Cytotoxic T cell-mediated immunotherapy of allergic airway inflammation

Naomi June Daniels
Malaghan Institute of Medical Research

A thesis submitted to the University of Otago for the degree of Doctor of Philosophy in Immunology

2015
Abstract

The worldwide incidence of atopic disease, including asthma, has increased dramatically over the last few decades. In New Zealand, asthma affects at least one in four children and one in six adults, a prevalence among the highest reported in the world. Up to half of these individuals suffer from exacerbations of disease that are driven by allergic airway inflammation. Key instigators of allergic airway inflammation are CD4+ Th2 cells that respond to normally harmless inhaled antigens presented by airway-surveying antigen presenting cells (APC). Th2 production of chemical mediators subsequently induces eosinophilia, mucus hypersecretion and airway remodeling; the characteristic features of allergic airway pathology. Allergen-specific cytotoxic T lymphocytes (CTL) can ameliorate airway inflammation in a model of acute allergic airway inflammation, however, the mechanism of their inhibitory activity is not fully defined. Furthermore, the effects of CTLs on established airway inflammation, like that which presents clinically, have not been elucidated.

In this thesis, a mouse model of allergic airway inflammation was established and characterised, where multiple allergen exposures stimulated significant airway pathology. Additionally, a model involving the adoptive transfer of in vitro-generated Th2 cells into naive hosts was developed to investigate the cellular interactions, phenotype, and disease-mediating functions of these cells during active inflammation. Pathology was examined by enumeration of lung- and airway-infiltrating inflammatory cells, assessment of the extent of mucus production, as well as the measurement of inflammatory mediators. Lung tissue was also examined microscopically to investigate the interaction of CTLs with key disease-mediating cells: Th2 cells and APCs – specifically, lung CD103+ and CD11b+ dendritic cells (DCs). The cellular targets of cytolytic CTLs were additionally investigated by use of fluorescent probes to detect the expression of active cell-apoptosis-associated caspases within dying cells.

Treatment with allergen-specific CTLs was found to suppress inflammation over multiple allergen challenges, and was additionally effective in the amelioration of established allergic airway inflammation. Therapeutic success was associated with reduced early cytokine
production by Th2 cells in lung, as well as diminishing their subsequent accumulation and production of IL-4 and IL-13. In addition, treatment with CTLs increased the proportion of caspase+ DCs in the lung-draining mediastinal lymph node and decreased the numbers of CD103+ and CD11b+ DC in the lung. The targeting of DCs was dependent on their ability to directly interact with CTLs through the presentation of antigen on MHC-I, with lung imaging revealing interactions not only with the CD103+ classical cross-presenting DCs, but also in the critical allergy-driving CD11b+ DCs. Therefore, allergen-specific CTLs deplete CD103+ and CD11b+ DC populations in the lung, which in turn reduces allergen presentation to disease-mediating Th2 cells, resulting in amelioration of allergic airway inflammation. Immunotherapy with allergen-specific CTLs thus represents a targeted treatment that may provide new hope in the fight against allergic exacerbations of atopic asthma.
Acknowledgements

I would like to express my sincere thanks to my supervisor, Prof Franca Ronchese. You have challenged me, supported me, and shown patience throughout my development as a researcher. I am honoured to have worked under a scientist with such a wealth of knowledge and aptitude for brilliance. Your mind for science is incredible, as is your passion and determination to give your all for this cause; you are an inspiration. Thank you.

I also thank Dr Lisa Connor and Prof Brett Delahunt for co-supervision, especially for the support and understanding offered by Lisa through the later stages of my PhD. I have been incredibly fortunate to be a part of the Malaghan Institute of Medical Research, which has established itself as world class in the field of Immunology through the continuing excellence of it's researchers and support staff, under the direction of Prof Graham Le Gros. Thank you, Graham, for your stubborn determination to accept nothing less than constant progress and innovation from all who become a part of MIMR; may the success of the institute exceed even that which you have envisaged.

I also gratefully acknowledge the support of Prof Alex McLellan and Prof Frank Griffin. It was your backing that initially enabled me to undertake this PhD, and I am especially thankful for the continued support and encouragement of Alex, going out of your way to facilitate my studies and always speak positively of me. I am incredibly grateful for training and assistance from Mark (Jianping) Yang and Evelyn Hyde. Your willingness to help with my extensive experimental end points was invaluable! I have appreciated all the members of the Immune Cell Biology group and thank you for your assistance, advice, input, and queries over the years. Thank you to the support staff of the Hugh Green Cytometry Core and Biological Research Unit who enable the success of our experiments. A special thanks to Apii Ulberg, who makes the Malaghan Institute feel more like a family. You have blessed all of us with your smile, your interest in our lives and research, and the way you find joy in all that you do.
I would like to express thanks to my fellow PhD students, past and present, with whom many discussions, immunological and otherwise, have challenged and shaped my own journey. A special thank you to Sabine Kuhn, Alanna Cameron, Cameron Field, Ryan Kyle, Catherine Plunkett and Connie Gilfillan. You have supported me, housed me, fed me during crazy experimental days, deliberated with me in difficult times and celebrated successes with me. Sabine, we shared much life and science over coffees, thank you for helping me to maintain a healthy headspace.

I am sincerely grateful for the financial support of the Rotary Club of Wellington, the University of Otago, the Wellington Medical Research Foundation, the Maurice Wilkins Centre for Molecular Biodiscovery and Lottery Health Research Grants for funding my project, my PhD scholarship, and travel awards that enabled me to present my work at international conferences.

To my friends and family. There are not words enough to express my gratitude to all of you. Thank you for believing in me, standing by me, and showing understanding when this endeavour required much from me- sometimes at your expense. Mandy, Anita, Anni, Renee, Jo, you have kept my mind in balance. My church families and lifegroups at Nations and The Street, you have kept my heart well. I am thankful for the incredible, determined, intelligent women who have gone before me and been my inspiration, my Mum, Grandma, and Nan. Mum and Dad, your support has been endless, your encouragement always sincere, and your wisdom and guidance have grounded me, I thank you for constantly blessing me. Josh, Sam, Ben, Megan, Emma, Chelsea, you bring so much joy to my life, I love you. Libby, thank you for always trying to keep me well; Brian, Kaori, Paul, Damien, Kim, thank you for your love.

To my husband, my best friend, my greatest encourager. You have never ceased to be positive, supportive and encouraging throughout my studies. You inspired me to remain strong to see the task through, endured the years we had to spend apart, and preserved my sanity along the way. Thank you for loving me and making me more than I could have been on my own...and for learning how to describe to someone what cytotoxic T cells get up to with dendritic cells- you’re amazing.
Finally, to The Author of Life, whose masterpiece has captured me. Thank you will never be enough. You inspired me and carried me. You are the reason I am who I am, and by grace may I continue to seek to fulfil your purposes in this life you have blessed me with. Thank you, with all that I am.
# Table of Contents

Abstract iii  
Acknowledgements v  
Table of Contents ix  
List of Tables xiv  
List of Figures xv  
List of Abbreviations xviii

## 1 Introduction  
1.1 Key players in the induction of immune responses 5  
1.1.1 Dendritic cells drive T cell responses 7  
1.1.2 CD4+ T cell subsets 14  
1.1.3 CD8+ T cells 19  
1.1.4 The generation of immunological memory 22  
1.1.5 Immune cells in the lung under steady state conditions 23  
1.2 Orchestration of Th2-mediated pathology 28  
1.2.1 Characteristics of allergens 28  
1.2.2 Priming Th2 immunity 30  
1.2.2.1 Sensitisation to aeroallergens 39  
1.2.2.2 Aeroallergen challenge: Th2 effector functions and cytokine production mediate the pathology of atopic allergic asthma 39  
1.2.2.3 Airway remodelling and chronic allergic airway disease 44  
1.3 CTLs in the treatment of allergic airway disease 46  
1.4 Modelling allergic airway inflammation 50  
1.4.1 Methods of inducing allergic airway inflammation 50  
1.4.2 Model allergens in the induction of experimental allergic airway inflammation 51  
1.4.3 Murine models of experimental allergic airway inflammation 53  
1.4.4 Acute and chronic models of allergic airway inflammation 55
1.4.5 Visualising allergic responses 56

1.5 Aims of this thesis 59

2 Materials and methods 61

2.1 Materials 63
   2.1.1 Labware 63
   2.1.2 Reagents and buffers 64
   2.1.3 Commercial kits 69
   2.1.4 Antibodies and fluorophores 71
   2.1.5 Mice 72

2.2 Methods 75
   2.2.1 In vitro methods 75
   2.2.2 In vivo methods 77
   2.2.3 Ex vivo methods 79
   2.2.4 Methods of data analysis 87

3 Allergen-specific CTLs have preventative and therapeutic effects in a multiple allergen exposure model of allergic airway inflammation 91

3.1 Introduction 93

3.2 Aims 96

3.3 Results 97
   3.3.1 A multiple airway allergen exposure model displays characteristic features of allergic airway inflammation 97
   3.3.2 CTLs suppress the development of allergic airway inflammation 102
   3.3.3 CTLs have therapeutic effects on established allergic airway inflammation 103
   3.3.4 CTLs decrease mastocytosis in the allergic airway 109
   3.3.5 CTLs have therapeutic effects in a multiple challenge model of allergic asthma that displays markers of established disease 114

3.4 Discussion 119
4 CTL-mediated suppression of allergic airway inflammation is associated with a reduction in disease-mediating Th2 cells in the lung and airways 127
4.1 Introduction 129
4.2 Aims 131
4.3 Results 132
   4.3.1 Optimisation of CD4+ T cell culture system 132
   4.3.2 Optimisation of an adoptive transfer model of allergic airway inflammation 133
   4.3.3 CTLs suppress inflammation in an adoptive transfer model of allergic airway disease in a manner dependent on perforin 138
   4.3.4 Time course of the infiltration of adoptively transferred CD4+ and CD8+ T cells into the lung and BAL 141
   4.3.5 CTL immunotherapy partially reduces the proliferation of disease mediating CD4+ T cells in a model of allergic airway inflammation 148
   4.3.6 The expression of activation markers on CD4+ T cells is not affected differentially by wild type CTLs, compared to PKO CTLs 150
   4.3.7 Number of total and Th2 cytokine-producing CD4+ T cells in the lung is significantly reduced by CTL immunotherapy 154
   4.3.8 T regulatory cell numbers in the lung are not affected by CTL treatment at the day 3 peak of lung inflammatory cell infiltrate 156
4.4 Discussion 160

5 Allergen-specific CTLs target CD103+ and CD11b+ lung DCs to suppress allergic airway inflammation 169
5.1 Introduction 171
5.2 Aims 173
5.3 Results 174
   5.3.1 CTL therapy increases the expression of pro-apoptotic caspases in DCs 174
   5.3.2 CTLs elicit a perforin-dependent decrease in lung DCs 176
   5.3.3 CTL therapy targets CD11c+CD11b+CD64- conventional lung dendritic cells 178
5.3.4 DCs form clusters with CTLs near small airways in the lung during allergic airway inflammation 183
5.3.5 CTLs and CD4+ T cells co-localise with both XCR1\textsuperscript{GFP}+ and CD11c+XCR1- DCs in the lung 186
5.3.6 OVA-specific CTLs abrogate allergic airway inflammation in OVA/HDM dually sensitised mice 191
5.3.7 A novel peptide conjugate vaccine elicits an allergen-specific CTL response that inhibits allergic airway inflammation 195
5.4 Discussion 200

6 General Discussion 211

6.1 The main findings of this thesis 213
   6.1.1 CTL-mediated suppression of allergic disease is associated with effects on disease-mediating CD4+ Th2 cells 213
   6.1.2 Allergen-specific CTLs counteract Th2 immune responses to suppress allergic airway inflammation 217
   6.1.3 Allergen-specific CTLs have therapeutic effects on established airway inflammation 220
6.2 Importance of findings in the understanding of allergy 224
6.3 Implications for clinical immunotherapy 226
6.4 Summary and conclusions 229
6.5 Future directions 230

References 235

Appendices 309
   A Distribution of cell frequencies in tissue samples 310
   B Publications 313
List of Tables

Table 2.1  Labware  
Table 2.2  Antibodies and fluorophores  
Table 2.3  RT-PCR Primers  
Table 4.1  Optimisation of CD4+ Th2 cell generation – conditions for culture
List of Figures

Figure 1.1 CD4+ T cell subsets 14
Figure 1.2 The allergic response 32
Figure 3.1 BAL analysis by flow cytometry 98
Figure 3.2 Multiple intranasal allergen exposures provokes significant immune cell infiltrate in BAL, comprised primarily of eosinophils and lymphocytes 99
Figure 3.3 Inflammatory infiltrate improves within 6 days and resolves by 10 days after intranasal challenge 101
Figure 3.4 Peak of cytokine production is day 1 after airway allergen challenge 102
Figure 3.5 CTLs prevent inflammation over multiple airway challenges 104
Figure 3.6 CTLs have therapeutic effects on allergic airway inflammation 106
Figure 3.7 CTLs have therapeutic effects on airway congestion and goblet cell mucus production 107
Figure 3.8 CTL immunotherapy reduces mobilisation of eosinophils from the bone marrow 108
Figure 3.9 Mast cell accumulation in tissue is prevented, IgE production unaffected by CTL therapy 110
Figure 3.10 Mast cell number, protease production and localisation in a chronic model of allergic airway inflammation 113
Figure 3.11 Allergen-specific CTLs have therapeutic and long term preventative effects on allergic airway inflammation 115
Figure 3.12 CTL immunotherapy resolves features of allergen-induced airway remodelling 117
Figure 4.1 Optimisation of CD4+ T cell culture conditions for adoptive transfer experiments 134
Figure 4.2 Airway inflammation induced by varying numbers of adoptively transferred CD4+ T cells 135
Figure 4.3  The induction and suppression of airway inflammation by adoptively transferred T cells 137
Figure 4.4  CTLs suppress inflammatory infiltrates in an adoptive transfer model of allergic airway inflammation in a manner dependent on perforin 140
Figure 4.5  Adoptive transfer of OTII cells recapitulates in vivo sensitisation models of allergic airway inflammation and the inhibitory effect of allergen-specific CTL 142
Figure 4.6  CTLs suppress the infiltration of adoptively transferred and host CD4+ and CD8+ T cells 144
Figure 4.7  Tissue infiltration kinetics of adoptively transferred CD4+ and CD8+ T cells in vivo 146
Figure 4.8  Partial suppression of CD4+ T cell proliferation in CTL-treated mice 149
Figure 4.9  The expression of activation markers on adoptively transferred CD4+ T cells in the absence of intranasal allergen exposure 151
Figure 4.10  The effects of CTL immunotherapy on the activation status of CD4+ T cells in vivo 153
Figure 4.11  4C13R CD4+ T cells recapitulate adoptive transfer model disease characteristics and suppression of airway inflammation is achieved by CTLs 155
Figure 4.12  The number of total and cytokine producing CD4+ T cells in the lung is significantly reduced by CTL immunotherapy 157
Figure 4.13  T regulatory cell numbers in the lung are not affected by CTL treatment, at the day 3 peak of lung inflammatory cell infiltrate 159
Figure 5.1  Increased activation of pro-apoptotic caspases in DCs after CTL therapy 175
Figure 5.2  CTLs elicit a perforin-dependent decrease in lung DCs 177
Figure 5.3  The effects of CTL immunotherapy on the activation status of DC populations in vivo 179
Figure 5.4  Evaluation of the degree of chimerism of mixed bone marrow chimeric mice 181
Figure 5.5  CTL immunotherapy targets CD11c+CD11b+CD64-conventional lung dendritic cells 182

Figure 5.6  XCR1\textsuperscript{GFP}+ DCs redistribute to form clusters in the allergic lung and MLN 184

Figure 5.7  CTLs are located sparsely around large airways in the lung; more predominantly clustering with XCR1\textsuperscript{GFP}+ and CD11c+XCR1-DC around small airways 187

Figure 5.8  CTLs interact with both XCR1\textsuperscript{GFP}+ DC and CD11c+XCR1-DC 189

Figure 5.9  CD4+ T cells also localise within DC/CTL clusters 190

Figure 5.10  HDM-induced allergic airway inflammation in C57BL/6 mice 193

Figure 5.11  OVA-specific CTLs abrogate allergic airway inflammation in OVA/HDM dually sensitised mice 194

Figure 5.12  A novel peptide conjugate vaccine inhibits allergic airway inflammation 197

Figure 5.13  A novel peptide conjugate vaccine prevents airway histopathology 199

Figure 5.14  Proposed mechanism of OVA-specific CTL-mediated suppression of OVA-and-HDM induced airway inflammation 206

Figure A.1  Cell frequencies and cytokine concentration in BAL and lung are consistent with Gaussian distribution 310
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>αGalCer</td>
<td>α-galactosylceramide</td>
</tr>
<tr>
<td>ABPAS</td>
<td>Alcian blue periodic acid-Schiff stain</td>
</tr>
<tr>
<td>ACT</td>
<td>Ammonium Chloride Tris</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperreactivity</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>AMCase</td>
<td>Acidic mammalian chitinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
</tr>
<tr>
<td>BAL-F</td>
<td>Broncho-alveolar lavage fluid</td>
</tr>
<tr>
<td>BATF3</td>
<td>Basic leucine zipper transcriptional factor ATF-like 3</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>CAE</td>
<td>Chloracetate esterase stain</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxy fluorescein succinimidyl ester</td>
</tr>
<tr>
<td>cIMDM</td>
<td>Complete Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine (C-X-C motif) receptor</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>dLN</td>
<td>Draining lymph node</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>Diphtheria toxin receptor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAP</td>
<td>Facilitated antigen presentation</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Fms-like tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA-binding protein 3</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GILT</td>
<td>Gamma-interferon-inducible lysosomal thiol reductase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>gp33</td>
<td>Lymphocytic choriomeningitis virus glycoprotein 33</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin stain</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Id2</td>
<td>Inhibitor of DNA protein 2</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTC4</td>
<td>Leukotriene C4</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLN</td>
<td>Mediastinal lymph node</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T</td>
</tr>
<tr>
<td>OTI</td>
<td>Ovalbumin transgenic-I</td>
</tr>
<tr>
<td>OTII</td>
<td>Ovalbumin transgenic-II</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>OVA_{257-264}</td>
<td>Ovalbumin MHC class I peptide (SIINFEKL)</td>
</tr>
<tr>
<td>OVA_{323-339}</td>
<td>Ovalbumin MHC class II peptide (ISQAVHAAHAINEAGR)</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death receptor 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycocyanin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PKO</td>
<td>Perforin deficient</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SPI-6</td>
<td>Serine protease inhibitor-6</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activator of Transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular helper T cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
An epidemic is occurring and we do not understand why. Atopic diseases such as asthma and atopic dermatitis now affect at least one in four children and one in six adults in New Zealand (1), with many more suffering from allergic rhinitis; a prevalence that is among the highest reported in the world. Furthermore, worldwide incidence of atopic disease has increased dramatically in the past 30 years.

Asthma is characterised by chronic airway inflammation with a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough. A diagnosis may be confirmed when these symptoms are accompanied by a variable and reversible limitation in expiratory airflow, measured as a reduction in the forced expiratory volume in 1 second, compared to the normal range for children or adults (2). These symptoms result from multiple episodes of contraction of the airways and over-production of mucus that restrict an individual’s ability to breathe. Even in the absence of recent symptoms, and with lung function in the normal range, episodic exacerbations may occur due to airway hyperresponsiveness to direct or indirect stimuli and underlying chronic inflammation. It is not understood why asthma resolves spontaneously in some individuals, while others experience long-term persisting disease.

A heterogeneous disorder, multiple factors may drive asthma, and many disease phenotypes have been identified(3-5). The most common phenotypes may be broadly divided into atopic and non-atopic asthma. Atopic asthma (also referred to as allergic or extrinsic asthma) is that which develops as a result of repeated allergic responses to environmental stimuli in the airway. Often commencing in childhood, allergic asthma is most often associated with a personal or family history of atopic diseases such as eczema, allergic rhinitis, or food or drug allergy. These patients commonly present with eosinophilic airway inflammation, evident by sputum analysis (3-5). Non-atopic asthma (also referred to as non-allergic or intrinsic asthma), conversely, is not associated with allergy, and in most cases the causative agent is unknown. Multiple factors may cause exacerbations including physical exertion, respiratory infection, work-place triggers, or exposure to chemicals and cigarette smoke. Airway inflammatory infiltrates in these patients may be primarily neutrophils or eosinophils, while in some cases few cells are present in sputum (3-5). Up to half of the individuals who suffer from asthma are of the atopic, allergic subtype. Further
work is required to understand the critical factors underlying the interaction of the immune system with the external environment that consequently result in allergy, which may lead to breakthroughs in the treatment of allergic asthma. This thesis is concerned with the pursuit of such studies, specifically related to the allergic asthmatic subtype.

The critical causative factors contributing to the development of allergy, including that which manifests in the lung, are not yet fully understood; it is not known why one particular individual may become allergic to the same stimulus that another will respond to with tolerance. However, multiple contributing factors have been recognised. While no specific gene has been identified that solely confers susceptibility to allergic asthmatic disease, a strong correlation exists with those who suffer from atopy: the genetic predisposition to mount mucosal immunoglobulin type E responses (6). It is unlikely that genetic factors alone, however, are sufficient to mediate increasing incidence with the striking rapidity that has been recently described. It has been hypothesised that our behaviour in relation to our environment is largely to blame. The “hygiene hypothesis” proposed that the decreased exposure of children to immune stimulating factors, such as infectious disease, has led to an increase in allergy (7-10). However, evidence has also emerged that the incidence of respiratory viral infection during early life, such as that caused by respiratory syncytial virus, predisposes to the development of chronic airway inflammation and asthmatic disease (11-14). In either case, these and many other studies have not yet fully answered the question: how is allergic disease to harmless inhaled constituents initiated and maintained? Further studies are needed to elucidate these factors.

For the rapidly growing number of individuals who suffer from allergy, another issue is paramount: the need for safe, effective therapies or, ideally, a cure. Currently corticosteroids are commonly prescribed for the treatment of allergic asthma and have been reported effective in alleviating asthma attacks in both children and adults (15, 16), showing reasonable safety when used as a short-term treatment during exacerbations only (17). However, many patients are prescribed long-term courses of corticosteroids for maintaining the control of asthma symptoms, and experience side effects: weakened antiviral immune responses in the lung, suppression of adrenal function and decreases in bone density(18, 19). Therefore, the non-specific nature of this treatment regime results in
additional undesired pathology. Corticosteroid-resistant disease has also emerged, prompting the requirement for new, and more targeted, therapies.

An alternative approach to broad suppression of immune responses is to redirect allergic responses towards tolerance. Subcutaneous and sublingual immunotherapies have been developed where small but incremental amounts of the allergen that an individual is allergic to are administered by subcutaneous injection or as a tablet under the tongue. As the skin and mouth are home to immune cells that preferentially drive tolerogenic responses, the aim of treatment is to stimulate these cells in the hopes that, gradually, tolerance will override allergy. This is successful for many individuals, and prevention of recurrent disease such as allergic rhinitis may persist for a number of years after the cessation of treatment (20-24). However, responses vary and some people do not experience an improvement in asthmatic symptoms, or a plateau of efficacy may be reached at certain concentrations, although this has not been widely assessed in asthma patients. In addition, the specific allergen that is driving the allergic response must be known to enable attempted desensitisation, which may be complicated to treat in the many patients that are sensitised to multiple allergens (discussed in Chapter 5). Symptoms may also return upon cessation of regular immunotherapy treatments. Furthermore, some individuals who are very highly allergic to particular allergens and prone to anaphylaxis cannot be exposed to this regime, and the majority of individuals who are undergoing sublingual or subcutaneous immunotherapy are still recommended to receive adjunct corticosteroid treatment (2). Therefore, an urgent need remains for the development of a targeted therapy for allergic asthma.

1.1 Key players in the induction of immune responses

The immune system involves an array of mediators, receptors, cells, tissues, and organs that work in concert to differentiate self from foreign, and harmless from injurious, and is commissioned with the task of responding appropriately to evade damage and maintain
homeostasis. Classically divided into innate and adaptive, the immune response can target foreign pathogens in a variety of ways.

A comprehensive and immediate response begins upon the sensing of pathogen-associated or danger-associated molecular patterns (PAMPs and DAMPs, respectively) by cells of the innate immune system including dendritic cells, macrophages and neutrophils; a rapid reaction ensues whereby the activation of signalling pathways and production of cytokines and chemokines function to eradicate the invader, or direct inflammation to the site of DAMPs of host origin. Innate immunity is broadly active against foreign antigens and, while enhanced innate immune responses may occur upon successive exposures, no specific immunological memory is generated. In contrast, adaptive immunity involves the specific recognition of antigens. Throughout the course of this response, effector cells are generated that carry out short-lived functions to eliminate the incident threat; additionally, memory cells are produced that remain after the contraction of the primary immune response. While adaptive immune cells are not mobilised as rapidly as innate responders, memory cells respond quickly upon secondary and subsequent encounters of the same antigen.

Exposed to the surrounding environment every time a breath is taken, the lung is accosted with a massive variety of inhaled aeroantigens. As such, maintaining health and homeostasis in the lung requires a competent surveillance system with the ability to discriminate harmless constituents as well as potential threats. To fulfil this role, innate and adaptive immune cells and chemical mediators collaborate to target invaders and protect the body from insult. This arsenal is arguably led and co-ordinated through the directorship of lung dendritic cells, possessing important roles in the realms of both innate and adaptive immunity.

In this chapter, the characteristics and functions of dendritic cells will be introduced. The focus will then be turned to key players in lung pathogenesis; CD4+ T cells. Their specific roles as well as the contribution of collaborating cells and factors in the orchestration of allergy in the lung will be examined. Finally, CD8+ T cells will be described, a population that may present hope for a novel immunotherapy for allergic asthma.
1.1.1 Dendritic cells drive T cell responses

Dendritic cells, liaising with innate factors, are central to the initiation and propagation of adaptive immune responses. Present in most lymphoid and non-lymphoid tissues, immature DCs carry out constant surveillance of their surroundings and display effective phagocytic function. Upon detection of PAMPs (or DAMPs) through pathogen recognition receptors (PRRs), maturation of DCs occurs; upregulation of co-stimulatory molecules occurs and cytokine production commences, with DCs migrating to the draining lymph nodes where interaction with adaptive immune cells can occur, and T cell responses may be initiated.

1.1.1.1 DC subpopulations

Under steady state conditions, two main subpopulations of DCs have been described in humans and mice: conventional (cDC) and plasmacytoid DCs (pDC). In the mouse, cDCs include lymphoid resident and peripheral migratory populations, within which further subsets may be derived on the basis of cell surface marker expression. During inflammation, monocyte precursors give rise to inflammatory DCs, also referred to as monocyte-derived DC (moDC). Different PRRs are expressed by each DC subset, and different cytokines and chemokines are secreted upon activation; as such, specialised DC subsets have distinct roles in antigen presentation and the initiation and regulation of T cell responses. These DC are introduced herein, while specific lung DC populations will be expanded on in section 1.2.

Lymphoid resident cDC

DCs resident in the thymus, lymph nodes and spleen include CD8α+CD11b- and CD8α-CD11b+ DCs (25). The development of these DC relies on the presence of FMS-related tyrosine kinase 3 ligand (Flt3-ligand), as demonstrated by their low frequency in Flt3-ligand null mice and exaggerated presence in normal mice upon administration of Flt3-ligand (26, 27). Additionally, inhibitor of DNA protein 2 (Id2) and IFN regulatory protein 8 (IRF8) drive the differentiation of the CD11b- subset. The CD8α+CD11b- DC are derived from bone marrow precursors that continuously seed the lymphoid organs; making up 20% of spleen DC and 70% of thymus DC (28-30). The CD8α+ and CD8α- subsets express several differentiating markers: while CD8α- DC express CD11b (28, 29), CD172a/Sirpα (31) and
TLR7 (32), CD8α+ DC express Clec9A (33-35), CD205/DEC205 (28, 30, 36), CD36 (37) and CD24 (30, 38) as well as TLR3 and TLR12 (32, 39). Some of these distinguishing markers, such as CD36 and Clec9A, are receptors that afford specialisations for antigen handling to CD8α+ DCs, which the CD8α- subset do not possess; CD8α+ DC are more efficient in the uptake and presentation of antigens obtained from dead cells (40-43), and are also better at cross-presenting cell-bound and soluble antigens on MHC-I (44, 45), especially during the steady state. Both subsets, however, may efficiently present antigens after receptor-mediated endocytosis (46, 47).

Under steady-state conditions, immune regulatory properties have been ascribed to CD8α+ DCs; they are more efficient than spleen CD8- DCs at inducing FoxP3+ regulatory T cells both in vitro and in vivo (48), largely due to their production of TGF-β. However, this is in contrast to the thymus where blood-derived CD8- DCs are more efficient at generating regulatory T cells than the CD8+ DC (49). An additional important role is served by CD8α+ DCs, in the maintenance of peripheral tolerance; antigen targeted to DEC205+ DC has been shown to induce deletional tolerance and generate regulatory T cell responses (50-52). Once activated, CD8α+ DC are major producers of IL-12p70 (53, 54) and stimulate inflammatory responses, importantly the development of Th1 cell responses is primarily directed by the CD8α+, but not CD8α-, DCs (55, 56). Their ability to produce IL-12 and cross-present antigens also provides the stimuli for driving CTL responses. This is associated with their particularly important role in the development of anti-viral immunity during infection by influenza virus, respiratory syncytial virus, herpes simplex virus type 1, vaccinia virus and lymphocytic choriomeningitis virus (57-63) as well as contributing to T cell priming for intracellular pathogens including *Listeria monocytogenes, Salmonella typhimurium, Toxoplasma gondii* and *Plasmodium spp* (39, 61, 64-66). These studies reveal that different lymphoid DC subsets are specialised to stimulate different immune responses, driving both tolerogenic and inflammatory responses according to the type of stimuli.

