofstra et al., 1999; Fort et al., 2001). Furthermore, the depletion of CD4+ cells or CD8+ cells with anti-CD4 or anti-CD8 mAbs did not alter the development of eosinophilia in the bone marrow and peripheral blood compartments in OVA/OVA STAT6-deficient mice. This indicates that other cells, such as NK cells, may also involve in the development of eosinophilia by producing of IL-5 and that this process is not exclusively regulated by CD4+ Th2 cells.

7.6 Conclusion

Investigations in this thesis on different inflammatory factors in models of allergic airways disease have highlighted the complex nature of the mechanisms that underpin the development of inflammatory responses. In particular, the data show that inflammation at a cellular and molecular level is regulated by an integrated network. The experiments described in this thesis have added to the accumulated knowledge linking eosinophilia with the development of AHR. Importantly, they extend our
knowledge by showing that eosinophils and eosinophil regulatory molecules may regulate IL-13 production from T-cells and subsequently AHR and mucus hypersecretion through IL-4Rα/STAT6 pathways. They also suggest that eosinophils are not just terminal effector cells but also act as immune modulators. Further investigations on the downstream pathways regulated by IL-13 and IL-4Rα/STAT6 may provide new knowledge on the development of defined pathogenic responses (AHR, mucus hypersecretion, eosinophilia, fibrosis and other features of airway wall remodeling) in allergic diseases and identify novel therapeutic targets. In particular, the regulation of NO and associated enzymes (in particular arginase I and iNOS) should provide a new understanding of the mechanisms that regulate the development of AHR.
References


References


References


Christie, M., B. Rutti and M. Brossard (2000). Cytokines (IL-4 and IFN-gamma) and antibodies (IgE and IgG2a) produced in mice infected with Borrelia burgdorferi sensu stricto via nymphs of Ixodes ricinus ticks or syringe inoculations. Parasitol Res 86(6): 491-6.


References


References


References


activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. J Immunol 157(12): 5613-26.


References


References


Hope, J. C., F. Campbell and S. J. Hopkins (2000). Deficiency of IL-2 or IL-6 reduces lymphocyte proliferation, but only IL-6 deficiency decreases the contact hypersensitivity response. Eur J Immunol 30(1): 197-203.


References


References


References


References


References


References


References


References


References

References


References
References