**Migratory cDC**

Migratory DCs, present in most peripheral tissues, include CD103+CD11b- and CD103-C11b+ subsets. In the intestinal lamina propria, CD103+CD11b+ DC also exist, and the skin epidermal layer is populated by langerin+ Langerhans cells (67-70). CD103+ DC, derived
from the same precursor cells as CD8+ lymphoid DC, are specialised for cross-presenting cell-associated antigens. Similarly, their development depends on Flt3-ligand, Id2 and IRF8, although the intestinal lamina propria CD103+CD11b+ DC do not require Id2 or IRF8. The CD103-CD11b+ subset, on the other hand, depends on Flt3-ligand and the M-CSF (macrophage colony-stimulating factor) receptor (70). Additionally, lamina propria and dermal DC require GM-CSF (granulocyte macrophage colony-stimulating factor) (71, 72).

Migratory DCs primarily function as tissue surveillance cells, sampling foreign antigens and transporting them to the draining lymph nodes where immune responses may be stimulated. Interestingly, while these DC are highly specialised for the uptake of antigen, they are not always efficient at antigen presentation under particular conditions, especially compared to lymphoid resident DC. It has been found that during lung infection with influenza virus, infected migratory CD11b+ DC trafficked to the MLN but were incapable of presenting antigen to CD8+ T cells (73); rather antigen was transferred to lymphoid resident cross-presenting CD8+ DC that effectively stimulate CD8+ T cells (58). A similar phenomenon has been implicated for skin-derived dermal DCs (74). Inter-DC antigen transfer has been reported both in vitro and in vivo, and has been suggested to be important for stimulating both regulatory and inflammatory immune responses (60, 74-76). Therefore, migratory DC are important for the uptake and delivery of antigens from peripheral tissues to lymph nodes where they can directly and indirectly stimulate T cell responses. Additionally, the inter-DC antigen transfer suggests that DCs with different specialisation and roles may co-operate to stimulate different immune response according to the nature of the antigen.

**Plasmacytoid DC**

Plasmacytoid DCs (pDC), unlike conventional DC, are CD11clow, and may be identified further as B220+CD11b-GR-1+. Developing under the control of the transcription factor E2-2, pDC are present in low numbers in the tissue during steady state conditions and accumulate in both lymphoid and non-lymphoid tissues under pathological conditions (77). These DC function to drive protective immunity as well as tolerance (78-81). During viral infection, pDC are activated in response to ssRNA and ssDNA through TLR7 and TLR9, respectively,
and produce significant amounts of type-I interferon (82), promoting anti-viral innate and adaptive immune responses. However, they are poor immunostimulatory DC (83, 84), and interaction with T cells most often results in the generation of T regulatory cells (85-87).

**Monocyte-derived DC**
Under inflammatory conditions, monocyte-derived DC (moDC) are generated and recruited into tissues by microbial or inflammatory stimuli (88); highly plastic and versatile cells that are influenced by, and co-operate with, local DC. Unlike conventional DC, moDC arise independently of Flt3-ligand and GM-CSFR, rather they are dependent on CSF-1R (89), and have thus recently been suggested to represent a distinct cellular population, rather than a true DC subset (90); nevertheless multiple functional capabilities are shared with cDC. Characterised as MHC-II⁺CD11c⁺CD11b⁺CD64⁺FceR1α⁺Ly6C⁺, moDC are crucial to the induction of protective Th1 immunity during infection with various pathogens and protect the gut from invading bacteria (91, 92). In a pathogenic model that is potentially the most well-described, *L. monocytogenes* infection induces rapid recruitment of moDC to the spleen in a CSF-1R dependent manner (89), followed by their secretion of high levels of TNF-α and iNOS (93). Interestingly, while this cytokine production was required for bacterial clearance, the absence of moDC did not affect the priming of CD4⁺ and CD8⁺ T cell responses (93). This finding suggests that direct effector function is the predominant role of moDC in this context, rather than T cell priming. Other studies, however, have demonstrated an important role for moDC as inducers of Th1 immunity during *Leishmania* infection, producing IL-12 and inducing antigen-specific CD4⁺ T cell priming (88). Their antigen-presenting and Th1-inducing functions during non-infections disease has also been demonstrated (91). The role of moDC in Th2 immunity will be discussed in section 1.2.

**1.1.1.2 Antigen processing and presentation to T cells**
Most nucleated cells present endogenous antigens on MHC-I as a way of displaying their cellular contents; these antigens are recognised by CD8⁺ T cells that can detect aberrant self or foreign infectious protein. Although many cells have also been ascribed specialised antigen presenting capability by various authors, few of these cells can take up exogenous antigens and effectively process and present them on both MHC-I and MHC-II to facilitate interaction with CD8⁺ T cells and CD4⁺ T cells. Macrophages are efficient in the uptake and
processing of antigens but weakly express MHC-II and costimulatory molecules, as such, they are predominantly ineffective in the activation of CD4+ T cells, although in the presence of IFN-γ, MHC-II upregulation can occur. Additionally, while macrophages can cross-present apoptotic cellular debris, they are unable to cross-prime naïve CTLs (94, 95). B cells are not as successful in antigen uptake and primarily activate memory CD4+ T cells; they are unable to stimulate naïve CD4+ T cells (96, 97). Conversely, DCs are highly effective at antigen uptake and processing, and can present exogenous antigens to both CD4+ and CD8+ T cells.

**Antigen uptake by immature DC**

Immature DCs use phagocytosis, macropinocytosis and receptor-mediated endocytosis to take up particulate and soluble antigens (98-100). In the absence of PRR stimulation, these antigens are processed gradually in lysosomes and loaded onto few MHC-II molecules (101-103), with weak expression of the co-stimulatory molecules CD80, CD86 and CD40 (36, 104). No associated pro-inflammatory cytokine production occurs (105, 106). As such, limited presentation of antigen to T cells occurs in the immature state, and in the absence of significant co-stimulation engaging T cell CD28 and CD40L, these interactions do not result in effector T cell differentiation, although some T cell proliferation may occur (50, 51, 107). This is not, however, a meaningless interaction. Rather, it has been shown that this process is important for the maintenance of T cell tolerance. Indeed, the depletion of immature DCs under steady-state conditions results in the development of autoimmune disease (108).

Upon encounter of antigens such as PAMPs, immature DCs become activated. DCs recognise a vast array of PAMPs through Toll-like receptors (TLRs) that activate intracellular signalling. This results in the increased synthesis and expression of MHC-II and upregulation of co-stimulatory molecules CD80, CD86 and CD40, as well as cytokine production (109, 110). DC may also indirectly sense pathogens through the presence of pro-inflammatory cytokines, such as TNF-α and IL-1β, which are additionally capable of inducing the upregulation of adhesion and co-stimulatory molecules (99, 111, 112).
Antigen processing and presentation

DCs, upon activation in peripheral tissues, migrate to draining lymph nodes. This migration occurs through upregulated DC expression of CCR7 which allows their homing to lymph nodes along a CCL19 and CCL21 chemokine gradient (113). Here, DCs enter the naïve T cell-rich paracortex region where antigen-specific T cells are normally found, facilitating effective antigen presentation. The presentation of antigens to naïve T cells by activated DCs then results in the activation and differentiation of fully functional effector T cells. It is possible for DC to restrict antigen presentation to the antigens internalised during maturation, which favours the stimulation of T cells that are specific for pathogen-derived antigens (112). Different DC subsets also express distinct cell surface molecules and secrete different cytokines, which also influence the type of immune response that is induced, as discussed in section 1.1.1.1. Therefore, depending on the stimuli, activated DCs drive the expansion of specific T cells, facilitating the required response. Evidence also exists to suggest that activated DCs may remain in peripheral tissues such as the lung, gut and skin, and present antigen locally, allowing for the induction of local T cell responses (114-117).

Classic antigen presentation

In classical antigen presentation pathways, exogenously-derived antigens may be directly presented on MHC-II to CD4+ T cells, while endogenous antigens are presented on MHC-I to CD8+ T cells. Additionally, NKT cells are stimulated through loading of lipid antigens onto CD1 molecules (118, 119). Alternatively, exogenous antigens may be cross-presented on MHC-I for interaction with CD8+ T cells. The mechanism by which cross-presentation occurs is not fully understood. Captured antigen may be degraded in cytoplasmic proteasomes and loaded onto MHC-I in a manner dependent on transport associated with antigen processing (TAP) molecules (120), or, endosomal degradation may occur, with antigen loading onto MHC-I in a TAP-independent manner (121).

Cross-presentation of antigen

Various antigen presenting cells (APCs) have been reported to cross-present antigens in vitro, however it appears that DCs are the primary cross-presenting APCs in vivo (122). The reason for this discrepancy is unclear, however it may be that high antigen concentration in some in vitro systems allows for the direct loading of antigen onto surface MHC-I molecules.
Particular DC subsets appear to be specialised in the efficient cross-presentation of antigens, although it is unclear whether the ability to cross-present is intrinsic, or rather conferred according to the nature of the antigen or cytokine environment encountered. The subsets of DCs widely recognised as proficient cross-presenting cells include CD8α+ lymphoid-resident DCs as well as CD103+ migratory DCs that have transported antigen to the lymph nodes from the lungs and skin (57, 73, 123-126). Cytokines such as GM-CSF promote the ability of DCs to cross-present antigens (127).

A recently discovered phenomenon describes a mechanism by which DCs may acquire intact antigen-MHC-I complexes from other nearby DC that are cross-presenting antigen. This process, termed cross-dressing, facilitates the expansion of the cross-presenting DC pool, and may also allow poor cross-presenting APCs to stimulate effective naïve and memory CD8+ T cell responses (76, 128-132).

Cross-presentation has multiple functionally important roles. Under steady state conditions, cross-presentation facilitates the generation of central and peripheral tolerance of CD8+ T cells to self and foreign antigens (133-137). A major role for cross-presenting DCs also exists in anti-tumour immune responses (138). Defective CD8α+ and CD103+ DCs are ineffective at generating CD8+ T cells to target tumours. In models of infection, DCs that are uninfected may acquire pathogen-derived antigens from infected cells and cross-present these to CD8+ T cells (139). This is critically important in the stimulation of particular antiviral immune responses; indeed, in basic leucine zipper transcriptional factor ATF-like 3 (BATF3)-deficient mice, which lack CD8α+ and CD103+ cross-presenting DCs, the CD8+ T cell response to Sendai virus and West Nile virus was diminished (138, 140). Additionally, Gamma-interferon-inducible lysosomal thiol reductase (GILT)-deficient mice, within which antigens may not be cross presented, display impaired CD8+ T cell responses to HSV-1 and influenza viruses (141). These evidences together highlight the significant roles of cross-presentation that are distinct from the direct presentation of antigen.
1.1.2 CD4+ T cell subsets

The roles of CD4+ T cells are incredibly diverse; from immune activation, to resolution and tissue repair. At least five subsets of T-helper cells have been well characterised: Th1, Th2, Th17, T-regulatory cells (Treg) and T follicular helper cells (Tfh). Additional subsets, such as Th9 cells, have also recently been described. As depicted in Figure 1.1, these subsets are characterised by their distinct transcription factors and specific cytokine production. T cell subsets are thus purposed with different functions in the regulation of immune responses. Major factors contributing to the determination of the effector subset fate of naïve Th cells are primarily the TCR signalling process and subsequent polarisation through the cytokine microenvironment.

![Diagram of CD4+ T cell subsets](image)

**Figure 1.1: CD4+ T cell subsets**

Upon recognition of antigen presented on MHC-II by APC, naïve CD4+ T cells differentiate into distinct subsets according to co-stimulatory factors and the cytokine microenvironment. A unique set of transcription factors is expressed by each subset and characteristic cytokine production enables them to carry out diverse roles in immune responses. The differentiation of four subsets, Th1, Th2, Th17 and Tregs, from naïve CD4+ T cells has been demonstrated; conversely it is less clear whether Tfh derive directly from naïve CD4+ T cells or from previously differentiated Th subsets, while Th9 may arise as a subset of functionally specialised Th2 cells.
 Upon recognition of their cognate antigen, naïve CD4+ T cells in vivo rapidly engage in a stable interaction with antigen-presenting DCs (142). The activation markers CD69 and CD44 are subsequently upregulated, and CD62L downregulated. In the fate decision between Th1 and Th2, the strength of TCR signalling has been implicated. While strong signals reportedly stimulate Th1 cells in vitro, through the inhibition of STAT5, weak TCR signalling has been reported to induce Th2 (143). The presence of IL-12 and IFN-γ in the cytokine microenvironment polarise naïve CD4+ T cells to a Th1 phenotype through the induction of STAT1 and STAT4 signalling that induces the master Th1 transcription factor, T-bet (144, 145). Production of IFN-γ characteristically ensues. Th2 differentiation occurs through the induction of the Th2 master regulatory transcription factor GATA-3. This may require IL-2-mediated activation of STAT5 and IL-4-driven STAT6 activation (146). The resulting Th2 cells produce IL-4, IL-5 and IL-13. The detailed development and functional characteristics of Th2 cells are further discussed in section 1.2.

Th17 cells have been proposed to differentiate as a result of TGF-β and IL-6 stimulation, in association with IL-23 (147, 148). TGF-β and IL-6 act in combination with TCR stimulation to induce the expression of the IL-23R and the Th17 master regulatory transcription factor RORγt through activation of SMADs and STAT3. The production of IL-17A, IL-17F and IL-22 then characterise the Th17 response (149, 150). The presence of IL-2 antagonises the generation of Th17 cells, overriding the effects of IL-6 and TGF-β if also present (151); instead, the differentiation of Treg cells is promoted by IL-2 and critically mediated through TGF-β-driven induction of the master Treg regulatory transcription factor, Foxp3 (152, 153). Tregs then produce TGF-β and IL-10 to regulate immune responses. Interestingly, TCR signal strength also plays a contributing role in the fate determination between Th17 and Treg differentiation. Upon weak TCR signalling, Th17-inducing cytokines fail to drive Th17 differentiation; instead, this TCR stimulation results in the expression of Foxp3 (154-156). Strong TCR signalling however, capable of blocking STAT5 activation and Th2 development as previously mentioned, may similarly result in the inhibition of STAT5-mediated Foxp3 activation and thus allow for the induction of Th17 cells (157, 158)
It is not clear whether Tfh cells differentiate from previously committed Th1, Th2 or Th17 cells, or whether they originate directly from naïve CD4+ T cells. As such, the cytokine signals involved in the differentiation of Tfh cells are not fully understood. However, their roles in the facilitation of T cell-dependent humoral immune responses have been recognised. Tfh cells promote the formation of germinal centres, immunoglobulin class switching, affinity maturation of B cells and maintain antibody responses (159). Their master regulator, B-cell lymphoma 6 (Bcl-6) is induced via STAT3 activation (160, 161).

Th9 cells, named due to their characteristic production of IL-9, were recently identified, and share a similar developmental pathway to Th2 cells (162, 163). Classic Th2-signalling IL-4/STAT6/GATA3, alongside TGF-β stimulation of PU.1, leads to the development of IL-9 producing cells (164). Identified more than 20 years after the delineation of Th1 and Th2 cells, the role of the Th9 subset was previously unappreciated, with IL-9 production being attributed to Th2 cells and Tregs (165). Indeed, cells previously committed to a Th2 profile were found to develop into Th9 cells upon TGF-β stimulation (162, 163). However, this subset can also develop directly from naïve CD4+ T cells in the presence of both IL-4 and TGF-β.

While specific CD4+ T cell subsets have been described, the delineation between these subsets is not completely black and white. It was originally thought that Th1 and Th2 cells became irreversibly committed to a terminal differentiation, insomuch as the cell would become incapable of reverting into a different subset. However, CD4+ T cells have been described that display features of more than one specific subset. Studies have identified cells that co-express Th1 and Th2 markers: GATA3+ IL-4+ and T-bet+ IFN-γ + cells (166). Th2/Th17 markers may also be co-expressed: GATA3+ IL-4+ and RoR γ t+ IL-17A+ (167). IL-9, characteristic of Th9 cells, may also be secreted by Th2 cells (163). Additionally, diminished expression of FoxP3 causes Tregs to revert back to effector T cells producing IL-2 and IL-4 (168, 169), while IL-12 can revert Th2 polarised cells to Th0/Th1 (170). These evidences reveal that fully differentiated CD4+ T cells are, to some extent, plastic and capable of adapting to their microenvironment as required.
It is my opinion that the described componentry of the immune system as we know it will likely not be entirely similar to that in time to come. The pathogenic threats that are ever-evolving under numerous natural and man-made pressures calls also for the diversification of immune responses. This could lead to the development of immune cell subsets that may not in fact have been historically misidentified, but rather represent a novel facet of the immune repertoire to counteract novel threats accordingly.

1.1.2.1 The role of CD4+ T cells

The roles of these functionally and phenotypically diverse CD4+ T cells in health and disease are many. These responses must be fine-tuned and tightly regulated such that a particular cell population does not overreact or insufficiently respond to stimuli, which in either case may lead to disease.

Type 1 immune responses (defined herein as those driven by cytokines classically, but not exclusively, attributed to Th1 cells, rather than referring to type 1 hypersensitivity reactions) and Th17 immune responses are initiated in response to bacteria, viruses, fungi and protozoa, for which Th1 and Th17 cells are key mediators. Th1 cells are important in cell-mediated immunity that drives delayed type hypersensitivity through the production of IFN-γ (171, 172), defending against intracellular parasites (173). Exaggerated effects, however, can lead to the development of autoimmune disease (174) such as Type 1 diabetes (175), and additionally has a pathogenic role in inflammatory bowel disease (176, 177). Th17 cells, through their production of IL-17, promote the maturation and chemotaxis of neutrophils that mediate defence against bacteria and fungi (178, 179). Additionally, Th17 cells are a source of IL-22, which has been shown to be indispensable in the stimulation of epithelial induced antimicrobial defence genes that protect against bacterial infection (180, 181). However, a role for Th17 cells has also been described in allergy; they are strongly associated with the development and maintenance of atopic dermatitis (182), and nickel allergy (183). IL-17 may also drive autoimmunity such as rheumatoid arthritis (184), systemic lupus erythematosus (185) and psoriasis (186), as well as transplant rejection (187).
The type 2 adaptive immune response, largely driven by Th2 cells, (and defined herein as those driven by cytokines classically, but not exclusively, attributed to Th2 cells, rather than referring to type 2 hypersensitivity reactions) mediates extracellular immunity in response to the invasion of specific pathogens; particularly important for protection against intestinal helminths. Th2 responses enhance mucosal barrier defences and mediate the expulsion or killing of parasites in a manner that is dependent on CD4+ T cells, IL-4/IL-13, IL-4R and STAT6 signalling (188). While IL-13 is critical for worm expulsion, exogenous administration of IL-4 is also sufficient to do so (189). The archetypal Th2 cytokines drive the activation of eosinophils, mast cells, and goblet cells, which stimulate inflammatory responses and the production of mucus at epithelial surfaces; while this response is necessary for protection against parasites, it can also be directed against harmless inhaled antigens (that is, those that are not normally disease-causing in healthy individuals) and consequently cause disease in the lung. These pathogenic Th2 responses are central to the development and maintenance of atopic dermatitis, allergic rhinitis and allergic asthma (discussed in section 1.2).

Foxp3+ T regulatory cells have many roles in the maintenance of immune homeostasis and are key in the regulation of effector T cell function in the periphery. Tregs, constitutively expressing CD25 and secreting IL-10 and TGF-β, are key in the tolerance of self-antigens and limiting overactive T cell responses (190, 191). In the realm of Th2 responses, Tregs are especially important for curbing the generation of disease. Tregs help restrict the onset and strength of Th2 responses during active helminth infection and limit the severity of intestinal disease (192, 193). The adoptive transfer of allergen-specific Tregs has also been shown to suppress allergic inflammation in sensitised mice (194). Foxp3+ Treg-derived cytokines suppress airway inflammation either through an IL-10-independent, TGF-β-mediated mechanism (195), or through IL-10-dependent mechanisms (194, 196). These cytokines may act on DCs to reduce DC number and activation (197), thereby reducing T cell stimuli, or directly suppress Th2 responses by decreasing IL-4 production, serum IgE and lung eosinophilia (198). Again, not all responses mediated by these T cells are beneficial; Tregs are also implicated in autoimmune diseases (199) and the generation of tolerance to tumours, preventing the induction of anti-tumour immune responses (200-202).
1.1.3 CD8+ T cells

In contrast to CD4+ T cells, naïve CD8+ T cells do not immediately engage in prolonged interactions with DC, but rather associate with multiple antigen-presenting DCs transiently, over a number of hours, before a stable interaction is formed (203). DCs then stimulate the differentiation of naïve CD8+ T cells into effector T cells with cytolytic function: cytotoxic T lymphocytes (CTL). DCs play a critical role in the development of fully activated CTLs (122, 204, 205) and this process is critically dependent on sufficient co-stimulation through DC CD80/CD86 and CD8+ T cell CD28 (206-208). To successfully prime naïve CD8+ T cells that differentiate into effective killer cells as well as long-lived memory cells, DCs must enlist the help of CD4+ T cells to provide the signals required to licence DCs (209, 210). This occurs through the engagement of DC CD40 by CD4+ T cell CD40L (211, 212). Licenced DCs are then capable of priming CTLs even after CD4+ T cell interaction has ceased (209, 213). DC-derived IL-12 also acts to promote the proliferation and survival of CTLs (214, 215).

1.1.3.1 CTL subsets

CTLs may be characterised as type 1 or type 2, termed Tc1 and Tc2. These subsets, similarly to Th1 and Th2 cells, produce distinctive cytokine repertoires resulting in different, or even opposing, functions. Tc1 cells secrete IFN-γ, while Tc2 cells produce IL-4, IL-5, IL-6 and IL-10 (216, 217). Cytokine signalling is important in the fate decision between Tc1 and Tc2; in vitro experiments have revealed that if IL-4 is present during CD8+ T cell priming, IL-4-producing CTLs are generated (216, 218, 219), conversely, IL-12 stimulation results in CTLs that produce IFN-γ (Curtsinger, J Exp Med, 2003). Similarly to the Th1/Th2 paradigm, the cytokine profile of Tc1 and Tc2 cells results in their differing effects on disease. Tc1 cells ameliorate allergic airway inflammation (220-226) whereas Tc2 exaggerate disease (227-229). Tumour-specific Tc1 cells provide protection against tumour challenge, while Tc2 do not do so efficiently (230, 231). The generation of a graft-versus-leukaemia response is promoted by Tc1 and abrogated by Tc2, conversely, higher numbers of Tc2 cells correlates with better humoral responses to influenza vaccination (232, 233), potentially due to the Tc2-derived IL-4 that can provide B cell help (234)
The CTLs generated undergo clonal expansion and migrate to peripheral tissues where pathogenic stimuli are present (235, 236). Here, CTLs mediate the elimination of infected cells.

**1.1.3.2 CTL-mediated killing of target cells**

Antigen-specific CTLs, upon recognition of their cognate antigen loaded on cellular MHC-I, mediate the killing of target cells. This is achieved either through direct CTL granule-mediated cytolysis, or through the engagement of target cell receptors that signal apoptosis. CTLs synthesise and store granules that contain proteins that induce rapid cytolysis, including perforin and granzymes. Upon CTL engagement with target cell antigen-MHC-I, the CTL releases the granules into a tight intercellular junction between the two cells, and signals apoptosis of the target cell. These phenomena are described in this section.

**Granule-mediated cytolysis**

It is not fully understood how perforin mediates cytotoxicity. The prevailing view is that perforin forms pores in target cell membranes, through which pro-apoptotic granzymes may be directed into the cytosol of the target cell, inducing cell death (237-242). Law and colleagues used X-ray crystallography to reveal the structure of perforin and generated a reconstruction of the perforin pore by cryo-electron microscopy to elucidate the mechanism of perforin pore formation (243). In brief, this is mediated by the binding of a C-terminal C2 domain on the perforin molecule with the target cell membrane, in a Ca²⁺ dependent manner. The perforin assembles into oligomers on the target cell surface, which then undergoes a conformational change that allows membrane insertion and formation of a transmembrane pore, through which granzymes are directed. The cylindrical pore model has been accordingly implicated in other studies (244, 245), with some demonstrating 15-20nm pores being formed, sufficient to allow the passage of a granzyme molecule, such as Granzyme B – 4.3nm (243, 246, 247). However, being a historically controversial topic, most recently this view has been again challenged; using concentrations of perforin that coincide with granzyme translocation and apoptotic cell death (246, 248), Metkar and colleagues report that pores of similar size to that described by others were not identified. Rather, protein “arcs” formed toroidal pores under these conditions (249). This finding suggested that cylindrical pores, still formed under higher perforin concentrations, may be
dispensable for granzyme delivery. The toroidal pores consist of inserted peptides and lipid that together form a water channel, in contrast to the cylindrical pores that are lined exclusively by protein and prevent the movement of phospholipids across the bilayer (250, 251); this water channel may provide more favourable conditions for the passage of highly cationic granzymes. Metkar and colleagues thus propose that cylindrical pores may serve a function distinct from the delivery of granzymes, which are likely to be transferred through the toroidal pores. Although controversy still exists in the mechanistic detail of this form of cytolysis, together, these studies are driving the refinement of our understanding of how perforin and granzymes mediate target cell death.

In any case, it has been established that perforin is critical for cytolysis, as revealed in the study of disease. CTLs from perforin knock out (PKO) mice show reduced cytotoxicity and a defective ability to induce membrane damage and apoptosis, compared to perforin sufficient mice (252). As such, perforin deficiency causes delayed clearance of viruses (253) as well as attenuated primary and drastically deficient secondary response to viral infection (254). The effects of perforin deficiency are more striking in humans: patients who suffer from familial haemophagocytic lymphohistiocytosis, where perforin is defective, experience uncontrolled activation of T cells and macrophages and the overproduction of inflammatory cytokines (255-258). This condition rapidly results in death. Together, these studies highlight the importance of perforin in the elimination of infected cells, as well as the critical requirement for perforin in the maintenance of human immune regulation and homeostasis.

Numerous serine proteases are delivered to target cells, of which granzymes A and B are the most well characterised. While granzyme A drives target cell death in a caspase-independent manner, granzyme B activates intracellular caspases to initiate apoptosis (259-261). Whether these granzymes are required for cytotoxic control of disease is not clear; their role varies according to the pathogenic stimulus. A requirement for granzyme A and B has been demonstrated in the suppression of poxvirus infection (262), however cytomegalovirus-infected or influenza-infected mice exhibit sufficient anti-viral immunity in the absence of granzymes A and B (263, 264). Some studies have questioned the role of granzymes in the CTL-mediated suppression of disease altogether; granzyme A and B may
not be required for anti-tumour immunity (265). Interestingly, it appears that the ability of granzymes to mediate apoptosis is critically dependent on perforin (266). These studies suggest specialised roles exist for granzymes in the control of disease, which may not be directly mediated by perforin, however, may remain under the control of perforin expression.

**Receptor-mediated cytolysis**

Receptor-mediated cytolysis is primarily driven by ligands and receptors belonging to the TNF-α superfamily: Fas ligand (FasL), which binds Fas, TNF-α that binds to the TNF-α receptor 1 and 2 and death receptor 3, while TNF-related apoptosis-inducing ligand (TRAIL) binds death receptor 5 and other receptors. Binding of CTL Fas ligand (FasL) to a target cell Fas receptor (241) results in the recruitment of Fas-associated death domain (FADD) protein and caspase-8 that bind to Fas and interact with the death domain (DD) and death effector domain (DED) to form a death-inducing signal complex (DISC) (267, 268). Similarly, signalling through the TNF-α receptors and TRAIL receptors results in the recruitment of FADD, or TNF-receptor 1-associated via death domain (TRADD), and subsequent formation of DISC (269, 270). The formation of DISC drives activation of caspase-8 molecules that further recruit and activate caspase 3 and caspase 7 which cleave cellular proteins and drive target cell apoptosis. Similarly to the requirement for perforin, Fas-FasL signalling appears to be crucial to immune homeostasis; deficiency or mutation in Fas or FasL can lead to lymphadenopathy or autoimmune disease (271).

**1.1.4 The generation of immunological memory**

The stimulation of effector T cell responses also results in the development of memory T cells. While the majority of activated effector cells apoptose upon withdrawal of antigen-stimulated cytokines and survival factors, a fraction of cells survive to become memory cells (272, 273). These cells may reside in tissues and are capable of rapidly responding upon secondary exposure to antigens. Populations of central and effector memory T cells are generated, distinguished by their expression of CD62L and CCR7. Cells that are CD62L<sup>hi</sup>CCR7<sup>hi</sup> preferentially home to lymph nodes and are labelled central memory T cells, conversely, CD62L<sup>lo</sup>CCR7<sup>lo</sup> cells home to nonlymphoid tissues and acquire effector function
more rapidly, termed effector memory cells (274-276). It has also recently been determined that a population of memory stem cells exists that may directly give rise to central memory, effector memory and effector T cells (277). These cells are specific for multiple antigens, foreign and self, and are capable of enhanced proliferation and reconstitution of immunodeficient hosts in comparison to traditionally recognised memory cell populations.

Interestingly, of the memory cell subsets, CD4+ and CD8+ memory T cells are retained differently. The memory phenotype and function of CD8+ memory T cells may be maintained for life (278), even in the absence of antigen stimulation (279-281). These memory cells may instead be maintained by nonspecific stimuli (224, 282, 283). Conversely, the persistence of CD4+ T cell memory has been debated. It has been shown that in vitro-differentiated Th1 and Th2 cells may be transferred into naïve hosts and retain their cytokine profile for a number of months (284, 285). On the other hand, it was also demonstrated that antigen-experienced cells may lose their enhanced helper function over time (286-288), thus suggesting that CD4+ T cell memory wanes without continuous antigenic exposure (289). These differences are likely a reflection of the immune system’s requirements for maintaining homeostasis. It is not, perhaps, that the development of CD4+ T cell memory is simply inferior to that of CD8+ T cell memory, per se, but rather the waning of CD4+ T cell memory may serve a protective purpose, so as not to allow sustained CD4+ T cell responses after the ‘threat’ of antigenic stimuli has been removed. This may also be important in maintaining Th1/Th2 balance such that tolerogenic, rather than allergic, responses prevail.

1.1.5 Immune cells in the lung under steady state conditions
The lung is a site of constant antigen exposure, and as such, multiple immune cell populations reside within the compartments of the lung where they interact with barrier epithelial cells to maintain homeostasis.
Lung macrophages

Under steady-state conditions, up to 95% of cells recovered from the lung by broncho-alveolar lavage are alveolar macrophages (AMs) (290). The role of AMs in the pathogenesis of allergic asthma, however, is not fully understood. Characteristically F4/80\(^+\)MerTK\(^+\)CD11c\(^+\)Siglec-F\(^+\)CD11b\(^lo\)MHCII\(^lo\) and displaying significant autofluorescence, studies have revealed both protective (291-293) and pro-inflammatory (294, 295) roles for these abundant cells, making it difficult to reconcile their involvement in asthmatic disease. AMs, through their production of IL-10, IL-12 and nitric oxide, decrease pulmonary DC antigen presentation (296, 297) and subsequent T cell proliferation (298, 299), as well as prevent the development of airway hyperreactivity (AHR) (291). Conversely, in response to IgE, AMs have been shown to drive pulmonary inflammation by facilitating the infiltration and activation of eosinophils and neutrophils through the up-regulation of adhesion molecules such as E-selectin, vascular adhesion molecule-1 and intercellular adhesion molecule-1 (300-304). A recent publication by Zaslona and colleagues aimed to shed some light on these apparently disparate roles (305); by selectively depleting either resident AMs or circulating monocytes- the latter of which the authors highlight as putative precursors to AMs- it was reported that resident AMs were in fact protective in asthma. In contrast, infiltrating monocytic cells recruited during lung inflammation promoted pathogenesis. The work of these authors addressed an important point: that the incongruent reports of AM roles may in fact be due to the differing activity of lung resident and recruited AMs under inflammatory conditions; though it was found that local proliferation, rather than recruitment, was important for the maintenance of the AM pool during the early phase allergic response. It must be noted, however, that the depletion of circulating monocytes would also prevent the infiltration of monocyte-derived inflammatory DCs. As critical mediators of allergic airway inflammation (306), moDC-derived proinflammatory chemokines promote the elicitation of allergic airway inflammation, and thus potentially effects the effective recruitment and development of AMs also. As such, the role of AMs in the asthmatic response is still yet to be conclusively elucidated.

Two other macrophage populations reside in the lung under steady state conditions: bronchial macrophages (BM) and interstitial macrophages (IM). While little is known about
the role of BMs, these cells possess substantial IL-10 receptors, but not IL-4 or IL-7 receptors, indicating a role in tolerance may exist (307). These cells display an altered phenotype in asthma patients; CD16 and CD64 are downregulated, compared to BMs from healthy subjects, and this results in poor phagocytic activity (307, 308). In contrast to AMs, IMs are generally considered antigen-presenting macrophages (309); they display enhanced MHC-II expression as compared to AMs (310, 311) and present antigens to interstitial T cells. IMs have, however, been shown to promote tolerance and prevent Th2 inflammation in the airway through production of IL-10 (311). It has been shown that AMs in fact originate from IMs; IMs are a necessary intermediate between blood monocytes and AMs (312).

Macrophages may be characterised into M1 and M2 subsets (313). Similarly to Th1 and Th2, M1 drive inflammation in response to intracellular pathogens while M2 phagocytose foreign pathogens and apoptotic cells (314-317). The presence of IFN-y and LPS polarises M1 cells and IL-4/IL-13 drive M2 polarisation. This is not always the case, however; innate cytokines such as IL-33 can induce both M1 and M2 (295, 318, 319), and IL-4 has also been shown to induce pro-inflammatory cytokine production by M1 macrophages (319). As such, the overlapping induction patterns and subsequent functions of M1 and M2 have made it difficult to assign strictly specific roles for these subsets. In asthma, M2 cells are primarily implicated through their production of FIZZ1 and Ym-1 in response to Th2 cytokines; these mediators normally function to maintain pulmonary homeostasis, however their excessive production during asthma drives lung injury (312).

**Lung DCs**

Although making up a lesser proportion of the bronchoalveolar lavage (BAL)-recovered cells, multiple DC populations reside in the airway and lung parenchyma. This primarily includes CD11c+CD11b+ and CD11c+CD103+ cDC, as well as pDCs. The CD11b+ subset are mainly located in the submucosa and perivascular regions (320, 321) and are functionally specialised for the presentation of antigens to CD4+ T cells to prime and restimulate effector responses (123, 322). In the steady state, CD11b+ DC have been shown to secrete chemokines that promote homeostasis, whereas CD103+ DC usually only secrete
chemokines under inflammatory conditions (322). CD11b+ DC perform critical roles in driving allergic inflammation in the airway (306, 323), as discussed in section 1.2.

The trachea and large airways are associated with CD103+ DC, specialised in the uptake of antigens for migration to the mediastinal lymph node and cross-presentation to CD8+ T cells (73, 123, 126, 324). The expression of tight junction proteins enables their traverse of the epithelium to acquire antigen (320). The localisation and function of these important CD8+ T cell-stimulating DCs have not been well characterised in the lung. One report has shown their steady state localisation to be primarily within the airway mucosa and arteriole walls (320), however this is yet to be demonstrated by other groups. Additionally, their redistribution and behaviour during inflammatory conditions is not known.

In terms of cell number CD11c^midCD11b^pDC, also expressing Siglec-H and bone marrow stromal antigen-1, are a minor population in the lung. However, an important role has been demonstrated for the tolerance of inhaled antigens (325). This may occur through their ability to suppress cDC-mediated stimulation of effector T cells (325, 326). Additionally, pDC can directly induce Tregs, which also promote tolerance (86, 327, 328).

**Additional immune cells in the lung**

Although few in number relative to macrophages and DCs, the steady state lung is also populated by innate lymphoid cells and tissue-resident mast cells. Of particular significance in the lung, a population of Type 2 ILCs has been described in both mice (329-336) and humans (330, 337); upon stimulation with IL-33, IL-2 and IL-7, these ILCs produce IL-5 and IL-13 but not IL-22, IL-17A or IFN-γ (329, 330). Similarly to CD4+ Th2 cells, ILC2 in the lung have been ascribed roles in protection against helminth infection, accumulating in response to IL-33, and producing IL-5 that is hypothesised to aid worm expulsion (333, 338). Conversely, pathological contributions to the development of allergy has also been described, with ILC2 implicated in the development of airway eosinophilia and mucus production in response to allergen challenge, and ILC-derived IL-13 reportedly crucial to the generation of AHR (331, 332, 336). It has, however, recently been shown that lung ILCs promote beneficial tissue repair in response to acute epithelial damage, such as that caused by viral infection with Influenza A (339). The healing response may be driven by ILC-
derived amphiregulin, a mediator produced in large amounts by ILC2 in the lung during viral infection (339), shown to be linked to tissue repair during acute epithelial insult as well as asthma (340, 341).

Mast cells may be classified, according to their localisation in the lung, into mucosal (MMC) and connective tissue (CTMC) subsets, although phenotypic differences in the protease expression profile of mast cells also exist. In the steady state, the MMC population reside in the respiratory mucosa (342-344), predominantly expressing chymases MCPT1 and MCPT2 and low levels of histamine and serotonin (345, 346). MMC may be dependent, at least in part, on T cells for population maintenance as well as expansion during inflammatory conditions (347, 348). Conversely, CTMC are found around large airways (344, 349), and express a range of mediators including chymase MCPT4, elastase MCPT5, tryptases MCPT6 and 7 and carboxypeptidase A3 as well as high levels of histamine and serotonin (345, 346). The maintenance and expansion of the CTMC subset is not dependent on T cells (347, 348). Although few in number in the steady state, the localisation of mast cells within the lung positions them for rapid response to invading pathogens. As such, mast cells have been shown to be important for the defence against bacteria, viruses and parasites (350-353). However, mast cells also play a role in pathological responses under particular conditions. Importantly, the damaging effects of mast cells have been implicated in Th2 disease, contributing to allergic airway inflammation as discussed in section 1.2.2.2.

The frequency and role of immune cells in the lung changes in response to various inflammatory stimuli. The generation of allergic airway inflammation results in the infiltration of multiple additional immune cell subsets that mediate disease as described in section 1.2.
1.2 Orchestration of Th2-mediated disease

The requirement for APCs in the initiation of T cell immunity is clear, however, for reasons that have not been fully elucidated, APCs may sometimes direct aberrant, insufficient, excessive or inappropriate immune responses that can lead to disease. The activity of multiple cell populations, costimulatory molecules, as well as the cytokine microenvironment may influence the development and progression of disease. Allergic responses, driven by the development of Th2 cells and associated cytokines, may develop at sites where antigens are encountered; commonly, the skin, gastrointestinal tract and the lung. In the airway, activation of antigen-specific Th2 immunity can lead to chronic inflammation characteristic of asthmatic disease.

1.2.1 Characteristics of allergens

It is well established that certain proteins commonly induce allergy. Recent decades have seen a striking rise in allergy, often associated with factors that may be encountered in everyday life; house dust mites, pollens, animal dander and various foods. What is less understood is how the interplay between these factors and the immune system drives allergy, as opposed to tolerance.

While varying in size, conformation, and allergenicity, common characteristics of allergens exist. Both B-cell and T-cell epitopes are required, allowing the binding of IgE and the initiation of a Th2 cellular response, respectively. Protease activity (commonly cysteine, serine or aspartic proteases) is frequently associated with allergens from HDM, cockroach, fungi and pollens (354-360). This property may facilitate allergen sensitisation through damaging epithelia, breaching primary defensive barriers and stimulating immune responsiveness (361). In a similar manner, respiratory viruses and air pollutants also impair barrier function by disrupting tight junctions (362, 363).

Glycosylation is also a predominant feature; major allergens from dust mites (Der p -1 and -2), cats (Fel d 1), dogs (Can f 1), cockroaches (Bla g 2), and peanuts (Ara h 1) are glycosylated, with 1-2, 1-3 and 1-6 mannose representing the dominant sugars on these
allergens (364-366). Cedar (Cry j 1) and cypress (Cha o 1) pollens and ovalbumin (OVA) allergens also possess mannans (367-369). Indeed, mannose receptors have been reported to facilitate the recognition and uptake of allergens by DCs (370, 371).

Allergens may engage TLRs (372, 373) or induce DAMPs by protease activity within tissues, providing stimulatory signals to initiate immune responses (374). Other biologically active constituents of allergens may include chitins and endotoxin, however the presence of these molecules does not necessarily drive Th2 immunity. The involvement of chitin in the pathology of allergy is a point of contention. When administered to the lungs of mice, chitin induces the accumulation of Th2-associated, IL-4 expressing cells (375). In response, acidic mammalian chitinase (AMCase) is produced by epithelial cells and macrophages in substantial amounts via a Th2 specific, IL-13 mediated pathway (376). However, when AMCase is inhibited, or transgenic or AMCase-deficient mice are used, allergen sensitisation and challenge leads to the development of similar features of allergic airway disease as control mice (377). As such, it appears that the activity of chitins and AMCase are not critical to the development of allergy, but may nevertheless aid allergenic simulation of Th2 responses.

Endotoxin, recognised by TLR4, has been detected in association with many common allergens. Eisenbarth and colleagues reported that low level LPS inhaled with antigens was necessary for the induction of Th2 responses through TLR4 signalling (372). Additionally, in vivo experiments using bone marrow chimeric mice that lacked TLR4 specifically on epithelial cells demonstrated that the allergic response to HDM was substantially ameliorated when endotoxin was not sensed (373, 378). The administration of high doses of LPS with antigen, on the other hand, results in Th1 polarised responses(372). The contribution of LPS to the allergenicity of protein antigens, then, depends, at least in part, on the concentration of endotoxin associated with the inhaled antigen.

While numerous factors appear to confer allergenic potential, many allergens are not intrinsically immunogenic and the mechanism of their allergenicity is unclear. Furthermore, it is still not understood why allergic sensitisation and Th2 immunity develop in particular individuals to particular allergens while others remain tolerant to the same proteins. These
findings suggest that “allergens” themselves are not the primary determinant of the development of allergy, but rather the individual’s predisposition to allergy is responsible for the initiation of Th2 disease.

1.2.2 Priming Th2 immunity

During the course of tissue surveillance, phagocytic and specialised APCs, upon allergen uptake, migrate to regional lymph nodes or local mucosal sites where interaction with T cells occurs (described in detail in 1.1.1 DC section and Figure 1.2 Allergic response). Upon encountering cognate antigen through their TCR, in association with appropriate costimulatory signals and cytokine stimulation, CD4+ T cells are signalled to differentiate.

Priming of Th2 responses to inhaled allergens

Epithelial cells of the skin, intestine and lung provide a physical barrier to pathogens, but are also capable of cytokine production upon encounter of allergens and helminth products (372, 379). In the lung, inhaled aero-constituents barrage the lung barrier epithelium frequently. It is becoming increasingly evident that the pulmonary innate immune system has a significant role in the generation of Th2 immunity in the lung, as well as contributing to associated asthmatic disease. Many allergens can directly or indirectly act on epithelial cells to stimulate the production of Th2-polarising cytokines including Thymic Stromal Lymphopoietin (TSLP) (380), IL-33 (381, 382) and IL-25 (383), as well as the production of GM-CSF (373) and stimulation of barrier permeability (384, 385). The Der p 2 component of one such allergen, house dust mite (HDM), directly binds epithelial cell TLR4, the expression and allergen-binding of which is necessary and sufficient for DC activation (373). Additionally, and in a manner not dependent on TLR4, in vitro studies have demonstrated the HDM-induced airway epithelial cell production of CCL20, chemokines attracting immature DCs (386-388).

Allergens may also gain access to DCs through the disruption of epithelial tight junctions (384, 385, 389) The stimulation of the innate immune system and targeting of lung epithelia are thus implicated as important initiating factors in the facilitation of Th2
priming. DCs then play a crucial role in bridging innate and adaptive immune responses, acting in concert with local cytokines to prime CD4+ T cells.

It has been demonstrated that CD11c+ DC are absolutely required for Th2 induction and development (390). Importantly, of the lung DC populations, it is CD11b+ DCs that are the key players in the priming of Th2 cells, conversely, CD103+ DC are not required (306). As such, the characterisation of a Th2-driving DC phenotype has been the subject of intensive investigation. Various costimulatory molecules on DCs have been proposed as key factors in providing the accessory stimulation required for priming Th2 cells. Essential for the activation and effector function of T cells, early interactions between B7-1/B7-2 (CD80/CD86) on DC and CD28 on T cells, as well as the interactions of CD40 on DC with T cell CD40L, have been targeted in attempt to elucidate their importance in mediating Th2 responses. Blockade of B7/CD28 was shown to reduce allergic inflammation by decreasing lung eosinophilia, type 2 cytokines, IgE and airway hyperreactivity (391-393). Conversely, targeting of CD40/CD40L in models of allergic airway inflammation produced discordant results between studies; in an acute model where OVA was administered twice on a single day, it was reported that CD40L-/- deficient mice display reduced airway eosinophilia and IL-4, though IL-5 was unaffected (394). In contrast, a study utilising CD40-/- mice exposed to multiple OVA challenges over 8 days revealed comparable inflammation to WT controls, despite a lack of IgE production (395). Therefore, these costimulatory molecules appear to play a role, albeit not fully understood, in the pathogenesis of Th2 responses in the airway.
Figure 1.2: The allergic response

(A) Sensitisation to allergens may occur upon initial exposure. Professional APCs, such as DCs, take up allergens from the airway, or through disrupted epithelial tight junctions. Innate cytokines produced by activated lung epithelial cells, such as IL-25, TSLP, IL-33 and GM-CSF, collaborate in the activation of local DC and ILC2; the latter being stimulated to produce Th2 cytokines IL-5 and IL-13. Activated APCs migrate to regional lymph nodes where interaction with T cells occurs. Upon encountering cognate antigen through their TCR, CD4+ T cells are signalled to differentiate into effector cells, migrating back to the site of allergen exposure. Activated CD4+ T cells also interact with antigen-specific B cells, with CD40/CD40L interactions and T cell-derived IL-4 driving the switching of B cells to IgE production. The IgE produced recirculates in the blood, preferentially binding high affinity FcεRI antibody receptors, present on tissue-resident mast cells as well as circulating basophils. In the lung, sensitisation culminates with allergen-specific IgE binding to mast cells of the airway epithelium and mucosa, rendering them capable of directly responding to allergens upon secondary exposure. Upon contraction of the primary response, a small proportion of T cells remain in the tissue as resident memory T cells (TRM), while cells expressing lymph-node-homing CCR7 and CD62L traffic to the lymph nodes as central memory T cells (TCM), capable of rapidly reacting to recurrent allergen-specific insults.

(B) In previously sensitised individuals, re-exposure to allergens drives an inflammatory response in the airway. During the early phase asthmatic reaction, inhaled allergens bind allergen-specific IgE on mast cells in the lung, leading to degranulation and release of stored and synthesised inflammatory mediators. The combination of these mediators drives mucus secretion, vasodilation and airway hyperreactivity, characterising immediate hypersensitivity. The late phase asthmatic reaction then predominates, with the recruitment of allergen-specific Th2 cells driven by DC production of chemoattractants including CCL17 and CCL22. Activated Th2 cells are then central to the induction of allergic airway inflammation, producing key cytokines IL-4, IL-5, and IL-13 that facilitate the activation and influx of multiple inflammatory cells, as well as stimulating airway hyperreactivity, goblet cell mucus production, and the activation and maturation of eosinophils that drive further pathology.
More recently, DC expression of the costimulatory molecule OX40 ligand (OX40L) has emerged as significant in the generation of Th2 immunity (396-398); although it has been demonstrated that OX40L does not polarise Th2 responses, but rather stimulates optimal Th2 priming and memory (399). While Th1 cell responses may also require OX40-OX40L interactions, a unique role for OX40L-mediated promotion of Th2 cell differentiation exists in the absence of IL-12 (400) (discussed further in the following section, *The cytokine micro-environment in Th2 differentiation*). The inducible nature of OX40L renders this costimulatory molecule of particular interest in the development of Th2 immune responses, as specific targeting may be possible. The upregulation of OX40L is induced by innate cytokines TSLP, IL-25 and IL-33 (400, 401), and contributes to the induction of T cell cytokine production as well as clonal expansion and the development of memory CD4+ T cells (402-405). Additionally, abrogation of allergic airway inflammation may be achieved through OX40L blockade (397). The role of OX40L co-stimulation is not critical in all models of Th2 induction, however; rather OX40L is primarily important in TSLP-driven Th2 differentiation. The priming of Th2 cells under TSLP-independent conditions, such as DCs stimulated by certain helminths, does not require OX40L (406). Therefore, OX40L represents an important co-stimulatory molecule but does not unilaterally direct Th2 responses.

**The importance of IL-4 for Th2 differentiation**

The effective priming and differentiation of Th2 cells is primarily dependent on the presence of IL-4 in the local microenvironment (143, 146, 407). *In vitro* studies revealed that engagement of the IL-4 receptor on CD4+ T cells activates a number of phosphotyrosine binding domain-containing proteins, of which signal transducer and activator of transcription 6 (STAT6) is necessary and sufficient to mediate both IL-4-driven Th2 differentiation and cell expansion (408-411). Subsequent upregulation of GATA-binding protein 3 (GATA3) expression ensues, which is the master regulator of Th2 cell differentiation and cytokine production (412-414). It has recently been shown that, in contrast to the critical requirement for STAT6 *in vitro*, some forms of Th2 cell differentiation can occur *in vivo* independently of the IL-4-IL-4R-STAT6 pathway; however, GATA3 activation remains essential (147, 148, 415, 416). The expression of GATA3 may be
induced by Notch activation (417), and activation of Wnt signalling by β-catenin and T cell factor 1 (418); as such, these factors have also been reported to be necessary for Th2 responses in vivo, however, it is not fully known whether this is through direct or indirect influence on GATA3 transcription. Suppression of Th1 and Th17 responses is also achieved through GATA3 expression, partly through upregulation of growth factor independent 1 that mediates transcriptional repression of IFN-γ and IL-17 (419). An additional protein kinase, mechanistic target of rapamycin (mTOR), plays a critical role in the regulation of T cell differentiation, and the deletion of it’s associated protein complex mTORC2 results in an inability to produce IL-4 leading to defective Th2 differentiation (420, 421).

**Cellular sources of IL-4**

The essential cellular source of IL-4 has been a point of some contention. Numerous potential candidates have been proposed including both adaptive and innate immune cells. CD4+ T cells can themselves produce IL-4, independently of IL-4 signalling, upon activation; peptide-stimulated, naïve CD4+ T cells from IL-4Rα-deficient mice have been demonstrated to secrete sufficient IL-4 for the induction of Th2 cell differentiation in an autocrine and/or paracrine manner (158, 422, 423). However, this has not been demonstrated in vivo.

An additional producer of significant amounts of IL-4 is the Natural Killer T (NKT) cell, capable of an immediate response upon TCR engagement(149). NKT cells have been identified in the BAL of asthma patients, implicating a role for NKT cell-derived cytokines in human asthma (424); it has also been reported that NKT cells are essential for allergen-induced airway hyperreactivity during effector Th2 responses (425). However, IL-4 production by NKT cells appears to be not critical for the priming of Th2 cells, as CD1d- and β2-microglobulin-deficient mice that lack NKT cells remain capable of Th2 cell differentiation and responses (425-427). Additionally, no significant recruitment of IL-4-producing NKT cells was observed in the lungs of *Nippostrongylus brasiliensis* infected mice, precluding their role in the local primary Th2 response; instead, CD4+ Th2 cells were the only IL-4 producing T cells identified in the lung in this study (147).

Cells of the innate immune system may also rapidly produce IL-4 upon antigenic stimulation. The newly identified ILC2s display similar functional characteristics to that of
CD4+ Th2 cells, including the production of IL-5 and IL-13. ILC2s have also been reported to produce IL-4 in humans (428), however, minimal IL-4 production has been demonstrated in experimental in vivo murine models (429); as such, the importance of ILC2-derived IL-4 is yet to be established.

Basophils have recently emerged as a potentially important IL-4 producing component of Th2 immunity. Although making up only 0.5% of steady state circulating blood cells, basophils readily secrete IL-4, as well as TSLP, in response to activation by protease allergens such as papain or HDM (430, 431) as well as Th2-inducing parasites (147, 148). Additionally, they have been proposed as possible professional antigen-presenting cells (432-434) supporting the notion that basophils may be critically important in Th2 immune responses. However, other authors have suggested their role in IL-4 production to be redundant and questioned the importance, even the existence, of basophil-mediated antigen presentation (390, 431, 435). Intranasal HDM exposure was shown to induce the recruitment of basophils to lung-draining lymph nodes, but they did not take up inhaled allergen, nor upregulate antigen presentation molecules; indeed basophil-mediated activation of T cells was not observed (431). The investigation of the importance of basophils has been complicated somewhat by particular experimental approaches. In support of a critical role for basophils in Th2 immunity, particularly in the lung, initial evidence was provided by use of a basophil-depleting antibody that targets the FcεRI (434), however, it was later discovered that inflammatory moDCs, also expressing this receptor, were depleted by the antibody (431). Indeed, it was determined by Hammad and colleagues that moDC, and not basophils, were crucial for the induction of Th2 immunity.

During primary Th2 responses, additional sources of IL-4 have been identified. Eosinophils may be recruited in massive number and are capable of producing IL-4 abundantly (147, 436, 437), also reportedly having antigen-presenting functions (438, 439). However, while a CD4+ T cell response may be inducible by eosinophils, anti-CCR3-mediated depletion of eosinophils does not affect Th2 cytokine production (437), neither do eosinophil-deficient mice have impaired Th2 responses(440), suggesting that the role of eosinophils is not critical. The contribution of γδ-T cell-derived IL-4 has also been reported (441) and mast cells participate significantly after chronic antigen exposure(442, 443). However, while
rapid activation may occur, these cells are not thought to provide the critical initial source of IL-4 required for the priming of Th2 cells (147, 444).

The crucial early source of IL-4 has to date not been identified; this may be brought to light in coming years. However, an alternative hypothesis may also be proposed: Th2 immunity, having diverse stimulating factors and purposes, may be inducible by the contribution of multiple redundant sources of IL-4, the involvement of each depending on micro-environmental factors, and of which none may be uniquely, universally, critical. Alternatively, IL-4 may indeed be unnecessary.

**The cytokine micro-environment in Th2 differentiation**

In addition to IL-4, the priming of Th2 responses also involves the interplay of cytokines such as IL-2, TSLP, IL-25 and IL-33; the involvement and relative importance of each cytokine varying depending on the Th2 model under investigation. Some authors attribute a critical role to IL-2, a growth factor produced rapidly by activated T cells and DCs, in the development of Th2 differentiation - primarily due to the importance of IL-2-mediated induction of STAT5 expression (445, 446); however, STAT5 is also promoted by the presence of IL-7 and/or TSLP (447). Importantly, the activation of STAT5 is crucial for the *in vitro* differentiation of IL-4-producing Th2 cells; *in vivo* neutralisation of IL-2 also inhibits IL-4 production (158, 445, 446). Somewhat surprisingly, however, *in vivo* TCR-driven clonal expansion of CD4+ T cells is not affected in the absence of IL-2 or functional IL-2 receptors (448-450), indicating that the critical effects of IL-2 on Th2 cell differentiation might be more related to preparing CD4+ T cells for Th2 cytokine production. Interestingly, where strong STAT5 activation is induced, a lower concentration of GATA3 is required for the induction of Th2 responses. However, in the absence of *Gata3*, constitutively active STAT5A is not sufficient to induce Th2 differentiation, again implicating a critical role for GATA3 (414).

TSLP, produced by epithelial cells, mast cells and basophils (430, 451-453), can act on DCs to preferentially prime the differentiation of naïve CD4+ T cells into Th2 cells as well as playing a significant role during effector Th2 responses (451, 454). This was proposed by some groups to be induced by TSLP-mediated upregulation of OX40L and suppression of DC
IL-12 production. Under physiologically non-allergic conditions, DCs activated in the presence of IL-4 or IL-13 would subsequently produce IL-12, purportedly as a mechanism of negative regulation of Th2 responses by redirecting the response to favour Th1 activity (455, 456). Defective IL-12 production, or TSLP-mediated suppression of IL-12, could thus allow the development of Th2 responses such as those resulting in allergy (457-460). However, when DCs are activated with human TSLP as well as CD40L stimulation, blocking of IL-12 production does not occur; nevertheless, Th2 differentiation may still proceed (461). Additionally, it was shown that IL-12-deficient mice, which are still capable of developing substantial numbers of CD4+ T cells in response to pathogenic stimuli, do not develop Th2 responses by default (462). Thus, it appeared likely that there were additional roles for TSLP in affecting DC functions that drive Th2. Indeed, it was recently discovered that STAT5 is required for TSLP-dependent DC activation. Bell and colleagues demonstrated that DC-specific deletion of STAT5 resulted in their inability to drive type 2 allergic responses in the lung as well as the skin (454); no effect on type 1 immunity was observed. The STAT5-deficient DC did not respond to TSLP, which prevented their activation, chemokine production and upregulation of costimulatory molecules CD80, CD86, and OX40L. Therefore, the ability of TSLP to drive Th2 immunity in the lungs and skin critically relies on STAT5 in DCs.

Upon PRR activation, epithelial cell production of IL-25 and IL-33 also promotes Th2 responses. Additionally produced by Th2 cells, IL-25, of the IL-17 family, and IL-33 of the IL-1 and IL-18 family, have been demonstrated to induce IL-4, IL-5 and IL-13, as well as IL-4-mediated IgE production, that may result in Th2 disease in the lungs and gastrointestinal tract (463, 464). Stimulated by IL-25 and IL-33, as well as TSLP, ILC2 represent an important component of this early Th2-polarising cytokine production (332, 334, 336, 465, 466). Interestingly, the requirement for ILC2 in the priming of some Th2 responses has been reported, but only in the context of local antigen exposure; experimental models involving systemic priming do not require ILC2 (467). Rather, a critical role for T and B cells in the development of the primary CD4+ Th2-cell mediated immune response is implicated (463). Indeed, epithelial cell-derived IL-25 (383) and IL-33 (464) can directly act on CD4+ T cells, resulting in IL-4-dependent Th2 cell differentiation. IL-33, additionally, stimulates
basophils to induce the production of IL-4 (468, 469), as well as basophil and mast cell release of histamine (464, 470, 471) potentiating the development of Th2 immunity.

1.2.2.1 Sensitisation to aeroallergens
Exposure to inhaled aeroallergens results in the sensitisation of an individual and consequential allergic airway inflammation upon subsequent exposure to the specific allergen(s) (See Figure 1.2 A – The allergic response). Central to the development of allergy is the priming of a Th2 response, as discussed in Section 1.2.2. Priming induces maturation, expansion and migration of Th2 cells to the site of allergen exposure (472, 473). Th2 cells then produce large amounts of IL-4 and IL-13; this cytokine microenvironment, as well as CD40/CD40L signalling, leads to B cell class switching of immunoglobulin production to IgE (474-476). It was initially thought that B cell immunoglobulin class switching occurred exclusively in germinal centres of the lymph nodes, however it has since been discovered that clonal selection and affinity maturation of IgE-producing B cells can also be induced in the respiratory mucosa (477) and gastrointestinal tract (478) of allergic individuals. IgE antibodies then circulate in the blood, or are trafficked locally, and preferentially bind the high affinity Fcε antibody receptor, FcεRI, present on tissue-resident mast cells as well as circulating basophils. In the lung, sensitisation culminates with allergen-specific IgE binding to mast cells of the airway epithelium and mucosa, rendering them capable of directly responding to allergens upon secondary exposure (479, 480). The effector Th2 response is also accompanied by the development of long-lived memory CD4+ T cells and B cells that migrate to lymphoid or inflamed tissue, such as the lungs, and rapidly react to recurrent allergen-specific insults in the airway (discussed in section 1.1.4).

1.2.2.2 Aeroallergen challenge: Th2 effector functions and cytokine production mediate the pathology of atopic allergic asthma
In sensitised individuals, the inhalation of aeroallergens leads to the orchestration of a rapid secondary immune response that mediates allergic airway disease (See Figure 1.2 B). This response comprises early, IgE-mediated, and late, T cell-mediated components.
Initially, inhaled allergens bind allergen-specific IgE on mast cells in the lung, cross-linking IgE and activating complex intracellular signalling pathways (480). Mast cells then rapidly degranulate and release stored histamines and proteases; eicosanoids, leukotrienes and prostaglandins are rapidly synthesised, and the production of cytokines commences; notably, IL-4, IL-5 and IL-13 as well as TNF contribute to allergic disease (481, 482). The combination of these mediators drives mucus secretion, vasodilation and airway hyperreactivity, characterising the early phase asthmatic reaction – or ‘immediate hypersensitivity’ (483). Four to six hours later, the late phase asthmatic reaction predominates, with allergen-specific Th2 cells driving allergic airway inflammation.

The recruitment of Th2 cells to the airways is crucial to the pathogenesis of allergic asthma (484, 485). After proliferation in the MLN (486), the homing of Th2 cells to the lungs is dependent on chemoattractant receptor signalling. STAT6 expression performs a critical master regulatory role in orchestrating the expression of Th2-attracting chemokines CCL11, CCL17, CCL22, and CCL24 and the subsequent recruitment of Th2 cells to the allergic lung (487, 488). Interestingly, the STAT6 required for chemokine expression and Th2 recruitment is critically sourced from bone marrow-derived myeloid cells, while airway lining cells are not necessary; specifically, CD11b+ cDC in the lung are a crucially important source of CCL17 and CCL22 (488). Chemokines act to recruit Th2 cells through chemoattractant receptors CCR3, CCR4, CCR8 and the PGD2 receptor CRTH2 (489). Attempts to determine which of these receptors plays a crucial role in the trafficking of Th2 cells in vivo, however, revealed that targeted deletion of any of these receptors individually did not prevent Th2 homing (490-492). These findings suggest that multiple receptors are commonly stimulated to induce Th2 cell homing.

After trafficking to the lung, Th2 lymphocytes recognise and respond to allergens presented by lung-surveying APCs. Importantly, it has been shown that the induction of secondary Th2 responses to aeroallergens, as well as maintenance of chronic inflammation, is under the essential influence of DC (493). CD11c+ DCs are necessary and sufficient for the induction of the effector Th2 immune response that leads to allergic asthma; their depletion during airway challenge abrogates the characteristic features of disease in previously
sensitised mice (321). Following allergen challenge of sensitised mice, CD11b+ DCs increase in number and localise within the vicinity of allergic airways (494). It has been reported that CD11b+ DC may be found presenting allergens in the airways up to 8 weeks after allergen exposure (114); perhaps indicating that inter-DC antigen transfer is occurring to prolong the duration of antigen presentation (60, 74-76). Specialised in presenting soluble antigens to CD4+ T cells (123, 322), CD11b+ DCs were recently reliably separated into CD64- conventional and CD64+ monocyte-derived DCs (306). The CD11b+CD64+ moDC, recruited to the lung under inflammatory conditions, were subsequently found to contribute significantly to the progression of Th2 immunity through the production of pro-inflammatory chemokines (306). Conversely, lung CD103+ DC and plasmacytoid DCs have not been implicated in the CD4+ Th2 cell-mediated allergic response; CD103+ DC are specialised in cross-presentation to CD8+ T cells (73, 126), while allergen-loaded plasmacytoid DCs have been demonstrated to suppress airway inflammation in sensitised mice (325).

Th2 cytokines mediate allergic disease

Activated Th2 cells produce key cytokines IL-4, IL-5, and IL-13 that facilitate the activation and influx of multiple inflammatory cells that mediate pathogenic effects on the airway (Figure 1.2 B). IL-10 may also be produced, however an inhibitory role has been described for IL-10 production in allergic disease (495-498). It is worth noting that after the priming of Th2 cells, IL-4 is not required for allergic airway inflammation to proceed; blocking of IL-4 during allergen inhalation challenge results in intact airway inflammatory responses, however, STAT6 signalling is still required for the recruitment of eosinophils and Th2 cells to the lung (499, 500). IL-4, however, works in concert with IL-5 and IL-13 to induce airway eosinophilia. In atopic allergic asthma, eosinophils are the major infiltrating cell population found in the lung tissue and bronchoalveolar lavage (BAL) fluid of asthmatic patients (501); the extent of eosinophilia correlates with disease severity (502). Th2 cell-derived IL-5, IL-13 and IL-4 act on lung epithelial and endothelial cells to produce eotaxin-1 (CCL11), -2 (CCL24) and -3 (CCL26) that act as chemoattractants to recruit eosinophils by interaction with eosinophil chemokine receptor CCR3 (503-506). IL-5 itself is also important for mediating the release of eosinophils from the bone marrow, as well as driving their differentiation, proliferation and maturation (507-509). Additionally, IL-4 plays a role in
enabling eosinophil migration across the endothelium (510, 511), and IL-3 and GM-CSF have been reported to play a role in facilitating eosinophil maturation (512). In the lung, primed eosinophils release a massive arsenal of proteins, lipids, chemokines and cytokines that generate characteristic asthmatic lung pathology. Of note, IL-4, IL-13, TGF-α, eosinophil peroxidase, cysteinyI leukotrienes and major basic protein contribute to the development of airway hyperreactivity through epithelial cell damage, smooth muscle contractility (513, 514), vascular permeability (515) and mast cell activation and survival (516, 517). The same mediators and additional fibroblast growth factors and TGF-β play a role in ongoing airway remodelling and chronic inflammation (518-523).

The primary effects of IL-13 on the allergic airway, additional to those already described, are the induction of the Mucin 5AC protein (Muc5AC) and goblet cell hyperplasia, that together lead to increased mucus production, as well as airway remodelling that drives smooth muscle hyperreactivity (524, 525). However, investigations by numerous authors have revealed differences in the requirement for IL-13 during the acute and chronic phase of disease. Transgenic overexpression of IL-13, or the direct administration of IL-13 to the airways has been shown to induce mucus secretion, IgE production, eosinophilia, and subepithelial fibrosis (526-528). IL-13 blockade or deletion of the IL-13 gene was reported to prevent or reverse these features of disease (529-531). However, in chronic airway inflammation IL-13, while capable of potentiating disease, may not have a necessary role. IL-13 blockade after allergen challenge has been shown ineffective; specifically, structural changes to the airway and associated AHR are not reversed once established (529, 532, 533). As it can be difficult to block cytokine production completely, it is possible that the studies utilising antibody blockade of IL-13 may not have observed fully effective suppression of IL-13; indeed this data was not clearly shown in these publications. However, it was clear that although the AHR and structural changes were not reversible in these chronic models of disease, the acute phase components of the allergic response, such as eosinophilia and cell infiltrates into the airway were in fact suppressed by anti-IL-13 treatment. Therefore, these results appear consistent with IL-13 having a redundant role in perpetuating the features of chronic airway inflammation.
**Mast cells in allergic airway inflammation**

Th2 cells are also responsible for the activation of mast cells through the production of IL-3, IL-4, IL-13 and IL-9 (481, 534-536); however, the IL-9 production classically attributed to Th2 cells may in fact be most significantly produced by recently described Th9 cells (162, 163). The role of mast cells in active allergic airway inflammation is not fully resolved. Hallmark features of chronic asthma such as AHR and eosinophilia may be generated in the absence of mast cells (444) or IgE (537, 538). However, mast cell-dependent (539) and IgE-induced (539, 540) pathways also exist for the development of AHR in mice. This discrepancy may have evolved from differences in experimental models in terms of the concentration of allergenic stimuli and method of exposure. Although participating in the early asthmatic response, it appears that mast cells are not critical for the induction of allergic asthma in models that induce strong eosinophilia (444). Chronic exposure to allergens, however, leads to an increase in mast cell numbers in the lung tissue (443) that then amplify antigen-dependent inflammation (541). Mast cells may be found associated with airway smooth muscle in human asthmatics whereby muscle cell contractility is increased, facilitating the development of airway resistance and AHR (542, 543). Upon activation, mast cells secrete a wide range of mediators including histamine, prostaglandin D2, TNF-α, mast cell proteases and leukotriene C4 (LTC4) that generate bronchoconstriction, mucus secretion and vascular permeability; additionally producing the key Th2 cytokines IL-4, IL-5 and IL-13 as well as pro-inflammatory TGF-β (544-546). These mediators promote the recruitment of Th2 cells, eosinophils, basophils, neutrophils and monocytes, as well as aiding their infiltration of the lung tissue during the late phase asthmatic response (547-549).

**Basophils in allergic airway inflammation**

Basophils share many common phenotypic and functional features with mast cells, importantly including FceR-I expression and the production of mediators such as histamine and LTC4. As such, some overlapping roles exist for these cells. However, numerous non-redundant activities are carried out by basophils to amplify allergic airway responses. While mast cells are long-lived, tissue-resident and primarily required for immediate hypersensitivity reactions, basophils are predominantly a short-lived circulatory population, homing to sites of allergic inflammation as part of the late phase response (550).
The recruitment of basophils to the allergic lung is not fully understood as reliable unique basophil-specific markers have not yet been described, however, it is known that T cells are critically involved in this process \((431, 551)\), whereas STAT6, IL-4 and IL-13 are not required \((147)\). IL-3, IL-5, nerve growth factor and GM-CSF as well as innate cytokines IL-25 and IL-33 may promote basophil influx and release of chemical mediators including histamine and LTC4 \((468, 469, 552, 553)\). Importantly, basophils – primed by IL-3 \((554-557)\) – are major producers of IL-4, which is secreted minimally by mast cells \((558, 559)\), as well as TSLP \((430)\), thus driving the amplification of Th2 immunity.

### 1.2.2.3 Airway remodelling and chronic allergic airway disease

Individuals that suffer from allergic asthma are often recurrently or persistently exposed to the allergens that elicit their allergic reactions. While multiple early phase and late phase reactions are induced and subsequently resolve, structural damage to the airway accumulates and persists long term; characteristic asthmatic airway remodelling thus becomes evident over time, and chronic disease develops. Distinguishing features of remodelling include increased numbers of mucus-producing goblet cells in the airway epithelium, subepithelial fibrosis, increased airway smooth muscle mass, proliferation of blood vessels, airway oedema and tissue destruction \((560-562)\). Together, these changes result in thickening of the airway wall.

Markers of epithelial repair are characteristically present alongside inflammatory features in chronic disease. Tenascin-c, normally absent or weakly expressed in the lung, increases under pathological conditions such as inflammation, infection, biomechanical stress and wound healing, and is an early initiator of airway wall remodelling \((563, 564)\). Expression is enhanced in the subepithelial extracellular matrix and correlates with the extent of bronchial mast cell and eosinophil infiltration \((565)\). Tenascin-c stimulates IL-5, IL-13 and IgE secretion from lymphocytes \((566)\), potentially through an indirect mechanism whereby tenascin-c stimulates the rolling and tethering of cells, provoking lymphocyte trafficking to inflamed mucosa where cells may be stimulated \((567-569)\). These mediators are significantly reduced in the BAL of allergen sensitised/challenged tenascin-c-deficient mice \((566)\); as such, an important role exists in the pathogenesis of asthma.
Matrix metalloproteinases (MMPs) are also involved in lung diseases that are characterised by airway remodelling. MMP-9, the predominant family member in asthma (570), actively degrades type IV collagen to allow inflammatory cell trafficking throughout the lung. Importantly, MMP-9 is required for the successful migration of DCs into the airway to take up antigens (571, 572), as well as playing an important role in the activation of TGF-β1 that drives subepithelial fibrosis (573).

Repeated allergen exposure can also result in the production of IgE to multiple epitopes of the same allergen, or to unrelated allergens. A mechanism through which this can arise involves facilitated antigen presentation (FAP) mediated by CD23, an IgE binding receptor of the C-type lectin superfamily, that can be expressed by cells such as epithelial cells, myeloid cells and B cells (574). Airway epithelial cell CD23 binds, and mediates the transcytosis of, IgE and antigen-IgE complexes across the epithelium, enabling the binding of this IgE to mast cells, basophils, macrophages and DC in the lung tissue (575). This process may also occur in the gastrointestinal tract (576). Antigen-IgE complexes can bind CD23 molecules on non-specific myeloid cells and B cells, which may then present the antigens to cognate T cells (577). This FAP-driven presentation of new epitopes or allergens to T cells in the presence of Th2 cytokines may promote their differentiation into Th2 cells, subsequently inducing the B cell production of IgE specific for the new allergens. IL-4 and IL-13 have also been demonstrated to increase the expression of CD23 on myeloid cells and B cells, enhancing the potential for this phenomenon to occur (476). Thus, when allergic individuals are exposed to multiple potential allergens simultaneously, FAP in a Th2 micro-environment can mediate sensitisation to the additional allergen specificities. Indeed, the majority of individuals who suffer from allergic airway inflammation are polysensitised (22, 578, 579).

**Respiratory viral infection drives exacerbation of asthma**
Common respiratory viruses such as rhinovirus, influenza, and respiratory syncytial virus can markedly exacerbate the symptoms of asthma (580-582). Rhinovirus infection has been
shown to enhance histamine release, eosinophil recruitment and AHR during allergen exposure (581, 582). While it is not fully understood how this occurs, exacerbations may be driven by the effects of viruses and viral replication on the airway epithelium and associated cells. Viral products have been demonstrated to act directly on mast cells, in humans and murine models, by inducing TLR signalling, especially through TLR-7 (583, 584). In chronic asthmatic disease, the elevated number of mast cells within the airway epithelium and smooth muscle layers positions them for rapid inflammatory responses (542, 543). These combined effects of viruses on key allergy-driving cells represent a mechanism through which viral infections result in exaggerated asthmatic responses.

1.3 CTLs in the treatment of allergic airway disease

The role of the CD8+ T cell in allergic asthma is not fully defined, and has been a point of some controversy. Multiple studies have revealed contexts in which CD8+ T cells exaggerate or abrogate allergic airway inflammation. CD8+ T cells may be of a Tc1 or Tc2 phenotype (as discussed in Section 1.1.3.1). Tc1 cells, characterised by their production of IFN-γ, and clearance of virus-infected cells, promote Th1 responses. Conversely, Tc2 cells produce IL-5 and IL-13, amplifying Th2 disease. As such, in models of allergic airway inflammation, CD8+ T cells have been shown to have either pro-inflammatory (227-229) or suppressive activity (220-226) on asthmatic disease. The pathway through which suppression is achieved by Tc1 has not been fully elucidated, although numerous potential mechanisms have been proposed.

A number of studies have highlighted the role of the key CTL effector cytokine IFN-γ in the suppression of allergic disease (221, 585); CD8+ T cells from IFN-γ knock out mice were unable to suppress inflammation like their WT counterparts. IFN-γ is known to inhibit IgE production (586, 587), presenting a mechanism through which allergic responses may be prevented. However, evidence surfaced that questioned the importance of CD8+ T cell-
derived IFN-γ in the suppression of IgE production. The adoptive transfer of IFN-γ−/− CD8+ T cells was shown to suppress IgE production similarly to WT cells (588). Additionally, both Tc1 and Tc2 cells are capable of suppressing IgE production, despite the limited IFN-γ production of Tc2 cells (589). The importance of suppressing IgE is particularly relevant during antigen exposure that may lead to sensitisation. Once adaptive immunity is generated in the form of an antigen-specific Th2 response, IgE-independent stimulation of allergic airway inflammation may occur. The effective suppression of established Th2 responses by CTLs must then require an alternative mechanism of action. Indeed, it has been demonstrated that CD8+ T cells can mediate suppression of the late allergic response including reduced eosinophilia and Th2-cytokines, without affecting the levels of antigen-specific serum IgE (220).

It has been suggested, alternatively, that CTL-derived IFN-γ alleviates allergic disease through the skewing of Th2 immunity towards a Th1 bias that subsequently antagonises Th2 responses. This may occur through the IFN-γ-mediated stimulation of DCs to produce IL-12. Indeed, adoptive transfer of antigen-specific CD8+ T cells in a model of allergic airway inflammation has been reported to result in a reduction in BAL eosinophilia, IL-4 and IL-5, with an associated increase in IL-12p70 (223). It was further demonstrated in vitro that CD8+ T cells primed CD8-α+DC for IL-12 production in a cell contact-dependent manner (590). In contrast, other authors have shown that while enhanced DC production of IL-12p70 antagonised IL-13-driven gene induction and STAT6 phosphorylation, it had no effect on Th2 cytokine production, BAL inflammatory infiltrates or IgE synthesis (591). Furthermore, additional studies reported successful amelioration of allergic airway inflammation by CD8+ T cells that did not involve detectable production of IL-12 (226). These conflicting results suggest that while IL-12 may be suppressive in some models of allergic inflammation, it is not a critical factor in CD8+ T cell-mediated amelioration of disease. Of note, the work of Wong and Wells involved the co-administration of endotoxin with antigen which may promote IL-12 production and Th1 skewing via DC TLR stimulation, independently of their interaction with CD8+ T cells (372). Lewkowich and Enomoto, on the other hand, used low-endotoxin allergen preparations (226, 591).
While IFN-γ may be the archetypal Th1-associated cytokine, classic Th1-polarising signals may not always counterbalance Th2 immunity. IFN-γ has also been shown to exacerbate allergic disease (592, 593) and Th1 cells adoptively transferred with Th2 cells contribute to, rather than suppress, airway inflammation (594).

While CTL-derived IFN-γ may have the capability to modulate Th2 inflammation as discussed herein, Enomoto et al demonstrated that adoptively transferred CTLs did not require IFN-γ to suppress inflammation in previously sensitised mice; neutralisation of IFN-γ still resulted in prevention of disease in an acute setting (226). The authors propose that this discrepancy in the requirement for IFN-γ, which is in contrast to the results generated using IFN-γ -/- CTLs, may stem from the fact that IFN-γ -/- CTLs have an impaired ability to kill target cells. This observation is in accordance with the findings of Tang and colleagues, who reported that IFN-γ -/- CTLs were poorly cytotoxic and in fact displayed a Tc2/Tc17-biased phenotype, rendering them capable of inducing and exacerbating eosinophilia and neutrophilia (595). This factor was deemed important by the work of Enomoto et al that highlighted the critical requirement for perforin in successful CTL-mediated amelioration of acute asthmatic disease, which suggested that cell(s) were targeted and killed as a means of suppressing the Th2 response. The identity of the critical target cells, however, has not yet been revealed.

The ability of CTLs to kill target cells relies on the recognition of their cognate antigen through TCR stimulation. Whether CD8+ T cells need to be allergen-specific to successfully suppress allergic airway inflammation, however, has been questioned. Some allergens, such as the Der p 2 component of HDM, may directly activate CTLs through TLR signalling in the absence of their cognate antigen (596-598), and large pools of activated CTLs may carry out effective immune responses to alternative antigen specificities during viral infection (224, 599-601). It has also been reported that resting memory CD8+ T cells may be activated during viral infection in a non antigen-specific manner. These cells respond to the cytokine environment in lymphoid tissue as well as the lung and liver, particularly through stimulation by IL-12 and IL-18 (224). The resulting CTL activation, however, primarily drives the production of IFN-γ that modulates immune responses somewhat non-specifically. Indeed, studies that have investigated the effects of non allergen-specific CTLs
on allergic airway disease revealed that IFN-γ was central to suppressive responses (221, 585). Interestingly, Enomoto and Ronchese discovered that CTLs of different allergen specificity could suppress airway inflammation only when endotoxin was present, in a presumably IFN-γ dependent fashion (unpublished observations). CTL-mediated killing of target cells, however, occurs secondary to TCR ligation; as such, only allergen-specific CTLs can suppress allergic airway inflammation in the absence of endotoxin. While conventional vaccines do not induce CTL responses, antigen-specific CTL-mediated immune responses may be elicited in vivo through the use of dendritic cell vaccines, as has been widely used in the stimulation of anti-tumour responses (602-605); in a model of allergic airway inflammation, this can achieve protection similarly to adoptive transfer of CTLs (226). An alternative method for the induction of CTL responses is to vaccinate using viral vectors to target delivery of antigenic epitopes into DCs. This has been carried out experimentally, commonly using adenovirus or vaccinia virus vectors, resulting in effective CTL responses including anti-tumour immunity (606-609). However, this method may not gain approval for use in patients due to infection risk. Recently, a novel vaccine that stimulates an allergen-specific endogenous CTL-mediated response was developed; this vaccine is described, and its effects on allergic airway inflammation investigated, in this thesis (Chapter 5).

A general trend may be observed in the findings of the studies presented herein, whereby two major mechanisms are described for the CTL-mediated suppression of airway inflammation. Firstly, IFN-γ from activated CTLs may counteract allergen-induced Th2 immune responses, occurring in a non-antigen specific manner in the presence of endotoxin. However, the requirement for IFN-γ is debatable, and it appears that it is not sufficient for the suppression of disease on its own. Secondly, antigen-specific killing of target cells may occur in a perforin-dependent manner, independently of endotoxin effects, and without the requirement for IFN-γ. The work involving IFN-γ has been useful to further the understanding of CTL-mediated immune responses and how Th1-skewing may be able to divert or re-program pathogenic Th2 immune responses, however, in terms of the treatment of allergic lung disease, the need for a targeted therapy that dampens only specific inappropriate responses is of utmost importance in preserving lung health and immune defence against infectious disease.
From these studies it is evident that CTLs are capable of suppressing allergic airway inflammation and, although this is yet to be tested in clinical studies, the potential for treatment of allergic asthma exists. However, investigations into the mechanism of suppression have produced conflicting, inconclusive results. Additionally, while many studies have shown the capability of CTLs to prevent inflammation during initial allergen challenge, it is not known whether suppression may be achieved in chronic disease, and how long this protection persists. Further work is also required to identify the targets of CTL-mediated killing and the localisation of CTL-target cell interactions. These insights could lead to the development of a novel therapy for sufferers of allergic asthma.

1.4 Modelling allergic asthma

Key to furthering understanding of the basic science of health and disease, animal models of disease provide a tool with which we may also investigate the effects of novel therapies. For animal experimental results to be potentially applicable and translatable to human health a model system must be developed where the pathology closely resembles that of the human disease. The characteristic features of allergic asthma may be generated in mice using \textit{in vivo} sensitisation techniques or the adoptive transfer of \textit{in vitro} activated Th2 cells. Different strains of wild type mice may be used and the type and amount of allergen exposure varies between studies. As such, the pathology developed in these models may differ and findings may not be fully comparable throughout the literature. Regardless of these disparities, well-characterised models of allergic airway inflammation allow for understanding of the limitations as well as the conclusions that may be deduced from each study and provide important insights that may be related to human health.

1.4.1 Methods of inducing allergic airway inflammation

To generate allergic airway inflammation in mice, allergen-specific Th2 responses may be primed through a number of routes. Commonly, intranasal (in.) or intraperitoneal (i.p.)
exposure to allergen is used to facilitate sensitisation. Model allergens may be mixed into aqueous solutions and delivered to the lung either through nebuliser-generated aerosols that are inhaled, or instillation of the solution directly into the nares or trachea of mice. The airway epithelium and associated cells then respond to allergens through the recognition of PAMPs by TLRs; as discussed in section 1.2, this leads to the activation of DCs that prime Th2 responses. While i.n. sensitisation to protease allergens can be successful, weaker allergens usually require additional Th2-polarising stimuli for the generation of allergen-specific IgE. This may be achieved intranasally through the co-administration of low doses of LPS (610), or by systemic sensitisation through i.p. injection of allergen co-administered with a Th2-promoting adjuvant such as aluminium hydroxide (Alum). Sensitisation via these different routes may elicit allergic airway inflammation that is initiated through differing pathways. Direct intranasal exposure results in the activation of innate immune cells such as APCs, ILCs, mast cells, and the airway epithelium (611, 612). In contrast, i.p. sensitisation subverts the involvement of the airway cells, activating peritoneal APCs that stimulate the differentiation of Th2 cells in draining lymph nodes. Nevertheless, both models reliably generate robust Th2 immune responses in the lung upon airway allergen challenge. As such, the model of sensitisation is largely developed in accordance with the protocol appropriate for the allergen of choice, to fulfil the purposes of different experimental questions.

1.4.2 Model allergens in the induction of experimental allergic asthma

Common experimental allergens used to induce Th2 immunity in the airways of mice include OVA, HDM, and cockroach extracts, Aspergillus fumigatus, Alternaria alternata, ragweed, cedar and cypress pollens. Predominantly, studies of allergic airway inflammation have utilised OVA or HDM; distinct differences exist in these allergens that can alter the phenotype of allergic disease.

The widespread use of OVA as a model allergen has led to extensive characterisation of OVA-induced asthmatic pathology. The OVA protein contains two main epitopes that are immunogenic in C57BL/6 mice, OVA257-264 which may be presented on MHC-I to stimulate CD8+ T cell responses and OVA323-339, presented on MHC-II for the stimulation of CD4+ T cells (613-616). Many different experimental protocols exist for the sensitisation and
challenge phases that lead to allergic disease, however the basic outline of these models is similar. Airway exposure to OVA, in the absence of prior sensitisation, leads to a tolerogenic response (586, 617) and as such, mice are usually sensitised systemically by i.p. administration of OVA protein along with a Th2-promoting adjuvant such as Alum. Commonly, two i.p. doses of OVA/Alum are administered some time apart to boost IgE production (618); this also lessens the chance of failed i.p vaccination as a result of technical error. Airway exposure to OVA is then performed either intranasally, intratracheally, or by inhalation of aerosolised OVA which drives a local Th2-mediated allergic response in the lung.

More recently, HDM is becoming a popular model allergen in the study of allergic asthma, as it commonly presents as an airway allergen in human disease (619). Different research groups have used preparations of either crushed whole dust mites, or select extracted components such as the major *Dermatophagoides pteronyssinus* group 1 or 2 epitopes to which 80-90% of mite allergic patients react: Der p 1 and Der p 2 (620, 621). These epitopes have different roles in the promotion of allergic sensitisation in the lung. Der p 1, a cysteine protease, acts on respiratory epithelial cells to induce the release of pro-inflammatory mediators (622). This is thought to be achieved by protease-mediated disruption of epithelial barriers and subsequent access to APCs (389, 623, 624). Der p 2, on the other hand, does not possess enzymatic activity, however it has been shown that Der p 2 sensitisation also leads to the generation of specific IgE as well as airway inflammation (596, 625). While it is not clear how this occurs in the absence of protease function, it has been shown that Der p 2, in concert with LPS, can activate bronchial epithelial cells via inducing TLR 4 signalling (598). Inflammation may be driven by the activation of intracellular MAPK and NF-κB signalling pathways which stimulate the production of pro-inflammatory cytokines as well as factors that facilitate the infiltration of immune cells into the lung (388).

Standardisation of models of HDM-induced allergic airway inflammation has been complicated by the fact that numerous immunogenic factors differ in the HDM-derived preparations utilised. The relative amount of Der p 1 to Der p 2 in crushed whole mites may vary and differing amounts of LPS are also naturally associated with the mites, and as such,
each particular HDM preparation may differ in the amount of endotoxin present. Due to the auto-adjuvanting properties of LPS, HDM-mediated allergic airway inflammation may be induced solely through airway exposure to the allergen (373). Multiple doses are administered intranasally or intratracheally to progressively stimulate allergic airway inflammation. The endotoxin component of HDM stimulates a neutrophilic component to airway infiltrates that is not present after endotoxin-free OVA allergen exposure (373, 626, 627). However, the eosinophilic inflammation induced by HDM exposure is much weaker than that elicited by OVA/Alum immunisation and thus may be more difficult to draw conclusions between treatment groups (experimental observation).

A criticism that has gained some momentum is the fact that models that utilise OVA, which is normally tolerated, rely on co-administration of adjuvant and systemic sensitisation which is not considered to mimic natural sensitisation as closely as the HDM protocols. However, this may not be an entirely flawless argument. In humans, the majority of inhaled antigens do not drive allergic responses, but rather are tolerated; and in non-allergic individuals, common allergens such as HDM are also tolerated upon exposure. As such, even “human relevant” allergens require adjuvanting cofactors, such as LPS, PAMPs and DAMPs, as well as host susceptibility, to produce allergic disease. Therefore, the use of adjuvants in OVA-induced allergic airway inflammation does not preclude these models from representing valid, physiologically relevant asthmatic disease. Importantly, as OVA has been well characterised, distinct CD4+ and CD8+ T cell epitopes defined, and endotoxin-free preparations generated, our understanding of the pathology generated by OVA is grounded in extensive literary reports of experimentation in this model. As such, the efficacy of novel therapies on specific parameters of allergic airway inflammation may be examined.

1.4.3 Murine models of experimental allergic asthma

Evidence is emerging in the literature that describes how genotypic differences in wild type mice may affect their responses to allergen sensitisation and challenge protocols. The most commonly used strains of mice in the modelling of allergic airway inflammation, C57BL/6 and BALB/c, differ in their tendency to drive Th2 responses. While allergic disease may be provoked in both C57BL/6 and BALB/c mice, it has been reported that BALB/c mice are more prone to Th2 bias, due to their intrinsically higher susceptibility to develop allergy
than C57BL/6 (628). Indeed, when exposed to the same experimental protocol, BALB/c mice experience enhanced AHR, eosinophilia and mast cell numbers in the lung tissue, as well as elevated levels of IL-4, IL-5 and IL-13 compared to C57BL/6 mice (500, 629-631). Other authors, however, have reported that C57BL/6 mice display higher BAL eosinophilia and neutrophilia, peribronchial eosinophilia, as well as increased expression of chemokines CCL11 and CCL5 in BAL (630, 632). As such, that BALB/c mice are “Th2 prone” does not necessarily reflect the complete picture; rather, they may display a greater propensity for the development of allergic disease that both BALB/c and C57BL/6 mice do in fact experience, with some variation in phenotype.

Differences in the ability to generate Th2 responses may stem from genetic variance and the different pathways through which allergic responses are initiated, however little information is available on what these specific factors are. It has been shown that CD4+ T cells derived from BALB/c mice show a bias towards IL-4 production in response to TCR stimulation (633). Additionally, decreased IL-12 receptor expression may reduce the promotion of Th1 and suppression of Th2 responses (634). The airway epithelium has been found to respond to antigen challenge differently in the two strains, with resulting NF-κB activation being more prominent in BALB/c mice (635). C57BL/6 mice, on the other hand, have elevated production of innate immunosuppressive molecules such as surfactant protein-D, which may confer some resistance to the generation of AHR (629).

As a result of these differences, studies attempting to understand the critical factors involved in asthma pathogenesis have revealed contrasting results. Experiments conducted on C57BL/6 mice report a critical requirement for eosinophils in the generation of AHR and goblet cell metaplasia (636, 637), whereas the opposite conclusion was drawn from experiments using BALB/c mice (492, 500, 638). Additionally, the activity of airway epithelial cells was shown to be critical for OVA-induced allergic airway inflammation in BALB/c mice, but not in C57BL/6 (635). As such, C57BL/6 models often require increased doses of allergen, or additional exposures, to mediate significant airway inflammation, due to their apparent reduced susceptibility to develop allergic disease. These findings highlight the significance of strain-dependent differences in the characteristics of allergic disease, and thus have implications for the deduction of conclusions from these models.
1.4.4 Acute and chronic models of allergic airway inflammation

A complete understanding of the pathogenesis of allergic asthma has not yet been grasped. The initiation of allergy and the further development of chronicity involve distinct immune pathology. As such, there is much that can be learned through the use of acute, short-term models of allergic airway inflammation as well as those that drive chronic, long lasting disease.

Many studies use experimental protocols involving the administration of a single airway allergen challenge to sensitised mice, or multiple exposures over a short number of days. Features of allergic inflammation are induced in the lung; BAL and lung eosinophilia, AHR, and Th2 cytokine production (639-641). These models are particularly useful in the examination of the early asthmatic response and late asthmatic response that occur during the first 8 hours after allergen exposure (642, 643). Early features of airway remodelling may be evident even in the acute setting, however inflammation may resolve rapidly upon discontinuation of allergen challenge, with limited residual disease (538, 639-641, 644). The value of these acute models is therefore applicable to the understanding of how allergic disease is initiated, and the changes that occur during early pathogenesis that may facilitate persistence of disease. Additionally, the preventative activity of novel vaccines may be examined in this context.

By definition, asthma is a chronic disease of the airways involving changes that persist even in the absence of allergic stimuli. As such, modelling chronic asthmatic disease involves the administration of multiple airway allergen challenges to sensitised mice, over a number of weeks or months. These protocols can drive chronicity (618, 632, 645), or may in fact lead to tolerance (646) depending on the co-stimulatory factors present during airway allergen challenge. Through the repeated stimulation of allergic inflammation and subsequent repair, the characteristic features of asthma may be replicated by these models (618, 647-649). Airway remodeling is evident through the presence of multiple contributing factors that result in thickening of the airway wall (discussed in section 1.2.2.3). As classic features of established airway inflammation are generated, the effects of therapeutic vaccines for the treatment of individuals who suffer from chronic asthma may be tested.
These *in vivo* sensitisation models culminate in the development of an allergen-specific Th2 response. It is difficult, however, to examine the activity of the critical CD4+ Th2 cells in allergic airway disease, as limited techniques exist for the tracking of endogenous allergen-specific T cells. Some studies have shown that the direct adoptive transfer of *in vitro*-cultured, allergen-specific Th2 cells into mice can establish allergic airway inflammation upon allergen exposure (650, 651). While the focus of these studies was primarily to investigate the capability of CD4+ T cells to produce inflammation in naïve hosts, this concept may be used to develop a standardised model of allergic airway disease within which the activity and whereabouts of CD4+ T cells may be studied. This could lead to a better understanding of the role and intercellular interactions of the CD4+ T cell that drive allergic disease.

### 1.4.5.1 Visualising immune cells and the allergic response

To develop an understanding of the involvement, localisation and intercellular interactions of key immune cells in the allergic response, visual tools are invaluable. Numerous transgenic mouse strains are now available where fluorescent reporter genes allow for the identification of critical allergy-driving cells such as DC and CD4+ T cells.

As important initiators of multiple immune responses, CD11c<sup>eYFP</sup> expressing mice were developed to examine the roles and interactions of DCs (652). In tissue sections, these fluorescent cells may be counterstained to reveal DC subpopulations; alternatively, some specific DC subsets may be identified through the use of additional fluorescent mice. XCR1-venus mice, originally generated by Yamazaki and colleagues (653) display fluorescence in the XCR1-expressing CD103+ lung DC population (654, 655). Using the brighter fluorophore, GFP, others subsequently generated XCR1<sup>GFP</sup> reporter mice (Generated by Okada, Kaisho et al; Daniels, Muc Immunol, 2015, *In Press*). Therefore, to differentiate the main lung DC populations, the progeny of crossed CD11c<sup>eYFP</sup> and XCR1<sup>GFP</sup> mice reveal CD11b+ and CD103+ DC subsets; CD11b+ DC are solely YFP+ whereas CD103+ DC fluoresce a combined signal of YFP+ and GFP+.
Many cell types from ubiquitously fluorescent mice may be isolated and adoptively transferred into non-fluorescent or differently fluorescent hosts to examine their fate. Fluorophores such as yellow, green, red, or cyan fluorescent proteins, and their numerous derivatives, have been integrated into cell nuclear genomic DNA by knock-in, or bacterial artificial chromosome (BAC) constructs that express fluorescence in the cell cytoplasm (656-659). These fluorophores are either constitutively expressed, or are under the control of specific promoter genes responding to particular stimuli. Cells of interest, including allergy-driving CD4+ T cells and allergy-ameliorating CD8+ T cells, may be sourced from these mice to investigate their roles in allergic airway inflammation. Of particular relevance to allergic disease, 4C13R mice have been developed by William E Paul and colleagues, within which AmCyan and dsRed report the production of IL-4 and IL-13, respectively (660). The production of one or both of these Th2 cytokines has been demonstrated in multiple cell types to varying degrees, evident by fluorescent reporting in T cells, ILC2, nuocytes and innate helper cells, as well as eosinophils and basophils (147, 332, 660-663). This tool is invaluable to further our understanding of the distinct roles of these key Th2-polarising cytokines.

1.4.5.2 Visualising lung inflammatory infiltrates
A key measure of allergic airway inflammation is the infiltration of immune cells into the lung and airway. A useful tool in the clinical setting, bronchoalveolar lavage (BAL) allows for the diagnosis of multiple lung diseases (664, 665). Similarly, experimental allergic airway inflammation can be examined in this way, although mice are usually sacrificed before the BAL procedure is carried out, and lungs are most often harvested for tissue analysis. Single cell suspensions of processed lungs and BAL fluid were classically examined by cytopsin of a small sample onto a slide for microscopic analysis. A Wright-Giemsa or Haematoxylin and Eosin (H&E) stain was then used to manually differentiate inflammatory cells. While this method was sufficient for the identification of eosinophils, lymphocytes, monocytes, and neutrophils, some difficulty is experienced in the differentiation of morphologically similar cells and there was no way to discriminate specific sub-populations of cells.
Recently, an automated method was proposed whereby the cellularity of BAL samples is analysed by flow cytometry (66). Using stains for CD11c, MHC-II, B220, CCR3 and CD3, the authors were able to reliably identify (directly or by exclusion) DCs, alveolar macrophages, eosinophils, neutrophils, T cells and B cells. This method has proven vastly superior to the cytospin technique as cells may be identified more accurately, less user error is involved, a larger volume of sample can be examined, and the analysis of individual samples is much quicker. Adaptations to this BAL flow panel can be made to assess multiple additional cells of interest or to subtype DCs, and lung samples may be examined in the same way.
1.5 Aims of this thesis

The pathogenesis of allergic asthma critically involves the activity of dendritic cells and CD4+ Th2 cells. It has been shown that CTLs are capable of ameliorating allergic airway inflammation in an acute model of disease, however it was not known whether CTLs may be effective long-term, or in the treatment of chronic disease such as that which presents clinically. It was also reported that perforin is crucial to CTL-mediated suppression of acute disease, suggesting that target cell-killing was an important mechanism through which disease progression may be subverted. The critical targets of CTL-mediated killing remained to be elucidated. The hypothesis of this thesis was that CTLs may target allergy-driving cells such as DCs, subsequently reducing the pool of allergen-presenting cells and culminating in the abrogation of CD4+ Th2 cell-mediated allergic disease.

The specific aims of this thesis were:

- To determine the therapeutic potential for CTLs in established allergic airway disease
- To investigate the effects of CTLs on disease-mediating CD4+ T cells during active inflammation
- To elucidate the mechanism of CTL-mediated suppression of allergic disease and identify the cellular targets of CTL killing.
Chapter 2

Materials and Methods
## 2.1 Materials

### 2.1.1 Labware

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical adhesive covers for Real-time PCR</td>
<td>Applied Biosystems, Foster City, CA, USA</td>
</tr>
<tr>
<td>Axygen sterile pipet tips 10, 200 and 1000μL</td>
<td>Axygen Scientific Inc., Union City, CA, USA</td>
</tr>
<tr>
<td>Axygen Micro Tubes 0.6 and 1.7mL</td>
<td></td>
</tr>
<tr>
<td>PCR thin wall tubes; 8 strips &amp; Caps, 0.2mL</td>
<td></td>
</tr>
<tr>
<td>BD Falcon™ polystyrene sterile conical tubes: 15mL and 50mL</td>
<td>BD BioSciences, Bedford, MA, USA</td>
</tr>
<tr>
<td>BD Falcon™ polystyrene sterile 5mL round bottom tubes</td>
<td></td>
</tr>
<tr>
<td>BD Falcon™ polystyrene sterile multiwall tissue culture plates: 6 well and 24 well</td>
<td></td>
</tr>
<tr>
<td>BD Falcon™ polystyrene sterile tissue culture flasks: 250 mL and 600 mL</td>
<td></td>
</tr>
<tr>
<td>Microtest™ U-bottom 96 well plates</td>
<td></td>
</tr>
<tr>
<td>BD Falcon™ polystyrene sterile serological pipettes: 5, 10 and 25 mL</td>
<td></td>
</tr>
<tr>
<td>1 mL tuberculin syringes and 10mL syringes</td>
<td></td>
</tr>
<tr>
<td>PrecisionGlide™ needles: 18, 20, 25 and 27.5 gauge</td>
<td></td>
</tr>
<tr>
<td>BD Falcon™ 70μm nylon cell strainers</td>
<td></td>
</tr>
<tr>
<td>Ultra-Fine™ needle insulin syringes (29G) 0.5 mL</td>
<td></td>
</tr>
<tr>
<td>BD Insyte™ 18G intravascular catheters</td>
<td></td>
</tr>
<tr>
<td>Superfrost™ Plus microscope slides</td>
<td>Biolab Ltd., Auckland, NZ</td>
</tr>
<tr>
<td>Cover Slips 22x22 mm, No. 1 thickness</td>
<td>Biolab Ltd., Auckland, NZ</td>
</tr>
<tr>
<td>Titertube™ Microtubes 0.5mL</td>
<td>Bio-Rad Laboratories, Hercules, CA, USA</td>
</tr>
<tr>
<td>Costar™ Stripette sterile serological pipettes: 5, 10 and 25 mL</td>
<td>Corning Incorporated, Pittston, PA, USA</td>
</tr>
<tr>
<td>Cryo's™ sterile cryotubes 2mL</td>
<td>Greiner Bio-One, Frickenhausen, Germany</td>
</tr>
<tr>
<td>Petri dishes 90mm</td>
<td>Labserv, Auckland, NZ</td>
</tr>
</tbody>
</table>
2.1.2 Reagents and Buffers

2-Mercaptoethanol (2-ME)
A 55mM solution of 2 ME in PBS was purchased from Sigma (St. Louis, MO, USA), which was stored at 4°C.

Agarose
Ultra-Pure™ agarose was purchased from Invitrogen (Auckland, NZ).

Alsever’s Solution
Dextrose (20.5mg/mL), NaCl (4.2mg/mL) and sodium citrate (8.0mg/mL) were purchased from BDH Laboratory Supplies (Poole, England), from which a solution was generated in-house by dissolving the reagents to the concentrations indicated in distilled H₂O. The pH of the solution was adjusted to 6.1 using 1M citric acid also purchased from BDH Laboratory Supplies (Poole, England). The solution was stored at room temperature.

Aluminium Hydroxide (Alum)
Alu-Gel-S alum (research grade) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany) and stored at room temperature.

Ammonium Chloride Tris (ACT) Lysis Buffer
NH₄Cl (0.16M) and Tris-HCl (0.17M) were purchased from Sigma (St Louis, MO, USA) and Merck (Darmstadt, Germany) respectively. Working buffer was prepared directly before use by mixing 9 parts NH₄Cl: 1 part Tris-HCl to yield final concentrations of 0.144M NH₄Cl and 0.017M HCl, and stored at room temperature.

BlueJuice™ Loading Dye
BlueJuice™ was purchased from Invitrogen (Auckland, NZ) and stored at 4°C.
Bovine Serum Albumin (BSA)
Low endotoxin BSA powder was purchased from ICPbio Ltd. (Auckland, NZ) and stored at 4°C.

Diethylpyrocarbonate (DEPC)-Treated Water
RNase-free, DNase-free, pyrogen-free filtered DEPC treated water was purchased from Ambion Inc. (Austin, TX, USA) and stored at room temperature.

Dimethyl Sulfoxide (DMSO)
DMSO was purchased from Sigma (St Louis, MO, USA) and stored at room temperature.

Discombe’s Solution
Discombe’s Solution was prepared by adding 5 volumes acetone to 5 volumes 1% eosin Y (both purchased from Sigma, St. Louis, MO, USA), and made up to 100 volumes in dH₂O, as described by G Discombe(667). Solution was stored at room temperature, protected from light.

DNase I
DNase I lyophilised powder was purchased from Roche (Mannheim, Germany). A working solution of 10mg/mL was prepared by dissolving the powder in IMDM and then stored at -20°C.

Dynabeads® Mouse T-Activator CD3/CD28
Dynabeads® were purchased from GIBCO, Life technologies (Japan).

Ethanol
100% ethanol (molecular grade) was purchased from Carlo Erba Reagents (Milan, Italy) and stored at room temperature.

Ethylendiaminetetraacetic Acid (EDTA)
EDTA powder was purchased from Sigma (St. Louis, MO, USA). Working solution of 0.5M were generated by dissolving in dH₂O and then stored at room temperature.
Fluorescence-activated cell sorting (FACS) Buffer
EDTA, NaN₃ (both from Sigma, St. Louis, MO, USA) and FBS (GIBCO, Invitrogen, Auckland, NZ) were mixed with PBS to generate a working buffer containing 10mM EDTA, 0.01% NaN₃ and 2% FBS and stored at 4°C.

Foetal Bovine Serum (FBS)
FBS (27EU/mL) was purchased from GIBCO (Invitrogen, Auckland, NZ) and stored in 25mL aliquots at -20°C. In-use aliquots were thawed and stored at 4°C for a maximum of 3 weeks.

Formalin Solution
10% neutral buffered formalin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The solution comprises 4% formaldehyde w/v, and was stored at room temperature.

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)
Recombinant GM-CSF (murine) was generated in house by culture of the modified murine X63 cell line in cIMDM. Culture supernatant was collected, filtered and titrated in bone marrow DC cultures to determine the amount required for optimal cell yield. This was stored at -80°C until used, and then stored at 4°C.

House Dust Mite (HDM)
Dermatophagoides pteronyssinus (HDM, crushed whole mite preparation) was purchased from Greer Laboratories (Lenoir, NC, USA) and stored at -80°C until used.

Interleukin 2 (IL-2)
Recombinant IL-2 (human) was generated in house by culture of the IL2L6 cell line. Culture supernatant was collected, filtered and titrated in bone marrow DC cultures to determine the amount required for optimal cell yield. Supernatant was stored at -80°C until used, then stored at 4°C.

Interleukin 4 (IL-4)
Recombinant IL-4 (murine) was produced by culture of a modified Chinese Hamster Ovary
cell line. Culture supernatant was collected, filtered and titrated in bone marrow DC cultures to determine the amount required for optimal cell yield. Supernatant was stored at -80°C until used, then stored at 4°C.

Iscove's Modified Dulbecco's Medium (IMDM)
IMDM supplemented with GlutaMAX, 25mM HEPES buffer and 3.024 mg/L NaHCO₃ was purchased from GIBCO (Invitrogen, Auckland, NZ) and stored at 4°C.

Iscove's Modified Dulbecco's Medium, Complete (cIMDM)
1% Penicillin-Streptomycin (GIBCO, Invitrogen, Auckland, NZ), 55μM 2-ME and 5% FBS were added to IMDM to generate cIMDM, and stored at 4°C for a maximum of 3 weeks.

Isopropanol
Isopropanol (analytical grade) was purchased from Scharlau Chemie (Barcelona, Spain) and stored at room temperature.

Liberase TL
Liberase TL lyophilised powder was purchased from Roche (Mannheim, Germany). A working solution of 1mg/mL was prepared by dissolving in IMDM, and 1mL aliquots were stored at -20°C.

Lipopolysaccharide (LPS)
LPS lyophilised powder from Escherichia coli, serotype 0111:B4, was purchased from Sigma (St. Louis, MO, USA). A working solution of 1mg/mL was prepared by dissolving in IMDM and stored at 4°C.

Neomycin trisulfate salt hydrate
Neomycin powder was purchased from Sigma (St. Louis, MO, USA). Before use, neomycin solution was generated by adding 2mg/mL to autoclaved distilled H₂O, and then stored at room temperature.
Ovalbumin protein (OVA)

Grade V OVA protein lyophilised powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). Powder was reconstituted in PBS to 1mg/mL aliquots, and stored at -20°C.

OVA protein, low endotoxin (EndoGrade OVA)

EndoGrade OVA (endotoxin levels < 0.1 EU/mg) lyophilised powder was purchased from Hyglos GmbH (Bernried, Germany). Powder was reconstituted in PBS to 1mg/mL and 10mg/mL aliquots, and stored at -20°C.

OVA$_{257-264}$ (SIINFEKL)

OVA$_{257-264}$ peptide lyophilised powder was purchased from Mimotopes (Victoria, Australia). The powder was reconstituted in PBS to 1mM, and stored at -20°C.

OVA$_{323-339}$ (ISQAVHAAHAEINEAGR)

OVA$_{323-339}$ peptide lyophilised powder was purchased from Mimotopes (Victoria, Australia). The powder was reconstituted in PBS to 1mM, and stored at -20°C.

Polymerase Chain Reaction (PCR) reagents

TaqMan® expression master mix and TaqMan® primers were purchased from Applied Biosystems (Foster City, CA, USA). The master mix was stored at 4°C and primers were stored at -20°C.

Penicillin-Streptomycin

Penicillin-Streptomycin (liquid state, 10 000 U/mL and 10 000 μg/mL, respectively) was purchased from GIBCO (Invitrogen, Auckland, NZ) and stored at -20°C.

Phosphate Buffered Saline (PBS)

PBS (CaCl$_2$ and MgCl$_2$ free) was purchased from GIBCO (Invitrogen, Auckland, NZ) and stored at room temperature until opened, then stored at 4°C.

RNAlater® solution for RNA stabilisation and storage

RNAlater® was purchased from Ambion (Auckland, NZ) and stored at room temperature.
RPMI Medium 1640 (RPMI)
RPMI supplemented with 4.5 g/L D-glucose, 1.5 g/L Sodium Bicarbonate, 1mM Sodium Pyruvate, 10mM HEPES and 300 mg/L L-Glutamine was purchased from GIBCO (Invitrogen, Auckland, NZ) and stored at 4°C.

Sodium Chloride (NaCl)
NaCl powder was purchased from Sigma (St. Louis, MO, USA). A working solution of 1.8% NaCl was prepared by dissolving powder in dH₂O and stored at room temperature.

SYBR™ Safe DNA Gel Stain
SYBR™ Safe DNA Gel Stain was purchased from Invitrogen (Auckland, NZ). The stain was added to agarose solution during gel preparation. In-use stain solution was stored at 4°C.

Tris-acetate-EDTA (TAE) buffer
TAE buffer containing 2M Tris acetate and 50 mM EDTA was purchased from GIBCO (Invitrogen, Auckland, NZ). A working buffer was prepared by diluting 50x with dH₂O and stored at room temperature.

Trypan Blue Live/Dead exclusion stain
Trypan Blue was purchased from GIBCO, Invitrogen (Auckland, NZ) and stored at room temperature.

2.1.3 Commercial kits

Ambion® DNA-free kit
For the removal of DNA contamination in RNA samples, the DNA-free kit was purchased from Invitrogen (Auckland, NZ). The kit was stored at 4°C.

Bio-Plex Pro™ Mouse Cytokine Group I, 7-plex Assay
For the detection of cytokines in BAL (IL-3, IL-4, IL-5, IL-10, IL-13, TNF-α and IFN-γ), these
cytokines were selected from the Bio-Rad mouse cytokine assay options, and put together in a Bio-Plex kit, purchased from Bio-Rad (Auckland, NZ). The kit was stored at 4°C.

CellTrace™ Violet (CTV) Cell Proliferation Kit
To investigate the proliferation of adoptively transferred T cells, a CTV stain was employed. The kit was purchased from Molecular Probes, Life Technologies (Auckland, NZ) and stored at -20°C, protected from light.

CCL11/Eotaxin Quantikine Kit
The Eotaxin ELISA kit was purchased from R&D (Minneapolis, MN, USA) for the detection of eotaxin in lung and serum samples. The kit was stored at 4°C.

Granzyme B ELISA
For the detection of granzyme B in cell culture supernatants, the granzyme B ELISA kit was purchased from Huntingtree Bioscience Supplies (Auckland, NZ), manufactured by eBioscience. The kit was stored at 4°C.

MCPT-1 (mMCP-1) ELISA
For the detection of mMCP-1 in serum samples, the mMCP-1 ELISA kit was used. This kit was manufactured by eBioscience and purchased from Huntingtree Bioscience Supplies (Auckland, NZ), and stored at 4°C.

Red SR FLIVO™ in vivo Apoptosis Kit
For the visualisation of pre-apoptotic cells in ex vivo lung and MLN samples, FLIVO™ was purchased from ImmunoChemistry Technologies (Bloomington, MN, USA). This kit was stored at 4°C.

Reverse Transcription Kit
SuperScript Vilo™ reverse transcription kit was purchased from Invitrogen (Auckland, NZ) for the reverse transcription of RNA samples extracted from lung tissue, for PCR analysis. The kit was stored at -20°C.
RNA Extraction Kit

NucleoSpin™ RNA L kit was purchased from Macherey-Nagel GmbH (Düren, Germany) for the extraction of RNA from lung tissue, for PCR analysis. The DNase powder was stored at 4°C, DNase solution stored at -20°C, and additional kit components stored at room temperature.

2.1.4 Antibodies and Fluorophores

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Label</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>6B2</td>
<td>PE-Cy7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD3ε</td>
<td>17A2</td>
<td>PE-Cy7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4</td>
<td>GK1.5</td>
<td></td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>RM4-5</td>
<td>APC eFluor 780</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>RM4-5</td>
<td>FITC</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>RM4-5</td>
<td>Pacific Blue</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PerCP</td>
<td>BD</td>
</tr>
<tr>
<td>CD8α</td>
<td>2.43</td>
<td>Alexa Fluor 647</td>
<td>Purified in-house</td>
</tr>
<tr>
<td></td>
<td>53-6.7</td>
<td>Alexa Fluor 700</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>53-6.7</td>
<td>FITC</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>53-6.7</td>
<td>PerCP Cy5.5</td>
<td>BD</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>FITC</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>M1/70</td>
<td>PerCP Cy5.5</td>
<td>BD</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>PE-Cy7</td>
<td>BD</td>
</tr>
<tr>
<td>CD11c</td>
<td>N418</td>
<td>PerCP Cy5.5</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>N418</td>
<td>APC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD19</td>
<td>1D3</td>
<td>APC-H7</td>
<td>BD</td>
</tr>
<tr>
<td>CD44</td>
<td>IM7</td>
<td>Alexa Fluor 700</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>IM7</td>
<td>APC eFluor 780</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>IM7</td>
<td>PE</td>
<td>BD</td>
</tr>
<tr>
<td>CD45</td>
<td>H130</td>
<td>Pacific Blue</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD45.1</td>
<td>A20</td>
<td>APC</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>A20</td>
<td>APC eFluor 780</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>A20</td>
<td>Cy-5</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD45.2</td>
<td>104</td>
<td>APC</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD62L</td>
<td>MEL-14</td>
<td>APC</td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>MEL-14</td>
<td>PE-Cy7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD64</td>
<td>X54-5/7.1</td>
<td>APC</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>X54-5/7.1</td>
<td>PerCP Cy5.5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD69</td>
<td>H1.2F3</td>
<td>Alexa Fluor 488</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>
### 2.1.5 Mice

#### 2.1.5.1 Maintenance and ethical approval

Mice were bred and maintained in the Biomedical Research Unit (BRU) at the Malaghan Institute of Medical Research or RIKEN Center for Integrative Medical Sciences. Sterile laboratory food and water were freely available to mice. Ethics approval was obtained for all experimental protocols from the Victoria University Animal Ethics Committee or RIKEN Animal Research Committee. Experimental procedures were carried out according to institutional guidelines.

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Antibody Code</th>
<th>Manufacturer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1.2F3</td>
<td></td>
<td>BioLegend</td>
<td>PerCP Cy5.5</td>
</tr>
<tr>
<td>CD86</td>
<td>GL1</td>
<td>BD</td>
<td>Pacific Blue</td>
</tr>
<tr>
<td>CD103</td>
<td>M290/M290</td>
<td>BD</td>
<td>Biotin PE</td>
</tr>
<tr>
<td>CCR3</td>
<td>B3103</td>
<td>BD</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>FcγRII/III (CD32/CD16)</td>
<td>2.4G2</td>
<td>BD</td>
<td>None</td>
</tr>
<tr>
<td>Gr-1</td>
<td>RB6-8C5</td>
<td>Invitrogen</td>
<td>Pacific Orange</td>
</tr>
<tr>
<td>ICOS-L</td>
<td>7E.17G9</td>
<td>BioLegend</td>
<td>PE</td>
</tr>
<tr>
<td>Ly6C</td>
<td>HK1.4</td>
<td>BioLegend</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>MHCII</td>
<td>3JP/3JP/M5/114.15.2/M5/114.15.2/3JP</td>
<td>BioLegend and Purified in-house</td>
<td>Alexa Fluor 488/Alexa Fluor 647/APC-Cy7/Pacific Blue/FITC</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>Purified in-house</td>
<td>None</td>
</tr>
<tr>
<td>PD-1</td>
<td>RMP1-30</td>
<td>BioLegend</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>Rabbit anti-GFP</td>
<td>MBL</td>
<td>Life Technologies</td>
<td>Alexa Fluor 488 (goat anti-rabbit IgG)</td>
</tr>
<tr>
<td>Siglec F</td>
<td>E50-2440</td>
<td>BD</td>
<td>PE</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>Streptavidin</td>
<td>BD</td>
<td>PE Texas Red</td>
</tr>
<tr>
<td>Vα2</td>
<td>B20.1/B20.1</td>
<td>BD</td>
<td>APC/FITC</td>
</tr>
<tr>
<td>Vβ5.1,5.2</td>
<td>MR9-4</td>
<td>BD</td>
<td>PE</td>
</tr>
</tbody>
</table>

4,6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) cell viability dye

For the exclusion of dead cells by flow cytometric analysis, DAPI lyophilised powder was purchased from Invitrogen (Auckland, NZ) and diluted in dH₂O to a final concentration of 200μg/mL. This was stored at 4°C.
2.1.5.2 Mouse Strains

Mouse strains were maintained by sibling breeding pairs. For all experiments mice were sex matched and 6-8 weeks old at the beginning of the experiments, with the exception of chimeric mice that were 12 weeks old upon chimera generation and used for experiments 8 weeks after that, to allow for regeneration of immune cells. All mice were housed in conventional caging, except chimeric mice, which were housed in individually ventilated cages to reduce the risk of infection.

C57BL/6 mice breeding pairs were obtained from the Jackson Laboratories (Bar Harbour, ME, USA). Cells from these mice express CD45.2 only.

CD45 congenic B6.SJL-Ptprc<sup>a</sup> Pepc<sup>c</sup>/BoyJ (B6-SJ) mice were originally from the Jackson Laboratory (Bar Harbor, ME, USA). Cells from these congenic mice express CD45.1.

OT-I and OT-II mice were obtained from Dr Sarah Hook, School of Pharmacy, Dunedin, NZ with the permission of Prof Frank Carbone, Melbourne University, Australia. These mice have T cells that express Vα2<sup>+</sup>Vβ5.1/5.2<sup>+</sup> TCRs that are specific for OVA<sub>257-264</sub> presented on H2K<sup>b</sup> (OT-I) and OVA<sub>323-339</sub> presented on I-A<sup>b</sup> (OT-II). These mice were crossed with B6 mice at the Malaghan Institute of Medical Research to produce congenic OT-I x B6.SJL-Ptprc<sup>a</sup> and OT-II x B6.SJL-Ptprc<sup>a</sup> mice.

4C13R mice were generously gifted by Dr William Paul, Laboratory of Immunology, NIH, USA. These mice were generated by use of a bacterial artificial chromosome (BAC) construct that expresses AmCyan during IL-4 production and dsRed when IL-13 is produced, as described by Roediger et al (Roediger et al, 2013). These mice are IL-4 and IL-13 sufficient. 4C13R mice were crossed with OT-II at the Malaghan Institute of Medical Research to generate mice with OVA<sub>323-339</sub> specific CD4+ T cells that report production of IL-4 and IL-13.

OT-I mice were crossed to C57BL/6-Prf1<sup>tm1Sdz</sup>/J mice to generate PKO OTI, or to B6;129S6-Gt(ROSA)26Sor<sup>tm14(CAG-tdTomato)Hze</sup>/J, and CAG-Cre mice to generate tdTomato+ OT-I.
XCR1\textsuperscript{GFP} mice were generated by Dr Takaharu Okada and Dr Tsuneyasu Kaisho and colleagues, at the RIKEN Center for Integrative Medical Sciences, Japan. For the generation of XCR1\textsuperscript{GFP} mice: A targeting vector was designed to replace the mouse Xcr1 coding sequences with a gene encoding GFP followed by a polyadenylation signal derived from bGHpA. A neomycin resistance gene (neo) driven by the MC1 promoter and flanked by the yeast FRT sequences was used as a selection marker. An HSV thymidine kinase gene (HSV-TK) was inserted for negative selection. C57BL/6J ES cells were transfected with the linearized targeting vector by electroporation and selected with G418. Then, correctly targeted clones were aggregated with BALB/c morulas. Chimeric male offspring were mated with C57BL/6J female mice. XCR1\textsuperscript{GFP}CD11c\textsuperscript{YFP} double positive mice were generated by crossing XCR1\textsuperscript{GFP} mice with established CD11c\textsuperscript{YFP} mice.

Foxp3\textsuperscript{GFP} breeding pairs were obtained from Prof. Alexander Y Rudensky, University of Washington, USA. Mice express enhanced green fluorescent protein fused to fully functional Foxp3 proteins upon translation of the Foxp3 gene. These mice were crossed with OT-II mice at the Malaghan Institute of Medical Research to yield mice that reported Foxp3 expression in OVA\textsubscript{323-339} specific CD4+ T cells.

Bone marrow from C57BL/6.H2K\textsuperscript{bm-1} (bm-1) mice was kindly provided by A. Prof. Alexander McLellan at the University of Otago, Dunedin, NZ. bm-1 mice have a mutation in the H-2K molecule rendering their cells unable to present OVA\textsubscript{257-264}. They are consequently unable to interact with OT-I cells, however they express wild type MHCII, thus retaining the ability to present OVA\textsubscript{323-339} to OT-II cells. Bone marrow was extracted from mice and frozen on site, then transported to the Malaghan Institute of Medical Research for the generation of bone marrow chimeras.
2.2 Methods

2.2.1 In vitro methods

Cell culture
All cultures were carried out at 37°C with 5% CO2 and 95% humidity.

2.2.1.1 Bone marrow dendritic cell (BMDC) culture
The femurs and tibias of euthanised C57BL/6 mice were removed, separated from surrounding tissue and briefly immersed in ethanol to sterilise. The ends of the bones were cut and bone marrow flushed out with IMDM using a 25 gauge needle and 10mL syringe. Bone marrow was collected in a 50mL tube, vortexed to disrupt clumps, and then filtered through a 70μm cell strainer. The solution was centrifuged at 250 g for 10 minutes to pellet the BM cells. The supernatant was removed and cells resuspended in 10mL IMDM; 10μL of this suspension was added to 90μL trypan blue and a sample loaded onto a haemocytometer to manually count live cells. Cells were again pelleted and resuspended in cIMDM, then plated at 0.4 x 10^6 cells/mL in 6-well plates. GM-CSF and IL-4 were added to the culture to final concentrations of 10ng/mL and 20ng/mL respectively. Cells were cultured at 37°C for 7 days. Nutrients were replenished on day 3 and 5 by replacing 2mL of culture supernatant with fresh cIMDM supplemented with GM-CSF and IL-4 to final concentrations of 10ng/mL and 20ng/mL respectively. During the last 24 hours of culture, 100ng/mL LPS was added to activate BMDC. For co-culture with OT-I or OT-II cells, BMDC were harvested from culture plates by lightly flushing the well contents using IMDM in a 10mL pipette. The cells were pelleted and resuspended at 1 x 10^6/mL and incubated with 0.1μM OVA_{257-264} or 0.01μM OVA_{323-339} peptide (for OT-I or OT-II respectively) for 4 hours at 37°C.

2.2.1.2 OT-I culture, BMDC stimulated
To generate OT-I CTLs, lymph nodes (axial, brachial, inguinal, cervical) were removed from OT-I.B6 or PKO OT-I mice. LNs were mashed with a syringe plunger and flushed with IMDM through a 70μm cell strainer into a 50mL tube. Cells were washed by centrifugation at 250g
for 10 minutes and resuspension of pelleted cells in fresh IMDM. A 10µL sample of this suspension was added to 90µL trypan blue and a sample loaded onto a haemocytometer to manually count live cells. Cells were again pelleted, and resuspended in cIMDM. Cells were co-cultured at 0.5 x 10^6/mL with 0.0625 x 10^6/mL OVA_{257-264}-loaded BMDCs in 6-well plates, incubated at 37°C for 4 days. On day 5, cells were harvested from culture plates by lightly flushing the well contents using IMDM and a 10mL pipette. Cells were centrifuged at 250 g for 10 minutes and resuspended in fresh cIMDM, counted on a haemocytometer, and adjusted to a concentration of 0.25 x 10^6 cells/mL. Cells were then cultured in sterile tissue culture flasks (250mL or 600mL) for 2 further days, with 100U/mL IL-2 were added to the culture each day.

2.2.1.3 OT-II culture, BMDC stimulated
To generate OT-II CD4+ Th2 cells, lymph nodes (axial, brachial, inguinal, cervical) were removed from OTII.B6 or 4C13R.OT-II mice. LNs were mashed with a syringe plunger and flushed with IMDM through a 70µm cell strainer into a 50mL tube. Cells were washed by centrifugation at 250 g for 10 minutes and resuspension of pelleted cells in fresh IMDM. A 10µL sample of this suspension was added to 90µL trypan blue and a sample loaded onto a haemocytometer to manually count live cells. Cells were again pelleted, and resuspended in cIMDM. Cells were co-cultured at 0.8 x 10^6/mL with 0.2 x 10^6/mL OVA_{323-339}–loaded BMDCs in 6-well plates. IL-2 and IL-4 were added to the culture at final concentrations of 20ng/mL and 62ng/mL respectively, and then incubated at 37°C for 5 days. Nutrients were replenished on day 2 and 4 by replacing 2mL of culture supernatant with fresh cIMDM supplemented with IL-2 and IL-4 (final concentrations of 20ng/mL and 62ng/mL respectively).

For experiments where the proliferation of CD4+ T cells in vivo was examined, 5-day cultured cells were stained with CellTrace™ Violet as per the manufacturer’s instructions. Briefly, cells were incubated in a 10µM solution of CellTrace™ Violet for 20 minutes at 37°C and then washed in cold PBS with 10% FCS. Cells were then manually counted for viability using a haemocytometer.
2.2.1.4 OT-I and OT-II culture, bead stimulated

Cell suspensions were obtained from LNs as previously described. OT-I or OT-II cells were resuspended at 5 x 10^6 cells/mL in RPMI media, with anti-CD3/CD28 T cell activating Dynabeads at a concentration of 0.2 x 10^6 beads/mL. Cells and beads were co-cultured in 90mm petri dishes (10mL per dish) for 5 days. Both cultures were supplemented with 20ng/ml IL-2, OT-II culture was also supplemented with 62ng/ml IL-4. On day 3, cultures were split by harvesting the 10mL of cells from each petri dish and resuspending them in 20mL RPMI with 20ng/ml IL-2 (OT-I and OT-II) and 62ng/ml IL-4 (OT-II only).

2.2.2 In vivo methods

2.2.2.1 In vivo sensitisation model of airway inflammation

C57BL/6 mice were sensitised by intraperitoneal injection of 2µg OVA (Grade V, Sigma-Aldrich) in 200µl (1.36mg) alum adjuvant on days 0 and 14. Some groups of mice received 5 x 10^6 OT-I CTLs (cultured as described in 2.2.1.2), injected into the lateral tail vein, on either day 23, 30, 37 or 44. In some experiments, mice were treated with αGalCer drug conjugates in the place of CTLs. On days 24, 31, 38, and in some experiments, days 45 and 52, 100µg low endotoxin OVA in 50µl PBS was administered intranasally to systemically anaesthetised mice. The solution was given drop-wise to a single nostril through a 200µL pipette tip. Control mice received 50µL PBS only. Mice were sacrificed by anaesthetic overdose 1 and 3 days after the final intranasal challenge and tissues were harvested for analysis. For time-course experiments, mice were sacrificed 1, 2, 3, 6 and 9 days after the final intranasal challenge. For experiments where apoptotic cells were identified, 100µl of FLIVO™, reconstituted as per kit instructions, was injected into the tail vein of each mouse 60 minutes before mice were sacrificed.

2.2.2.2 Adoptive transfer model of airway inflammation

OT-I CTLs and OT-II CD4+ T cells were cultured as described (see 2.2.1.2 and 2.2.1.3). Cultured cells were harvested and washed thrice by centrifugation of cells at 250g, followed by removal of supernatant and resuspension in sterile PBS. 5 x 10^6 CD4+ T cells with or
without 5 x 10^6 CTLs in a final volume of 200µL PBS were injected into the lateral tail vein of C57BL/6 mice on day -1. On day 0, a single challenge of 100µg low endotoxin OVA in 50µl PBS was administered intranasally, as previously described. Control mice received 50µL PBS only. On days 1 and 3, mice were sacrificed by anaesthetic overdose and tissues were harvested for analysis.

### 2.2.2.3 House Dust Mite model of airway inflammation

C57BL/6 mice were sensitised by intranasal instillation of 100µg HDM in 50µL PBS on day 0. Challenges were carried out on days 14, 15 and 16 by intranasal administration of 25µg HDM in 50µL PBS. Control mice received 50µL PBS only. On day 17, mice were sacrificed by anaesthetic overdose and tissues were harvested for analysis.

### 2.2.2.4 Generation of mixed bone marrow chimeras

Bone marrow was obtained as previously described (see 2.2.1.1) from naïve bm-1 and naïve wt B6 donor mice. Naïve recipient B6 mice (12 weeks old) were depleted of CD4+ and CD8+ T cells as well as NK cells by intraperitoneal injection of anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43) and anti-NK1.1 (clone PK136) (all generated in-house), 200µg of each in a total volume of 200µl PBS, on day-1 and fasted overnight. On day 0, recipient mice were irradiated with two rounds of 550 Rads, 3 hours apart. Immediately after the second round of irradiation, a mixture of 6 x 10^6 bm-1 and 4 x 10^6 B6 bone marrow cells in 200µL IMDM were injected into the lateral tail vein of recipient mice. A further CD4+, CD8+ T cell and NK cell depletion was carried out on day 1. Mice were maintained on 2mg/mL neomycin supplemented autoclaved distilled water and housed in sterile Individual Ventilated Cages. Eight weeks was allowed for regeneration of immune cell populations in recipient mice, during which time mice were closely monitored for signs of bone marrow rejection. Mice were then tested for success of bone marrow chimerism by taking peripheral blood samples from the tail vein and analysing the %bm-1 (CD45.2+) and %wt B6 (CD45.1+) of the white blood cells by flow cytometry. Mice were excluded from experiments if chimerism was not achieved – defined as more than 60% of cells originating from one bone marrow source.
The adoptive transfer model of airway inflammation was then tested in chimeric mice (as described in 2.2.2.2).

2.2.2.5 Testing of αGalCer Drug Conjugates in airway inflammation models

Drug conjugates were developed through collaboration by Dr Ian Hermans at the Malaghan Institute of Medical Research, Wellington, NZ, with Dr Regan Anderson and Dr Gavin Painter at the Ferrier Research Institute, Victoria University of Wellington, NZ, as described (668). Briefly, CN152 consisted of αGalCer conjugated to SIINFEKL peptide and CN178 consisted of αGalCer conjugated to gp33. An unconjugated mix of αGalCer and SIINFEKL peptide was used as a control. For experiments, the molecular equivalent of 500ng SIINFEKL peptide was administered for each drug conjugate; 1360ng for CN152, 1400ng for CN178 and 445ng αGalCer + 500ng SIINFEKL (unconjugated). The respective amounts of each compound were diluted in 200μL PBS and injected into the lateral tail vein of mice. Drug conjugates were tested in place of CTLs in the in vivo sensitisation model as well as the HDM model of airway inflammation (described in 2.2.2.1 and 2.2.2.3).

2.2.3 Ex vivo methods

2.2.3.1 Processing of tissues

2.2.3.1.1 Bronchoalveolar Lavage

To perform BAL, mice were administered a lethal overdose of anaesthetic, and an incision was made into the skin covering the lungs, cutting upwards towards the head. The skin was pulled back and tissue covering the trachea removed. A small hole was cut into the trachea and an 18G catheter tube (needle removed) attached to a 1mL syringe of PBS was inserted into the hole towards the lungs. BAL was performed by flushing PBS into, and out of, the lungs three times. BAL fluid was then collected in a 15mL tube, recovery volume recorded, and kept on ice. Tubes were centrifuged at 250 g for 5 minutes to pellet cells. Supernatant was collected in 2mL cryotubes and stored at -20°C for later cytokine analysis. Cells were
resuspended in FACS buffer, manually counted on a haemocytometer and then stained for FACS analysis.

2.2.3.1.2 Lungs

Preparation of single cell suspensions
After BAL, lungs were removed from mice and placed in individual wells of a 24-well plate, each well containing 1mL IMDM with 0.5mg Liberase TL and 0.5mg DNase I. Tissue was finely diced with scissors and then incubated for 45 min at 37°C. In the last 5 minutes of incubation, 20μL of 0.5M EDTA was added to halt digestion. The suspension was passed through an 18G needle (attached to 5mL syringe) to break down remaining tissue fragments and filtered through a 70μm cell strainer into 50mL tubes. Cells were pelleted by centrifugation at 250 $g$ for 5 minutes, and resuspended in 3mL ACT buffer. Tubes were incubated for 4 minutes at 37°C to lyse red blood cells, then neutralised by adding 7mL PBS. Cells were pelleted again and resuspended in FACS buffer. Total cells were counted manually on a haemocytometer, and samples were then kept on ice and stained for FACS analysis.

Preparation of sections for histological analysis
After BAL was performed, lungs were removed and fixed by immersion in 10% formalin. Both lobes of the lung were then embedded in paraffin wax. Airway histology was examined by staining of 4μm thick sections on microscope slides with Haemotoxylin and Eosin (H & E), mucus-producing goblet cells were detected by Alcian Blue and Periodic Acid-Schiff (AB-PAS) stain, mast cells were visualized with Chloracetate Esterase (CAE) stain and collagen deposition was identified using a Masson’s Trichrome stain. Slides were examined and photographed using the Olympus BX51 compound microscope.

Preparation of sections for fluorescent and confocal microscopy
Unstained fresh tissue sections (from inherently fluorescent XCR1GFP mice) were prepared by manually cutting thin sections of lungs using a sterile scalpel blade. Sections were then placed in a petri dish and immersed in PBS, then directly examined using a the BZ-9000 (Keyence) fluorescent microscope.
Fluorescently labelled sections were prepared by fixing lungs in 4% paraformaldehyde for 2 h. Fixed tissues were washed with PBS for 3 h, placed in Tissue-Tek OCT compound (Sakura), and snap-frozen in dry ice and ethanol. Cryosections 12µm in thickness were affixed to MAS-GP-coated slides (Matsunami Glass) and stained at room temperature for 2 h with APC-conjugated anti-CD11c (clone N418, eBioscience) and rabbit anti-GFP (MBL), which was detected by Alexa Fluor 488-conjugated goat anti–rabbit IgG (Life Technologies). Nuclei were stained with 3µM DAPI (Sigma-Aldrich). Images were acquired on BZ-9000 (Keyence, Osaka, Japan), and processed with Pixelmator (2.1.2, Pixelmator Team Ltd).

Confocal microscopy was carried out on fresh tissue sections prepared as described. Whole sections were stained with Cy5-conjugated anti-CD45.1 (clone A20, eBioscience), and imaged with the TCS SP5 confocal laser microscope (Leica Microsystems) by collecting emission signals of 499-514 nm (488 nm excitation), 519-540 nm (488 nm excitation), 574-622 nm (568 nm excitation), and 639-680 nm (647 nm excitation).

2.2.3.1.3 Lymph nodes
Lung-draining mediastinal lymph nodes were removed from sacrificed mice and collected in 24-well plates containing 1mL IMDM, 0.1mg Liberase TL and 100µg DNase. To extract DCs, LNs were disrupted by teasing apart with 18G needles, and digestion was carried out for 25 minutes at 37°C. Digestion was stopped by adding 20µL of 0.5M EDTA to each well. LNs were then mashed through 70µm cell strainers using a syringe plunger, and collected in 50mL tubes. Cells were washed by centrifugation at 250 g and resuspension in FACS buffer. Cells were then manually counted using a haemocytometer, kept on ice and stained for flow cytometry analysis.

2.2.3.1.4 Spleen
Spleens were removed from sacrificed mice and collected in 24-well plates containing 1mL IMDM, 0.1mg Liberase TL and 100µg DNase in each well. The solution was injected into spleens to aid the release of DCs from the parenchyma, and digestion carried out for 25 minutes at 37°C. Digestion was stopped by adding 20µL of 0.5M EDTA to each well. Spleens
were then mashed through 70μm cell strainers using a syringe plunger, and collected in 50mL tubes. Cells were centrifuged at 250 g and resuspended in 3mL ACT buffer to lyse red blood cells. Tubes were incubated for 4 minutes at 37°C, then neutralised by adding 7mL PBS. Cells were pelleted again and resuspended in FACS buffer. Total cells were counted manually on a haemocytometer, and samples were then kept on ice and stained for FACS analysis.

2.2.3.1.5 Blood
Blood was obtained from the lateral tail vein of live, restrained mice, by nicking the tail vein with a sterile scalpel blade and collecting drops of blood. Alternatively, submandibular bleeding was carried out by piercing the facial vein with a sterile scalpel blade and collection of drops of blood. From sacrificed mice, the descending aorta was punctured by a 27G needle and blood was drawn into a 1mL syringe.

Whole blood
Blood was immediately added to 500μL Alsever's Solution in 1.7mL tubes, to prevent clotting. Samples were centrifuged at 420 g for 2 minutes, supernatant removed, and resuspended in 1mL ACT buffer to lyse red blood cells. Tubes were incubated at 37°C for 4 minutes then again pelleted. Lysis was repeated if many red blood cells remained. Cells were then resuspended in FACS buffer, stored on ice, and stained for FACS analysis.

Serum
Blood was collected directly into empty 1.7mL tubes, left overnight at 4°C to clot, and centrifuged the next morning at 420 g for 2 minutes. Serum was removed by pipetting and transferred into a fresh 1.7mL tube, stored at -20°C until analysed.

2.2.3.2 Flow Cytometry

2.2.3.2.1 Staining of cells
Single cell suspensions were obtained from tissues as described (see 2.2.3.1) and 0.5 – 2 x 10^6 cells were transferred into a 96-well plate. Plates were centrifuged at 320 g for 2
minutes and then flicked to remove supernatant. Cells were resuspended by light vortexing of plates, and then incubated with 2.4G2 antibody for 10 minutes on ice to block non-specific binding. Fluorescent antibody mixes were added (at dilutions determined by titration) to the wells, and plates incubated for an additional 10 minutes on ice, protected from light. Cells were then washed, by pelleting and resuspension in FACS buffer, to remove unbound antibodies. Where secondary fluorescent antibodies were required, these were then added and plates incubated for a further 10 minutes on ice, protected from light. Cells were then washed twice and individual samples resuspended in 200μL FACS buffer with 0.008μg/mL of the cell viability dye DAPI.

2.2.3.2.2 Staining of controls
Single stain compensation controls were generated using OneComp eBeads (eBiosciences, Huntingtree Bioscience Supplies, Auckland, NZ). Separate tubes of eBeads were stained with a single fluorescent antibody (according to experiment) in FACS buffer, at a 10-fold dilution to that used to stain cells. Tubes were incubated for 10 minutes at room temperature, protected from light. Cells were then pelleted and resuspended in 300μL FACS buffer.

Fluorescence minus one (FMO) controls were prepared by excluding one of the antibodies from the complete antibody panel, and staining a sample of the cells of interest. Cells were stained as described in 2.2.3.2.1.

2.2.3.2.3 Acquisition and analysis
Acquisition of data was carried out using a LSRII SORP or LSR Fortessa flow cytometer (Beckton-Dickinson, San Jose, CA, USA) and analysis was performed using FlowJo software (version 9.6.1, Treestar Inc., CA, USA). Unstained cells were used to set channel voltages and single stained controls were used to compensate for any spectral overlap occurring between fluorophores. FMO controls were used to identify positively stained populations where this population was not easily defined (due to weakly positive cells, or a rise in background fluorescence). Doublets were excluded by gating out aberrant cells on a forward scatter (FSC) area versus height plot and subsequently on a side scatter (SSC) area versus height plot. Dead cells were excluded by their uptake of DAPI viability dye, revealing
live cells that were used for all further population discriminations.

2.2.3.3 Real-time PCR for analysis of gene expression

2.2.3.3.1 Extraction of RNA

RNA was extracted using the NucleoSpin™ RNA L kit (Macherey-Nagel GmbH), according to the manufacturers instructions. In brief, tissue was disrupted by use of a homogeniser and cells lysed by incubation in a chaotropic ion lysis buffer, which also inactivates RNases. The cell solution is then centrifuged through a filter tube, where RNA remains attached to the filter membrane. An rDNase solution is applied to the membrane to remove contaminating DNA and two additional buffers are used to remove salts, metabolites and large cellular components. RNase-free water is finally added to elute RNA through the membrane.

The concentration and quality of RNA samples were then measured on a ND-100 spectrophotometer by absorption at 230, 260, 280 and 340nm. The 260:280 and 260:230 ratios were analysed, with a ratio greater than 2 revealing contamination of the sample from DNA or protein, respectively, while absorption at 340nm should be zero.

Purity was also determined by running RNA on a 2% agarose gel. Agarose was mixed with TAE buffer and microwaved until dissolved. SYBR™ Safe DNA Gel Stain was then added and gel left to solidify with a comb inserted to produce microwell chambers for sample loading. A 1kbp DNA size ladder (Invitrogen, Auckland, NZ), and RNA samples (2μg) mixed with BlueJuice™ loading dye were pipetted into microwells in the gel. Gel electrophoresis was then carried out at 100V for 45 minutes. The gel was then place in a Bio-Rad Gel Doc imaging system to visualise RNA. Samples showed clear bands on the gel for 18S and 28S ribosomal RNA; DNA contamination was visible by the appearance of additional bands, or by samples being stuck in the loading chamber.
2.2.3.3.2 DNase digestion
Additional DNase treatment was carried out on contaminated samples (260:280 ratio < 2, additional bands on agarose gel) using the Ambion® DNA-free kit. In brief, DNase was added to RNA and incubated at 37°C for 30 minutes to digest DNA. The reaction was then stopped and samples were centrifuged. Supernatant was collected (containing RNA) and transferred to fresh tubes, and re-analysed for concentration and purity using the spectrophotometer and agarose gel.

2.2.3.3.3 Reverse transcription
RNA samples were then reverse transcribed into cDNA by use of the SuperScript Vilo™ reverse transcription kit, according to the manufacturer’s instructions. Briefly, 10μg RNA samples were added to the kit's reaction mix, enzyme mix, and made up to 20μL with DEPC-treated H2O. Samples were added to PCR 8-strips and reverse transcription carried out for 60 minutes at 42°C, and then a 5 minute enzyme inactivation step at 85°C. cDNA samples were then stored at -80°C until RT-PCR was performed.

2.2.3.3.4 Real-time PCR
All RT-PCR was carried out using Taqman expression master mix and Taqman primers (Applied Biosystems, Foster City, CA, USA), and run on the Applied Biosystems 7500 Fast Real-Time PCR System. Primers and probes (lyophilised) were reconstituted and combined with the master mix in 96-well PCR plates. RNA samples (20 – 100ng) were added to the wells and DEPC-treated water was used to make up a final volume of 25μL. RT-PCR was then carried out by incubation at 50°C for 2 minutes and 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold (CT) value for each primer was determined by the system software, from which ΔCT values were manually calculated by normalising samples to the 18S control and comparing with the PBS-treated controls.

2.2.3.3.5 Primers for Real-time PCR
All primers and probes were TaqMan® Gene Expression Assays, purchased from Applied Biosystems (Foster City, CA, USA).
### Table 2.3: RT-PCR Primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S ribosomal RNA</td>
<td>Mm03928990_g1</td>
</tr>
<tr>
<td>Matrix metalloproteinase 9 (Mmp-9)</td>
<td>Mm00442991_m1</td>
</tr>
<tr>
<td>Tenascin-c (Tnc)</td>
<td>Mm00495662_m1</td>
</tr>
<tr>
<td>Acidic mammalian chitinase (AMcase)</td>
<td>Mm00458221_m1</td>
</tr>
<tr>
<td>Mucin 5, subtypes A and C (Muc5ac)</td>
<td>Mm01276718_m1</td>
</tr>
</tbody>
</table>

#### 2.2.3.4 ELISA assays

ELISA kits were used to measure serum IgE (generated in-house by coating 96-microwell plates with 0.5µg/well anti-IgE (clone 6HD5) capture antibody), mMCP-1 (Huntingtree Bioscience Supplies, Auckland, NZ), and eotaxin (R&D, Minneapolis, MN, USA), according to the manufacturer’s instructions. Briefly, 96-microwell plates coated with capture antibodies for the analyte of interest were blocked with 10% FBS/PBS for 2 hours. Serum was diluted 1:2 and 1:10 and added to the plates to incubate overnight at 4°C. Detection antibody was incubated in wells for 2 hours, then streptavidin HRP was then added for 1 hour and colour developed by addition of substrate reagent, with reaction stopped by 0.25M H₂SO₄. Absorbance was measured at 450nm on the Infinite M1000 Pro plate reader (Tecan).

#### 2.2.3.5 Cytokine analysis assays

Cytokines in BAL were measured using a Bio-Plex Pro™ Mouse Cytokine Group I, 7-plex Assay (Bio-Rad, Auckland, NZ), according to the manufacturer’s instructions. Briefly, 96-microwell filter plates were pre-wet with assay buffer, and samples and standards were then added to the plate. Cytokine detection beads were added to each well and incubated on a plate shaker, protected from light. Plates were then washed, with liquid removed using a vacuum manifold (Bio-Rad, Hercules, CA, USA). A biotinylated detection antibody mix was added to each well, again incubated on a plate shaker. Streptavidin-PE was then used to stain samples. After washing the plate three times, samples were transferred to a flat-bottom 96-well plate and analysed on a Bio-Plex™ Suspension Array System (Bio-Rad,
The system software automatically calculated the concentration of the cytokines in each sample by comparison with the kit standards.

### 2.2.3.6 Enumeration of eosinophils in peripheral blood

Blood was taken by abrasion of the lateral tail vein of mice with a sterile scalpel blade and collection of a 5μl drop using a pipette. The blood was immediately mixed with 45μl Discombe’s staining solution. Bright orange stained eosinophils were counted manually using a haemocytometer.

### 2.2.4 Methods of data analysis

#### 2.2.4.1 Data analysis software

FlowJo software (version 9.6.1, Treestar Inc., CA, USA) was used to analyse cell populations by flow cytometry. Each population was calculated as a percentage of live cells and tabled using the software. The data was then copied into Microsoft Excel 2011 for Mac OS (Microsoft Corporation, Washington, USA) and GraphPad Prism 5.0 Macintosh Version (GraphPad Software Inc., California, USA) to generate graphs and carry out statistical analysis.

#### 2.2.4.2 Calculation of cell numbers

The total live cell number in each sample was counted manually by trypan blue exclusion as previously described (see 2.2.1). The percentage of each cell population within a sample was determined by flow cytometry. To then calculate the number of cells of each population, the percentage was used to determine cell number from the manual total live cell count.

#### 2.2.4.3 Determination of representative histology

All stained histological specimens were examined thoroughly, with at least 3 consecutive sections of tissue examined per sample. Each section was labeled with a reference number, rather than treatment group, to enable blinded analysis of the tissue. Photographs were
then taken (as included in figures), that accurately depicted the most appropriate field of view, representative of the trend across the whole tissue. Some stained sections were also examined externally by a Clinical Pathologist, blinded to treatment groups, and graded according to extent of pathology; this was found to correlate well with the measurable inflammation reported by graphical enumeration of lung cellular infiltrates in this thesis, therefore validating the selection of representative histology.

### 2.2.4.4 Statistical analysis

Data was tested for normality using the D’Agostino-Pearson omnibus test. Results showed that the percentages of different cell types in BAL, mast cell numbers in histological sections of lung, and cytokine concentrations in BAL were found to be consistent with a Gaussian distribution (Appendix A). Experimental data from similar experiments to those found to show Gaussian distribution were analysed for statistical significance. Where two groups were compared, analysis was conducted using a Student’s t-test. In experiments where three or more groups were compared, one-way Analysis of Variance (ANOVA) with Bonferroni’s post test was used. In experiments that had small sample sizes, Gaussian distribution could not be determined; these data were compared using the Kruskal-Wallis test with Dunn’s post test (does not assume normal distribution). Bonferroni and Dunn’s post tests were used to correct for errors introduced when comparing multiple groups. Mean and standard deviation (SD), or standard error (SE), are reported as used in figures.
Chapter 3

Allergen-specific CTLs have preventative and therapeutic effects in a multiple allergen exposure model of allergic airway inflammation
3.1 Introduction

Previous work from our Lab (described in section 1.3, Chapter 1) showed that in an acute model of allergic airway inflammation, allergen-specific CTLs, administered to sensitised mice, can prevent airway inflammation upon allergen exposure (226). It has been reported, however, that mice exposed to multiple airway allergen challenges over time may generate a lung-resident population of memory Th2 cells (669). As such, it was not known whether CTLs may be effective in the suppression of allergic airway inflammation in a model of multiple allergen exposures (administered at time intervals), where established disease is generated in the airway prior to treatment with CTLs.

The hallmarks of allergic airway inflammation can be generated experimentally using in vivo sensitisation models that vary considerably in the literature. The type and amount of allergen, the number of allergen exposures, as well as the use of adjuvants may all be modified; the characteristic features of disease differ accordingly, as does the resolution phase (500, 610, 670). The pathological characteristics of a particular model, once defined, serve as a platform for assessing the effects of treatment interventions.

The complexity of the allergic immune response is such that increasing amounts of allergen or additional allergen exposures do not necessarily result in a chronic condition. Additional challenges may lead to increased tolerance (646) or chronicity (618, 632, 645), depending on the immune environment. As such, key pathological features of disease are examined including the extent of airway histopathology and remodelling, and the induction of mastocytosis. A number of factors contribute to airway remodelling, defined broadly as changes in the composition and organisation of the airway wall. Structural changes include goblet cell hyperplasia, subepithelial fibrosis, increased airway smooth muscle (ASM) mass, proliferation of blood vessels, airway oedema and tissue destruction, primarily leading to thickening of the airway wall (560-562). The extent of mastocytosis may also be indicative of the development of chronic disease (443, 541).

In the model of acute asthma described by Enomoto and colleagues, it was shown that CTLs require perforin to mediate amelioration of allergic airway inflammation (226), suggesting
the killing of target cells is a key mechanism of suppression. Whether this approach would be equally effective in established disease was not known. While the airway pathology of persistent allergic inflammation is vastly different from early acute disease, the immunology, in terms of the cells crucially involved in effector responses, has common characteristics.

Central and effector memory T cells are developed during sensitisation, resulting in lymph node- and lung-resident allergen-specific T cells that rapidly respond to challenge both in the early and later phases of disease. Upon successive allergen challenges, more widespread pathology is generated (272-276). The long-term persistence of allergen-specific memory T cells in the tissue during allergy, however, has not been fully elucidated. These cells are activated by lung-resident APCs upon exposure to allergen. It was initially thought that while naïve cells required professional APCs such as DC to induce priming, the recall response of primed cells could be induced by any MHC-II expressing APC (671-673). While this is not untrue, it has recently been found that the induction of allergic responses during the challenge phase is also critically dependent on allergen presentation by DCs (321, 493).

The investigation of the role of the airway epithelium and innate cells such as ILC2 in allergic asthma originally revealed their importance in the initiation of the allergic response (332, 334, 336), as discussed in section 1.2. In recent months, however, these cells have also been implicated in the progression of allergic disease to chronicity; mediating persistence of multiple features of immunopathology through interrelated feedback loops involving epithelial-derived IL-33 and ILC2-derived IL-13(674). Substantial collaborative interplay between innate cells and Th2 cells has also been described in the exacerbations of allergic asthma (675).

Perhaps a significant difference between acute and established allergic disease in humans is the magnitude of the immediate hypersensitivity response mediated by allergen-specific IgE. Generated through the sensitisation phase, IgE persists throughout the progression of disease. Over successive allergen exposures, however, IgE is generated to multiple epitopes of the specific allergen and higher titres of IgE with greater affinity for the allergen are produced (676). Together, the increased complexity, affinity and concentration of allergen-
specific IgE correlates with increased efficiency of facilitated antigen presentation (FAP); translating to strengthened T-cell proliferative responses as disease progresses (677).

Collectively, these results reveal that while higher affinity responses may develop through the progression of disease, critical cell populations including innate cells, DCs and CD4+ Th2 cells are implicated in allergic exacerbations in both the early and later stages of disease. It is possible then, that the mechanism of CTL-mediated suppression - presumably the targeting of critical allergy-driving cells - may be as effective in established disease as it is in the amelioration of inflammation upon initial allergen exposure.

The development of a multiple allergen exposure model of allergic airway inflammation is reported in this chapter, within which the effects of CTL immunotherapy are determined. The generation of key features of CD4+ Th2 cell-mediated inflammation are examined, including the infiltration of inflammatory cells into the lung and the production of characteristic Th2 cytokines IL-4, IL-5 and IL-13. Lung histological examination identifies goblet cell hyperplasia that leads to increased mucus production, and airway remodelling that drives hyperreactivity. To assess the infiltration of inflammatory immune cells into the diseased airway, conventionally, white blood cells in bronchoalveolar lavage (BAL) samples were manually differentiated under a microscope. As some key inflammatory cells (for example, eosinophils and neutrophils) may be difficult to distinguish in murine blood samples, Van Rijt et al recently proposed an automated method by which BAL cells may be more reliably identified, utilising specific fluorescent conjugated antibody stains and flow cytometric analysis (666). This method has been adapted for the examination of airway inflammation herein.
3.2 Aims

The purpose of the experiments described in this chapter was to develop a multiple allergen exposure model of allergic airway inflammation in which to test the effects of CTL immunotherapy on airway disease. The hypothesis was that the known effect of allergen-specific CTLs preventing acute allergic disease may also translate to therapeutic effects on established airway inflammation. Furthermore, if CTL immunotherapy mediates suppressive activity on key allergy-driving immune cells, this may facilitate long-term abrogation of allergic airway inflammation.

The specific aims were:

- To develop and characterise a multiple allergen exposure model of allergic airway inflammation
- To assess the preventative effects of CTL immunotherapy over multiple airway allergen exposures
- To assess the therapeutic effects of CTL immunotherapy in the context of established airway inflammation
3.3 Results

3.3.1 A multiple airway allergen exposure model displays characteristic features of allergic airway inflammation

One of the key features of allergic airway inflammation is the infiltration of immune cells into the diseased lung and airway. A panel of fluorescent conjugated antibodies was developed to identify key inflammatory immune cells in BAL by flow cytometry. This method was adapted from that described by Van Rijt et al. (666). Cells were stained with antibodies to CD11c, Siglec F, Gr-1, CD3, MHCII and CD19 to identify alveolar macrophages, eosinophils, T cells, neutrophils, DCs and B cells (Fig 3.1). Cell doublets were gated out using FSC-A vs FSC-H and SSC-A vs SSC-H plots, and then DAPI-negative live cells were gated for further analysis. Alveolar macrophages were identified as high FSC-A, CD11c+ cells, while eosinophils were lower FSC-A and Siglec F+. T cells were identified by CD3+ staining. After exclusion of alveolar macrophages, neutrophils were identified as Gr-1+, while DCs were CD11c+MHCII+. Cells expressing MHCII, but negative for CD11c, were further gated to identify CD19+ B cells.

To examine changes in the airway inflammatory infiltrate over multiple airway allergen exposures, a model of in vivo sensitisation and subsequent allergen challenge was developed (Fig 3.2 A). Mice were sensitised by intraperitoneal (i.p.) injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Airway challenges were carried out either 1, 2 or 3 times, by intranasal instillation of OVA on days 24 and/or 31, and/or 38; control mice received PBS. BAL was performed three days after the final intranasal challenge for each group.

The total cell number in BAL (Fig 3.2 B) was increased in all OVA/Alum-primed and OVA challenged mice compared to priming with OVA/Alum and PBS challenge, which was not sufficient to induce immune cell infiltrate. Similarly, Alum-primed mice did not show an increase in BAL cellularity when challenged with additional OVA exposures. Therefore, mice must be both primed with OVA/Alum and challenged with OVA to induce the infiltration of
Figure 3.1: BAL analysis by flow cytometry

BAL was performed on mice and cellular inflammatory infiltrate obtained by centrifugation. Cells were stained with antibodies to CD11c, Siglec F, Gr-1, CD3, MHCII (3JP) and CD19. Cell doublets were gated out using FSC-A vs FSC-H and SSC-A vs SSC-H and DAPI-negative live cells gated for population analysis. Percentages represent the proportion of a specific cell type in each depicted histogram. Representative flow gating and determination of populations are depicted.
Figure 3.2: Multiple intranasal allergen exposures provokes significant immune cell infiltrate in BAL, comprised primarily of eosinophils and lymphocytes

(A) Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Airway challenges were carried out either 1 (day 38), 2 (days 31 and 38) or 3 times (days 24, 31 and 38), by intranasal instillation of OVA; control mice received PBS. BAL was performed on day 41. (B) Total cells, eosinophils, lymphocytes, neutrophils, alveolar macrophages and DCs were enumerated by flow cytometry. Mean cell number in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group. **p < 0.01, ****p <0.0001 as assessed by one way ANOVA with Bonferroni’s post test.
inflammatory cells into the BAL. Eosinophils and lymphocytes (including both T cells and B cells) showed a dose-dependent trend towards increase in BAL in OVA/Alum primed mice as additional OVA challenges were performed, compared to Alum only mice (Fig 3.2 B); this was statistically significant after two OVA challenges. While neutrophil infiltration was low overall, significant numbers of neutrophils were present in both the acute, primary setting after a single intranasal challenge, as well as after three challenges in OVA primed and challenged mice, compared to Alum primed mice. This was not a consistent trend, however, as two challenges did not result in significant neutrophilia, which suggests variation exists in neutrophil infiltration. Alveolar macrophages did not appear to be significantly increased in this experiment, nor were DCs. Thus, three intranasal allergen exposures provokes the strongest immune cell infiltrate in BAL, comprised primarily of eosinophils and lymphocytes.

It was necessary to characterise the resolution phase of the inflammatory response to determine how long the infiltration of BAL inflammatory cell populations persisted before resolving on its own. To this end, a time course was carried out where mice were sensitised with OVA/Alum or Alum only and challenged with 3 OVA intranasal exposures as previously described (Fig 3.2 A). Groups of mice were culled and BAL inflammatory infiltrate examined on days 41, 44 and 48: 3, 6 and 10 days after intranasal allergen exposure. On day 3, in accordance with previous findings (Fig 3.2 B), total cells, eosinophils and lymphocytes were significantly increased in OVA/Alum treated mice, compared to Alum controls (Fig 3.3). By day 6, there was a marked decrease in all cell populations shown in the BAL in OVA/Alum treated mice, while still remaining significantly elevated in number compared to Alum treated mice, with the exception of lymphocytes. Day 10 cell numbers were similar in both groups. Thus, these data show that inflammatory infiltrate improves within 6 days and resolves by 10 days after the last intranasal challenge. This also suggests a 3 day (day 41) time point for assessing differences in cellular infiltrate in BAL where the efficacy of an additional treatment intervention is being tested.

Another hallmark of allergic airway inflammation is the production of Th2 cytokines. As the cytokine response usually precedes substantial cellular infiltration into the tissue, it was hypothesised that the peak of measureable cytokine production in the BAL would be earlier
Figure 3.3: Inflammatory infiltrate is maximised at day 3, improves within 6 days and resolves by 10 days after intranasal challenge

Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Three OVA challenges were carried out on days 24, 31 and 38. Groups of mice were culled and BAL performed on days 3, 6 and 10 after airway allergen challenge. Total cells, eosinophils and lymphocytes were enumerated by flow cytometry. Mean cell number in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 3 mice per group. *p < 0.05, **p < 0.01, ****p <0.0001 as assessed by two way ANOVA with Bonferroni’s post test.

than day 3. Mice were sensitised with OVA/Alum or Alum only and challenged with 3 OVA intranasal exposures as previously described (Fig 3.2 A). Groups of mice were culled and cytokines were measured in BAL on days 39 and 41; 1 day and 3 days after intranasal allergen exposure. On day 1, there was a significant increase measured in key Th2 cytokines IL-4 and IL-5, as well as a trend towards increase in IL-13, in OVA/Alum groups compared to Alum only (Fig 3.4). Cytokines indicative of alternative immune responses, IL-10, IFN-γ and IL-17 were not measurable for either treatment group. By day 3, there was no difference in the concentration of any of the cytokines in the OVA/Alum group compared to the day 1 Alum control. Therefore, it is better to measure Th2 cytokine levels on day 1 post challenge in this model of allergic airway inflammation.
Figure 3.4: Peak of cytokine production is day 1 after airway allergen challenge
Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Three OVA challenges were carried out on days 24, 31 and 38. Groups of mice were culled and BAL performed on day 1 and day 3 after allergen challenge. IL-4, IL-5, IL-13, IL-10, IFN-γ and IL-17 in BAL were measured by Bio-Plex. Mean concentration in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 3-5 mice per group. **p < 0.001 as assessed by one way ANOVA with Bonferroni’s post test.

3.3.2 CTLs suppress the development of allergic airway inflammation

To determine the ability of allergen-specific CTLs to prevent allergic airway inflammation over multiple airway allergen challenges, mice were sensitised with OVA/Alum or Alum only and challenged with 3 OVA intranasal exposures as previously described (Fig 3.2 A). OVA-specific CD8+ OT-I T cells were activated in vitro and 5 x 10⁶ cells were administered in the tail vein of mice one day before the first allergen challenge - on day 23. BAL was performed for cytokine analysis on day 39 (1 day after allergen challenge), and both BAL and lungs were harvested on day 41 (3 days after allergen challenge) for assessment of inflammatory infiltrate and histological sectioning, respectively.
On day 41, there was a decrease in BAL total cellularity in CTL treated mice compared to OVA/Alum groups (Fig 3.5 A), with a strong inhibition of eosinophilia and lymphocytosis. Alum only treated mice did not display significant cell infiltration, and the number of alveolar macrophages were similar in all groups, as were neutrophils.

The Th2 cytokines IL-4, IL-5 and IL-13 were increased in concentration in OVA/Alum groups compared to Alum only (Fig 3.5 B), although not significantly so for IL-13. There did not, however, appear to be strong inhibition of IL-4 in CTL-treated mice in this experiment, though IL-5 and IL-13 were decreased in concentration, again this did not reach statistical significance. Histological sections of lungs were stained with H&E and AB PAS to examine airway congestion and mucus production (respectively). The H&E staining revealed that the OVA/Alum mice had strong inflammatory infiltrate and congested alveolar spaces compared to Alum controls. In contrast, mice treated with CTLs at day 23 had airways that remained clear after the three allergen exposures (Fig 3.5 C, upper panel). Goblet cell hyperplasia and mucus production (visible as dark purple-stained cells) were similarly affected by CTL treatments, with reduction in the strongly AB PAS-positive airway goblet cells seen in CTL treated groups, compared to the OVA/Alum group (Fig 3.5 C, lower panel). These data suggest that CTL therapy can prevent key features of allergic airway inflammation over multiple airway allergen exposures.

### 3.3.3 CTLs have therapeutic effects on established allergic airway inflammation

After observing preventative effects of CTL therapy throughout multiple challenges, it was necessary to determine what the effect of CTLs may be on established airway inflammation, as individuals suffering from allergic asthma present clinically after airway inflammation has already developed. In an experimental model similar to that previously described, mice were sensitised with OVA/Alum or Alum only and challenged with three OVA intranasal exposures (Fig 3.6 A). 5 x 10⁶ CTLs were administered to different groups of mice at one of two therapeutic time points; either before the second intranasal challenge, on day 30, or before the third intranasal challenge, on day 37.
Figure 3.5: CTLs prevent inflammation over multiple airway challenges

Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Three OVA challenges were carried out on days 24, 31 and 38. $5 \times 10^6$ *in vitro*-activated OT-I CTLs were administered into the tail vein of mice on day 23.
Groups of mice were culled and BAL performed on days 1 and 3 after allergen challenge. **(A)** Total cells, eosinophils, lymphocytes, alveolar macrophages and neutrophils were enumerated on day 3 after allergen challenge by flow cytometry. Mean cell number in BAL + SEM are shown. **p < 0.01, ****p <0.0001 as assessed by one way ANOVA with Bonferroni’s post test. **(B)** IL-4, IL-5 and IL-13 were measured in BAL on day 1 after allergen challenge by Bio-Plex. Mean concentration in BAL + SEM are shown. **(C)** Lungs were removed on day 3, embedded in paraffin wax and sections were stained with hematoxylin and eosin (H&E) for assessing general infiltrate, and with alcian blue periodic acid Schiff stain (AB PAS) to visualise goblet cell hyperplasia. Multiple consecutive sections of lung tissue were examined thoroughly, and photographs most clearly representing the trend across the whole tissue were included in figures to depict histology. Scale bars show 500μm (upper panel) and 200μm (lower panel). Data are from one representative experiment of three that gave similar results, each with 5 mice per group.

Day 41 BAL samples showed reduced total cellularity, eosinophilia and alveolar macrophages in mice treated with CTLs at either day 30 or day 37, compared to OVA/Alum mice (Fig 3.6 B). Interestingly, the decrease in these cells was more pronounced in mice treated with CTLs at day 30 than day 37, suggesting that earlier treatment may be more effective. Lymphocyte numbers in BAL were not significantly different between OVA/Alum treated mice, with or without CTL treatment. IL-4, IL-5 and IL-13 were increased in BAL in OVA/Alum groups compared to Alum only controls (Fig 3.6 C). A lower concentration of all three cytokines was measured in both day 30 and day 37 CTL-treated groups, in comparison with OVA/Alum mice, although this only reached statistical significance for IL-5, as depicted.

The airways of OVA/Alum treated mice showed widespread infiltration of immune cells (Fig 3.7, left panel) and extensive goblet cell mucus production (Fig 3.7, right panel) that was not seen in the Alum controls. Treating with CTLs on day 30 resulted in effective maintenance of clear air spaces and minimal mucus production. Mice treated with CTLs on day 37 also presented with ameliorated histopathology compared to OVA/Alum mice, however the improvement was not as marked as the day 30 treated mice. These data show that CTLs are capable of preventing further inflammation in mice that have experienced previous episodes of allergic airway inflammation. Additionally, BAL and histology results collectively suggest that earlier treatment may result in a better therapeutic outcome.
Figure 3.6: CTLs have therapeutic effects on allergic airway inflammation

(A) Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Three OVA challenges were carried out on days 24, 31 and 38. CTLs were administered i.v. on day 30 or day 37. Groups of mice were culled and BAL performed on days 39 and 41. (B) Total cells, eosinophils, lymphocytes, alveolar macrophages and neutrophils were enumerated on day 41 by flow cytometry. Mean cell number in BAL + SEM are shown. *p < 0.05, **p < 0.01, ***p < 0.001 as assessed by one way ANOVA with Bonferroni’s post test. (C) IL-4, IL-5 and IL-13 were measured in BAL on day 39 by Bio-Plex. Mean concentration in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group.
Figure 3.7: CTLs have therapeutic effects on airway congestion and goblet cell mucus production.

Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Three OVA challenges were carried out on days 24, 31 and 38. CTLs were administered i.v. on day 30 or day 37. Lungs were removed on day 41, embedded in paraffin wax and sections were stained with hematoxylin and eosin (H&E) for assessing general infiltrate, and with alcian blue periodic acid schiff stain (AB PAS) to visualise goblet cell hyperplasia. Multiple consecutive sections of lung tissue were examined thoroughly, and photographs most clearly representing the trend across the whole tissue were included in figures to depict histology. Scale bars show 500μm (left panel) and 200μm (right panel). Data are from one representative experiment of two that gave similar results, each with 5 mice per group.
As IL-5 has a role in stimulating the proliferation, differentiation and subsequent release of mature eosinophils from the bone marrow into the blood (678), it was assessed whether the decrease in IL-5 in BAL was also affecting the mobilization of eosinophils from the bone marrow. Mice were treated as described in Fig 3.2 A, and blood was collected from the tail vein on days 39, 40, 41 and 44. A lower number of eosinophils was observed in the circulating blood of CTL-treated mice compared to OVA/Alum mice at all time points measured (Fig 3.8 A); becoming statistically significant on day 41 (corresponding to the peak of BAL cellular infiltration) which showed the most marked decrease in eosinophil numbers between these groups. Lungs were harvested on day 39 and homogenised, then tested for the presence of eotaxin, a potent eosinophil chemoattractant cytokine (503). Eotaxin did not appear to be expressed significantly differently in the lungs of mice from any of the treatment groups at this time (Fig 3.8 B).

**Figure 3.8: CTL immunotherapy reduces mobilisation of eosinophils from the bone marrow**

Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Three OVA challenges were carried out on days 24, 31 and 38. (A) Blood was collected from the tail vein of mice on days 39, 40, 41 and 44 and eosinophils were identified using Discombe’s solution. Mean cell number per mL of blood + SEM are shown. **p < 0.01 as assessed by two way ANOVA with Bonferroni’s post test. (B) Lungs were harvested on day 39 and homogenised, ELISA was then carried out to determine the concentration of eotaxin in lung homogenates. Data are from one representative experiment of two that gave similar results, each with 5 mice per group.
3.3.4 CTLs decrease mastocytosis in the allergic airway

Mast cell numbers increase in tissue with chronic exposure to allergens (443). Thus, the presence of increasing numbers of mast cells in lungs can reveal progression to chronic allergic airway disease. Histological examination was carried out to determine when a significant increase in mast cells could be observed in lung tissue. Mice were sensitized and given 1, 2 or 3 OVA challenges, with lungs harvested 3 days after each challenge at day 27, 34 or 41 respectively (Fig 3.9 A). Sections were stained with chloracetate esterase (CAE) to visualize mast cells, which appear as large pink/red cells accumulating around airways in the lung, as represented in Fig 3.9 B. It was found, by light microscopy analysis, that the number of mast cells per field of view increased mildly after 1 and 2 challenges, but was most markedly increased after 3 allergen exposures (Fig 3.9 C).

The effect of CTLs on the induction of mast cells was also examined. Mice were exposed to three OVA challenges and administered CTLs at either day 23, day 30, or day 37. Histological sections revealed that few mast cells were present in any of the CTL-treated groups (Fig 3.9 D). These results demonstrate that CTL treatment results in decreased mastocytosis in the lungs of allergen sensitised and challenged mice. However, as mast cells only begin to appear in significantly high numbers after the third allergen challenge, it was not conclusively shown whether mast cell influx in a chronic condition may be reversed by CTL therapy, or rather instead, that mast cells did not increase in number.

The effect of CTL therapy on total IgE and OVA-specific IgE in serum was then examined, as IgE is known to sensitize mast cells leading to degranulation upon allergen exposure. Mice were sensitised and challenged as previously described (Fig 3.9 A), with some mice receiving CTLs at day 23, before intranasal allergen exposure. On day 41, after three intranasal challenges, mice were sacrificed and blood was collected from the descending
Figure 3.9: Mast cell accumulation in tissue is prevented, IgE production unaffected by CTL therapy

(A) Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. One, two or three OVA challenges were carried out on days 24, 31 and 38. Lungs were taken 3 days after each intranasal challenge, sectioned and stained with CAE to visualize mast cells in tissue. (B) Representative staining shows mast cell accumulation around airways, scale bar 200μm. (C) CAE+ cells were counted in lung tissue taken 3 days after 1, 2 or 3 OVA intranasal exposures. Bar graphs show mean + SEM number of CAE+ cells per 10 fields of view under a 40x objective lens. (D) Mice were exposed to three OVA challenges and administered CTLs at either day 23, day 30, or day 37. Bar graphs show mean + SEM number of CAE+ cells per 10 fields of view under a 40x objective lens. Data are from one representative experiment of two that gave similar results, each with 3 mice per group. (E) Blood extracted from mice treated with CTLs at day 23, or no cells, was tested for total and OVA-specific serum IgE 3 days after the final of 3 intranasal challenges. Bar graphs show mean + SEM of total ng/ml of IgE in serum, and the relative absorbance (OD) of OVA-specific IgE measured at 450nm. Data are from one representative experiment of two that gave similar results, each with 4-5 mice per group. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001, as assessed by one way ANOVA with Bonferroni’s post test.

As questions remained about the effects of CTLs on mast cells, it was necessary to perform an extended model of allergic airway inflammation to further elucidate whether mastocytosis was reversible as a therapeutic outcome of CTL therapy. To this end, a 5-challenge model of allergic airway inflammation was carried out as shown in Fig 3.10 A, where CTLs were administered before the first allergen challenge, or after the third challenge. The number of mast cells in lung tissue was increased four-fold in OVA/Alum aorta from which serum was obtained. All OVA/Alum primed mice showed a similar increase in both total IgE (Fig 3.9 E, left panel) and OVA-specific IgE (Fig 3.9 E, right panel), compared to mice primed solely with Alum. The IgE response was not exacerbated by intranasal OVA challenges, as OVA/Alum primed mice that were challenged with OVA showed similar levels of serum IgE as OVA/Alum primed mice that received PBS intranasally. Additionally, IgE was not induced in mice given solely intranasal exposure to OVA (Alum primed group). This suggests that the OVA/Alum priming regime alone is sufficient to induce an IgE response. Treatment with CTLs on day 23 did not affect IgE levels in serum. Therefore, CTL immunotherapy affects the number of mast cells in lung tissue, but may not be effective in preventing activation of mast cells by IgE.
mice compared to Alum only mice, after the 5 intranasal allergen exposures (Fig 3.10 B). In CTL-treated groups, mast cells were similarly low in both the day 23 and day 44 CTL treatments, and only marginally higher than in mice treated with alum only. As the data in Figure 3.9 C show that mast cell numbers were already high after the 3rd challenge, these results suggest that CTL therapy can reverse the accumulation of mast cells that occurs over successive allergen exposures.

The activity of mucosal mast cells was additionally examined by measuring the concentration of mouse mast cell protease – 1 (mMCP-1) in serum 4 hours after the third, fourth, and fifth intranasal challenges; on days 38, 45 and 52 respectively. On day 38 and 45, mMCP-1 levels were variable, and there did not appear to be a clear difference between CTL-treated groups and non CTL-treated groups (Fig 3.10 C). After the fifth allergen challenge, there was an increase in serum mMCP-1 in OVA/Alum mice compared to Alum only, and this was ameliorated in mice treated with CTLs at either day 23 or day 44; at this time point, the amount of mMCP-1 in serum reflected the mast cell numbers reported in B.

The overall trend revealed that CTL-treated groups had a decrease in mMCP-1 over successive intranasal allergen exposures, whereas OVA/Alum mice that did not receive CTLs showed an increase in mMCP-1 over time. This may be due to changes in lung mast cell populations with consecutive allergen exposures; in earlier time points, mMCP-1 may be produced primarily by lung resident mast cells, but as additional allergen challenges are carried out, the induced mast cell population increases in OVA/Alum mice, (though not in CTL-treated mice) and consequently, the production of mMCP-1 increases.

The localisation of mast cells in lung tissue revealed that numerous mast cells were present within, or directly in contact with, the airway smooth muscle layer (Representative histology, Fig 3.10 D). In this location, mast cells have been reported to promote a more contractile phenotype of airway smooth muscle cells, contributing to the development of AHR (542, 543).
Figure 3.10: Mast cell number, protease production, and localisation in a chronic model of allergic airway inflammation

(A) Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Five OVA challenges were carried out on days 24, 31, 38, 45 and 52. CTLs were administered i.v. on either day 23 or day 44. Lungs were taken on day 55, sectioned and stained with CAE to visualize mast cells in tissue. (B) CAE+ cells were counted in lung tissue. Bar graphs show mean ± SEM number of CAE+ cells per 10 fields of view under a 40x objective lens. (C) The concentration of mouse mast cell protease – 1 (mMCP-1) in serum was measured 4 hours after the 3rd, 4th and 5th intranasal challenges, by ELISA analysis. (D) Representative histology shows numerous mast cells localised within, or directly associated with, airway smooth muscle, scale bar 100μm. Data are from one representative experiment of two that gave similar results, each with 3-5 mice per group. *p < 0.05, ****p < 0.01 as assessed by one way ANOVA with Bonferroni’s post test.
3.3.5 CTLs have therapeutic effects in a multiple challenge model of allergic asthma that displays markers of chronicity

As the 5-challenge model showed evidence of increased chronicity, in terms of significant mastocytosis, we wanted to determine the potential for CTL immunotherapy to ameliorate allergic airway inflammation under these conditions, as well as examine the long term preventative effects of CTLs. Utilising the model described in Fig 3.10 A, mice were sensitised and challenged with OVA allergen, with CTLs administered on either day 23 or day 44. Mice were culled on day 53 and 55 and BAL obtained for analysis of cytokine production and cellular infiltrate, respectively.

It was found that a single CTL treatment on day 23 could suppress airway inflammation even after 5 allergen challenges. There was a decrease in BAL cellularity (Fig 3.11 A), with a strong inhibition of eosinophilia as well as a decrease in lymphocyte infiltration in CTL-treated groups, compared to OVA/Alum treatment. The numbers of alveolar macrophages were similar in all groups. In mice treated with CTLs on day 44, a time when a robust inflammatory response was already evident (see Fig 3.5), BAL cellularity, eosinophils and lymphocytes were reduced to a level comparable to mice treated with CTL on day 23. These data suggest that one treatment with CTL is sufficient to achieve long lasting prophylaxis, as well as an effective therapeutic treatment for reducing Th2 responses in a model of allergic airway inflammation with features of chronic disease.

In a similar experiment, airway histology revealed that the OVA/Alum mice had strong inflammatory infiltrate and congested alveolar spaces. In contrast, mice treated with CTLs at day 23 had airways that remained clear after the repeated allergen exposures (Fig 3.11 B, upper panel). The mice treated with CTL on day 44, also presented on day 55 with reduced infiltration of immune cells in the alveolar spaces. Thus, treatment with CTL on day 44 could reverse established inflammation. Goblet cell hyperplasia and mucus production
Figure 3.11: Allergen-specific CTLs have therapeutic and long term preventative effects on allergic airway inflammation

Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Five OVA challenges were carried out on days 24, 31, 38, 45 and 52. CTLs were administered i.v. on either day 23 or day 44. (A) Total cells, eosinophils,
lymphocytes and alveolar macrophages were enumerated on day 55 by flow cytometry. Mean cell number in BAL + SEM are shown. **p < 0.01, ***p < 0.001, ****p <0.0001 as assessed by one way ANOVA with Bonferroni’s post test. (B) Lungs were removed on day 55, embedded in paraffin wax and sections were stained with hematoxylin and eosin (H&E) for assessing general infiltrate, and with alcian blue periodic acid schiff stain (AB PAS) to visualise goblet cell hyperplasia. Multiple consecutive sections of lung tissue were examined thoroughly, and photographs most clearly representing the trend across the whole tissue were included in figures to depict histology. Scale bars show 200μm (upper panel) and 100μm (lower panel). (C) RNA was extracted from lungs and Muc5AC, AMCase, Tenascin-c and MMP-9 RNA were measured by RT-PCR. Relative expression of RNAs between treatment groups were examined by comparing RNA expression from lungs of OVA/Alum + CTL-treated mice to that of OVA/Alum mice that did not receive CTL treatment. Data are from one representative experiment of two that gave similar results, each with 4-5 (A and B) or 3 (C) mice per group.

were similarly affected by CTL treatments, with reduction in the strongly AB PAS-positive airway goblet cells in both day 23 and day 44 CTL treated groups, compared to the OVA/Alum group (Fig 3.11 B, lower panel). Due to variation in samples, no statistically significant differences were observed in the expression of RNA for the epithelial damage, remodelling and repair markers, AMCase, Tenascin-c, Muc5AC and MMP-9, however AMCase and Tenascin-c did show a trend towards reduction by CTL therapy (Fig 3.11 C). Together, these data show that immunotherapy with CTLs prevents congestion of airways caused by immune cell infiltrate and overproduction of mucus, and is additionally capable of reversing pathological effects on the airway after multiple episodes of inflammation have occurred.

Additional to mastocytosis, chronic allergic airway inflammation is characterised by airway remodelling including thickening of the airway smooth muscle layer and increased collagen deposition (560-562). These features of disease were examined, and it was discovered that significant airway remodelling was induced in OVA/Alum mice, with a thickening of the airway smooth muscle layer (Fig 3.12, left panel, ASM thickness highlighted) and an increase in collagen deposition evident as darker, more dense blue staining (Fig 3.12, right panel), compared to Alum controls. In prophylactic day 23 CTL-treated mice, ASM remained a similar thickness to that seen in Alum mice, and therapeutic day 44 treatment successfully retracted ASM to a baseline level. Therefore, CTL treatment results in the healing of airway
Figure 3.12: CTL immunotherapy resolves features of allergen-induced airway remodelling

Mice were sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum) on days 0 and 14. Five OVA challenges were carried out on days 24, 31, 38, 45 and 52. CTLs were administered i.v. on either day 23 or day 44. Lungs were taken on day 55, sectioned and stained with H&E and Massons Trichrome. Representative sections show ASM (left panel, thickness indicated) and collagen deposition (right panel, blue staining), scale bars 200μm. Multiple consecutive sections of lung tissue were examined thoroughly, and photographs most clearly representing the trend across the whole tissue were included in figures to depict histology. Data are from one representative experiment of two that gave similar results, each with 5 mice per group.
smooth muscle hyperplasia. Fibrosis around airways did not follow the same trend, however; while day 23 treated mice had similar collagen deposition to Alum controls, the day 44 mice appeared to have more collagen than both the day 23 treated mice as well as Alum mice, although not as much as the OVA/Alum untreated mice. This result suggests that collagen deposition may be prevented by CTL immunotherapy in a prophylactic context and prevented from worsening in a therapeutic context, however repair of fibrotic tissue is not achieved by CTL treatment.
3.4 Discussion

The critical shortcoming of many models of acute allergic airway inflammation is that treatments are only be tested for prophylactic effects. For clinical relevance, a model of allergic airway inflammation with features of chronic disease is preferable to determine the therapeutic potential for novel treatments. This chapter aimed to establish a multiple challenge model of allergic airway inflammation in which to assess the preventative and therapeutic potential for CTL immunotherapy.

Developing a multiple challenge model of allergic airway inflammation

Defined as a chronic inflammatory disease of the airways, asthma is a complex disease and the physiological and immunological changes associated have not been fully elucidated. As such, it is difficult to replicate all the features of pathology in an experimental murine model; numerous models exist where the mouse strain, method of sensitisation, number of challenges and amount of allergen administered are widely varied. This makes comparing results across studies difficult (500, 610). It is possible, however, to develop a model of allergic airway inflammation in which important measureable traits of established disease may be well characterised, such that specific effects of treatment interventions may be examined.

In this chapter, a model was established that comprised three or five airway allergen exposures after systemic sensitisation with OVA. This led to a robust lung immune cell infiltrate, eosinophilia and lymphocytosis as well as Th2 cytokine production. The nature of the resolution of this inflammation was such that immune cell infiltrate was no longer detected in BAL 10 days after the last allergen challenge. Nevertheless, the timecourse carried out also revealed that BAL cellularity continued to be elevated 6 days after allergen exposure, so inflammation was still present when the subsequent allergen challenge was administered. It has been argued that inflammation that rapidly resolves is not truly reflective of a chronic asthmatic condition (500, 610, 645). However, this acute inflammatory phase also occurs, and resolves, repeatedly in chronic disease and many patients suffer from acute exacerbations of asthmatic disease. It is, then, appropriate to
assess lung histopathology to determine whether changes have developed where airway remodelling and AHR may remain long after exposure to allergen has ceased (645, 679).

Extensive histological pathology was identified in the model of allergic airway inflammation described in this chapter; tissue destruction, goblet cell hyperplasia, increased airway smooth muscle and collagen deposition, and an increase in mast cell numbers in the lung were observed, replicating the key features of airway remodelling (560-562). It could be concluded, then, that in this model of airway allergy, several important features of chronic disease were effectively replicated.

A number of the parameters examined in the experiments in this chapter also reflect changes in the airway that may be indicative of airway hyperreactivity. Firstly, an increase in IL-13 was consistently measured in OVA/Alum groups. It has been widely reported both in murine experimental models, as well as human asthmatics, that IL-13 has a critical role in the development of AHR (525, 680-683). Additionally, there was an increase in airway smooth muscle (ASM) observed. While the relationship between ASM and AHR is not clear (that is, a greater mass of ASM does not necessarily equate to increased potential for contractility), it has been found that the removal of ASM in asthmatic patients by bronchial thermoplasty (the delivery of radiofrequency energy to the airway wall to heat tissue in a way that reduces muscle mass) leads to an improvement in AHR, suggesting increased ASM plays a pathological role (500, 610, 684). Interestingly, another study has found that the presence of mast cells within ASM is a key factor in the development of airway resistance and hyperreactivity in human asthmatics (542, 645), and contributes to AHR by increasing contractility of ASM cells (543, 645, 679); this pattern of mast cell localisation has not previously been reported in murine models, but was identified in the present study. A limitation of this study was that AHR, airway resistance and other parameters of physiological lung function were not directly measured. However, the findings discussed here suggest pathological changes that induce AHR may be present in this model.

**CTL-mediated immunotherapy of allergic airway disease**

The suppression of acute airway inflammation by allergen-specific CTLs has been reported (226, 560-562). The present study evaluated the ability of CTLs to prevent allergic airway
inflammation over multiple allergen exposures, as well as the therapeutic potential during established inflammation. It was clear that CTLs mediated significant effects on airway immune cell infiltrate, Th2 cytokine production and airway histology.

The characterisation of BAL cellularity revealed that mice treated with CTLs, in both preventative and therapeutic contexts, had significantly reduced eosinophilia. As eosinophils are critical mediators in the deposition of collagen, airway smooth muscle and mucus production (506, 525, 638, 680-683), this finding was an acute-phase primary indicator that a reduction in airway remodelling could become evident. A reduction in lymphocytosis was also observed with both preventative and therapeutic CTL treatment, however in some experiments where CTLs were given 4 – 11 days before mice were sacrificed, lymphocytes remained elevated. As host lymphocytes and adoptively transferred CTLs were not differentiated in these experiments, it was possible that the lymphocytosis observed in CTL-treated groups comprised both of these populations, and that CTLs administered closer to the time of the experimental end point may be present in the lung in higher numbers. This hypothesis is examined and reported on in Chapter 4.

A reduction in key Th2 cytokines IL-4, IL-5 and IL-13 was consistently seen in therapeutically CTL-treated groups, however the variation of measured concentrations within OVA/Alum groups meant that statistical significance was not reached. An anomaly encountered in some experiments was the inability of CTLs to suppress IL-4 production when CTLs were given in a prophylactic manner. This could be due to differences in cytokine production by key cell populations between the primary acute response and subsequent responses in the established Th2 inflammatory environment, and the effects of the introduction of CTLs before or after the initiation of these events.

There are a number of cells involved in the pathology of allergic airway inflammation that may produce IL-4 during allergen challenge. In addition to CD4+ T cells, basophils and NK cells have been reported as important producers of this key cytokine in both helminth and allergen-induced models of Th2 immune responses. Basophils have been deemed a key early source of IL-4 in Th2 immunity, (430, 433, 434) however, the priming and differentiation of Th2 cells occurs independently of basophils (435, 550, 685). The
requirement for NK and NKT cells in allergic asthma has been disputed, with studies using various knock-out models revealing both essential and non-essential roles (149, 424, 686-688). Recent work has more definitively shown that while these cells may amplify effector responses, the contribution of NK and NKT cells is not crucial for the initiation of the Th2 response (425), and may only be important in acute responses rather than in the chronic setting (689). Eosinophils and mast cells additionally produce IL-4 under Th2 conditions, in a redundant manner (690-693). As IL-4-producing populations were not enumerated in these experiments, their contribution to disease and subsequent response to CTL immunotherapy in this model cannot be defined, however the lack of suppression of IL-4 in these experiments did not translate to increased pathology in terms of BAL inflammatory infiltrates or airway histopathology, so amelioration of disease was still evident.

The observed reduction in IL-5 prompted further investigation of the systemic effects of CTL immunotherapy, as IL-5 is important for the proliferation and differentiation of eosinophils as well as their release from bone marrow (678). The reduction in peripheral blood eosinophils that was observed mirrors the response seen in corticosteroid treatment that is associated with improved clinical outcome (694, 695). This finding suggests CTL therapy reflects clinically relevant parameters in the treatment of allergic airway disease.

It is clear that lung histopathology was significantly improved by CTL immunotherapy, with reductions in tissue destruction, goblet cell hyperplasia, airway smooth muscle and collagen deposition, and mast cell infiltration. What is more difficult to ascertain is whether CTLs are capable of reversing airway pathology, or rather that pathology is prevented from worsening after the time of CTL administration. It appears that mastocytosis and goblet cell hyperplasia were, in fact, reversed in CTL treated mice. The pathology of three allergen challenges showed significant mucus production and mast cell numbers in the lung tissue at this time point; subsequent administration of CTLs resulted in the reduction of mast cell numbers and regression of goblet cell hyperplasia to the level of healthy controls, despite further airway allergen exposures. Others have reported that mast cells may rapidly return to baseline levels after treatment interventions (696-698), and goblet cell hyperplasia may also be reduced (699). Airway function may be significantly enhanced by an improvement in these parameters, however aspects of long term damage may remain; CTL therapy was
capable of preventing collagen deposition, and therapeutic administration of CTLs resulted in fibrosis not continuing to worsen, however lung tissue did not return to a healthy pre-challenge state in the time frame of these experiments. This is in accordance with what is known about healing and repair in chronic disease. Significantly fibrotic lung parenchyma loses the ability to be regenerated into physiologically functional tissue, and there is currently no effective therapeutic intervention that can lead to repair of tissue fibrosis (699-701). It would be useful to measure AHR, airway resistance and other parameters of lung function in this model to translate these findings into functional readouts of lung physiology. Nevertheless, the combined reduction of airway inflammatory infiltrate, Th2 cytokines, and mucus production represent significant parameters that correlate with improved lung function (525, 680-683, 694, 695).

It was interesting that a reduction in mast cells was observed with CTL immunotherapy, while IgE remained unaffected. The roles of mast cells and IgE in allergic airway inflammation have not been fully elucidated. Kobayashi et al suggest that two distinct pathways exist for the development of AHR: an IgE/mast cell dependent mechanism, as well as an eosinophil/IL-5 dependent mechanism (702). However, Williams and Galli argue that the Kobayashi model was “weak” (in the induction of eosinophilia) and thus does not prove a mast cell dependent mechanism for AHR (541). In a model that induces substantial eosinophilia, mast cells were shown not to be critical for the induction of the hallmark features of airway disease (444). However, mast cells are recognized as amplifiers of antigen-dependent chronic inflammation in the airway (541). Therefore, CTL therapy effectively reduces the amplification of chronic inflammation. Importantly, and directly related to mast cell activation, IgE has an important role only in experimental protocols where strong eosinophilia is not induced (540). Thus, the presence of IgE in serum is less indicative of, and has less impact on, disease severity in our model than the suppression of eosinophilia, which is effectively achieved by CTL immunotherapy.

Alterations in the temporal administration of CTLs revealed important insights into the mechanism of action of immunotherapy. The suppression of allergic airway disease appeared to be least effective when CTLs were administered immediately before the final of three airway allergen challenges, 4 days before mice were sacrificed. This finding seemed to
initially suggest that with additional airway challenges, inflammation is less well suppressed; however, this was tested in experiments where CTLs were administered after three challenges, with two further challenges then carried out – the result of which showed that inflammation did not become worse. In light of these results, it appeared that the reduced efficacy of CTL therapy was not related to the number of allergen challenges, but potentially due to the administration of treatment being closer to the end point of the experiment, allowing less time for CTL-mediated resolution of inflammation. However, if we consider that mice treated preventatively, 4 days before the end point, in an acute setting show effectively reduced airway inflammation (226), this suggests that CTL-mediated suppression of disease does not require extended resolution time- at least in the acute context. The kinetics of CTL immunotherapy in the context of acute vs. established airway inflammation is, then, the remaining factor to consider.

In chronic disease, there is an increase in DC numbers in lung tissue, with increased maturation markers and allergen-presenting capability (703-706). Additionally and subsequently, an increase in CD69 expression has been reported on CD4+ T cells, indicative of increased antigen presentation (706). In a model of multiple allergen exposures, then, a larger pool of allergen-presenting DCs stimulate CD4+ T cell responses, compared to the acute model where there are fewer allergen-presenting cells, yet a strong primary immune response is still induced. If the suppressive effects of CTL immunotherapy are mediated by the killing of DCs, as has been proposed (226), this could explain why in the acute scenario (where fewer allergen-presenting DC are present) CTLs may be able to diminish allergen-presenting DC populations more efficiently, such that inflammation is resolved quickly; the progression of allergic inflammation after multiple allergen challenges may be more difficult to restrain, as appears to be the case in mice treated with CTLs one day before the final of three challenges. This does not, however, fully explain why mice treated with CTLs after three challenges have strongly abrogated airway inflammation even after additional allergen challenges; a result that suggests CTL immunotherapy may be more effective over time. A possible explanation for this phenomenon is that successive allergen exposures stimulate increased CTL proliferation and activation allowing for the targeting of greater numbers of DCs. The work of other authors has described similar effects of treatment during early versus later stages of chronic disease (699). Alternatively, as the targeting of
DCs would require cell-cell contact with CTLs, it is worth considering that the localisation of DCs in tissue may affect the ability of CTLs to interact with and target allergen-presenting DC populations. These factors are explored in Chapter 5.

**Conclusions**

In this chapter, allergic airway inflammation was induced by multiple allergen exposures that led to increased immune cell infiltrate into airways and lungs, the production of Th2 cytokines, and airway remodelling evident as goblet cell hyperplasia, increased collagen deposition and airway smooth muscle. Within this model, allergen-specific CTLs were found to mediate suppression of all features of allergic disease in long-term preventative as well as therapeutic contexts. These results suggest that CTL-mediated immunotherapy may affect key allergy-driving cells, leading to the amelioration of allergic airway inflammation.
Chapter 4

CTL-mediated suppression of allergic airway inflammation is associated with a reduction in disease-mediating Th2 cells in the lung and airways
4.1 Introduction

The preventative and therapeutic effects of allergen-specific CTLs on established allergic airway inflammation have been described (Chapter 3). However, the mechanism by which CTLs ameliorate inflammation was not revealed through the examination of lung pathology. The ability of CTLs to suppress hallmark features of Th2 disease suggests that CTLs may be mediating direct or indirect effects on key drivers of the allergic response: CD4+ Th2 cells.

CD4+ T cells are widely recognised as critical mediators of allergic pathology, (472, 707-710), although their role and activity during allergic inflammation is still not fully understood. Additionally, the direct or consequential effects of CD8+ T cells on CD4+ T cells during allergic airway inflammation, especially in a context critically dependent on perforin, have not been thoroughly examined. Interestingly, in asthmatic patients, an increase in CD4+ T cells in the lung and BAL is directly related to the severity of airway hyperreactivity, while an inverse correlation exists with CD8+ T cells (708, 711). These studies suggest there may be an important balance between the activity of CD4+ T cells and CD8+ T cells that maintains airway homeostasis, and potentially implicates CD8+ T cells in the diminution of human atopic asthmatic disease.

The ex vivo analysis of antigen-specific CD4+ T cells and CD8+ T cells is difficult, due to their low frequency; indeed, even upon successful identification of these cells, the meagre population may not be sufficient to draw meaningful conclusions about their phenotype and function during inflammatory disease. An alternative method may be employed: the utilisation of exogenously enriched antigen-specific T cells that may be identified based on cell surface expression of unique markers. The adoptive transfer of in vitro-cultured CD4+ Th2 cells into naïve mice has been shown to induce lung eosinophilia and recapitulate the characteristics of allergic disease upon airway allergen exposure (472, 650, 651, 707-710). In this context, adoptively transferred T cells migrate to peripheral tissues where antigen is present (486, 711, 712). CD8+ and CD4+ T cells from congenic OT-I x B6.SJL-Ptprc and OT-II x B6.SJL-Ptprc mice, respectively, may be injected into C57BL/6 hosts without compatibility issues; the CD45.1+ congenic lymphocytes may then be isolated and distinguished from C57BL/6 host cells that are CD45.2+. This concept provides the basis for
the development and characterisation of a model of allergic airway inflammation in which adoptively transferred, allergen-specific CD4+ and CD8+ T cells may be tracked and examined.

Recently, 4C13R mice were developed by William E Paul and associates, providing a useful model for studying CD4+ T cell production of IL-4 and IL-13 \textit{in vivo}. The fluorescent markers AmCyan and dsRed report expression of IL-4 and IL-13, respectively. Generated using a bacterial artificial chromosome construct, these mice remain IL-4 and IL-13 sufficient – a significant advantage over previously available IL-4 reporter mice (660). Additionally, as IL-4 and IL-13 have distinct roles in asthma pathogenesis (680), the dual reporting capability of 4C13R mice is a valuable tool in revealing the production of these cytokines that are not always regulated in the same way. In an adoptive transfer model, CD4+ T cells from these mice could be utilised to investigate cytokine production on a per-cell basis.

The adoptive transfer model of allergic airway inflammation, as proposed, would primarily replicate the T-cell-driven late asthmatic response. During this phase, allergen exposure in the airway drives Th2 cell activation, proliferation, migration to the lung and production of key cytokine mediators IL-4, IL-5 and IL-13. These factors, in concert with subsequently infiltrating inflammatory cell subsets, drive characteristic pathology of allergic airway inflammation (503, 504, 510, 511, 524, 525). Examination of the effects of allergen-specific CTLs on these parameters of the effector Th2 cell response may therefore be useful to determine the mechanism by which disease is ameliorated.
4.2 Aims

The purpose of this chapter was to develop and characterise an adoptive transfer model of allergic airway inflammation to track and examine the activity of CD4+ T cells and CD8+ CTLs, and investigate the effects of these CTLs on the disease-mediating CD4+ T cells. The hypothesis was that the activation, proliferation, migration and cytokine production of CD4+ T cells may be affected by CTLs due to the observation (Chapter 3) that CTLs are capable of suppressing and treating the pathological outcomes of allergic airway disease that are largely driven by a Th2 response.

The specific aims were:

- To develop and characterise an adoptive transfer model of allergic airway inflammation that replicates the pathology observed after *in vivo* sensitisation
- To investigate the effects of CTLs on the proliferation of CD4+ T cells and their number in the lung and airways
- To assess the effects of CTLs on the activation status of disease-mediating CD4+ T cells
- To determine whether CD4+ T cells have a diminished ability to produce characteristic Th2 cytokines in the presence of CTLs
4.3 Results

4.3.1 Optimisation of CD4+ T cell culture system

To identify and investigate the in vivo mechanisms of action of CD4+ T cells, an adoptive transfer model of allergic airway inflammation was developed. CD4+ T cells were isolated from the lymph nodes of OTII mice and co-cultured with OVA_{323-339}-pulsed DCs in IL-2 and IL-4 supplemented media to generate OVA-specific Th2 cells.

To optimise the yield and activation status of CD4+ T cells, in vitro culture conditions were varied by modifying the ratio and number of CD4+ T cells and DCs in co-culture. Additionally, some cultures were split at day 4, while others were cultured continuously for 5 days (Conditions 1-4 outlined in Table 4.1). The cell yield and expression of activation markers CD44, CD69 and CD62L were examined to determine optimal culture conditions. The cell yield at the completion of culture varied considerably between conditions examined; a 1:4 ratio of DC:CD4+ T cells resulted in the largest increase in CD4+ T cell numbers, compared to 1:2 ratio (Table 4.1). Continuous 5 day culture of 1 x 10^6 DC with 4 x 10^6 CD4+ T cells showed an approximately 5-fold increase in cell numbers, and cultures of 0.5 x 10^6 DC with 2 x 10^6 CTL that were split at day 4 increased 7-fold.

<table>
<thead>
<tr>
<th>Initial cells per well</th>
<th>Yield at day 5, per well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio</td>
</tr>
<tr>
<td>1</td>
<td>1:2</td>
</tr>
<tr>
<td>1</td>
<td>1:4</td>
</tr>
<tr>
<td>3</td>
<td>1:2</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
</tr>
</tbody>
</table>

Table 4.1: Optimisation of CD4+ Th2 cell generation – conditions for culture

CD4+ T cells were isolated from the lymph nodes of OTII mice and co-cultured with OVA_{323-339}-pulsed DCs in IL-2 and IL-4 supplemented media to generate OVA-specific Th2 cells. The ratio and number of CD4+ T cells and DCs in co-culture were varied as indicated. Some cultures were split at day 4, while others were cultured continuously for 5 days. Data are representative of three separate cell culture experiments, within which the yield of 3 wells was averaged.
Cells were analysed by flow cytometry to assess the proportion of live cells by DAPI exclusion, of which FSC-Hi, CD4+, OVA-specific T cells (Vα2+, Vβ5.1,5.2+) were identified (representative flow gating, Fig 4.1 A). The proportion of live, antigen-specific cells generated through culture was best where fewer cells per well were plated initially (Table 4.1). The activation status of cells was additionally examined by the expression of activation markers CD44, CD69 and CD62L (Representative flow gating, Fig 4.1 A). An increase in CD44, CD69 and decrease in CD62L, characteristic of T cell activation, was observed to a similar extent in all conditions tested (Fig 4.1 B), compared to FSC-Hlo non-activated cells from the same culture well; however, CD44 was notably higher in cells cultured at a 1:4 ratio, with 1 x 10^6 DC and 4 x 10^6 CD4+ T cells. Considered together, the results of culturing CD4+ T cells under the conditions described showed that a 1:4 ratio of DC:CD4+ T cells was superior to 1:2. While 0.5 x 10^6 DC with 2 x 10^6 CD4+ T cells split at day 4 produced a high cell yield and proportion of live antigen-specific cells, the CD44 expression was most increased in cells cultured at 1 x 10^6 DC with 4 x 10^6 CD4+ T cells – a condition that showed reasonably similar cell yield after 5 days continuous culture. Therefore, a method of 1 x 10^6 DC co-cultured with 4 x 10^6 CD4+ T cells for 5 days was employed in all further experiments.

**4.3.2 Optimisation of an adoptive transfer model of allergic airway inflammation**

To determine the number of CD4+ T cells required to induce strong, reproducible allergic airway inflammation *in vivo* a titration was carried out, initially by administration of 2 x 10^6, 5 x 10^6 or 10 x 10^6 CD4+ T cells. The cells were injected intravenously into naïve C57BL/6J mice and a single airway allergen challenge was carried out one day later (Fig 4.2 A). BAL was performed 3 days after intranasal challenge and the inflammatory cell infiltrate assessed by flow cytometry as described in Chapter 3, Fig 3.1.

The number of total cells and eosinophils in BAL was increased significantly in mice that received 5 x 10^6 and 10 x 10^6 cells compared to naïve controls, however no significant increase was present in mice that received 2 x 10^6 cells (Fig 4.2). Administration of 5 x 10^6 CD4+ T cells provoked higher numbers of total cells and eosinophils to infiltrate than administration of 2 x 10^6 cells; no further increase was observed in mice given 10 x 10^6 cells.
Figure 4.1: Optimisation of CD4+ T cell culture conditions for adoptive transfer experiments

CD4+ T cells were isolated from the lymph nodes of OTII mice and co-cultured with OVA\textsubscript{323-339}-pulsed DCs in IL-2 and IL-4 supplemented media to generate OVA-specific CD4+ Th2 cells. Cells were cultured under conditions outlined in Table 4.1. (A) Cultured cells were examined by flow cytometry for viability, activation and Vα2,Vβ5.1,5.2 TCR specificity. (B) The expression of activation markers CD44, CD69 and CD62L on activated Vα2,Vβ5.1,5.2+ cells identified in (A) were then examined and compared to non-activated FSC-H\textsuperscript{lo} cells within the same sample. Staining and marker expression are representative of three separate cell culture experiments, within which the cells of 3 culture wells per condition were examined.
Figure 4.2: Airway inflammation induced by varying numbers of adoptively transferred CD4+ T cells

**A** 2 x 10^6, 5 x 10^6 or 10 x 10^6 CD4+ T cells were injected into naïve mice and a single airway allergen challenge carried out one day later. BAL was performed 3 days after intranasal challenge. **B** Inflammatory cell infiltrate was assessed by flow cytometry as described in Fig 3.1 to enumerate eosinophils, neutrophils, alveolar macrophages and DCs in BAL. Mean cell number in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ns= not significant as assessed by one way ANOVA with Bonferroni’s post test.
Neutrophils were not elevated in number in mice that received $2 \times 10^6$ CD4+ T cells compared to naïve controls, however a higher number were counted in mice treated with $5 \times 10^6$ or $10 \times 10^6$ CD4+ T cells; this only reached statistical significance in the $10 \times 10^6$ group, which had significantly stronger neutrophilia than mice treated with $5 \times 10^6$ cells. Compared to the influx of eosinophils, however, the number of infiltrating neutrophils was much lower. The number of lymphocytes, alveolar macrophages and DCs, while increased in number in all CD4+ T cell treated groups compared to naïve controls, were not statistically significantly higher in these experiments. These results show that $5 \times 10^6$ CD4+ T cells are sufficient to induce a strong allergic response, and the administration of $10 \times 10^6$ cells only provokes a significantly stronger response in terms of neutrophilia. As neutrophils may be more closely linked with tissue damage rather than indicative of an amplified allergic response (713), eosinophils are the more pertinent measure of allergy, as critical mediators of collagen deposition, airway smooth muscle thickening and mucus production (506, 638).

As $5 \times 10^6$ CD4+ T cells mediated a robust inflammatory infiltrate in BAL, it was not known whether this response would overwhelm the effectiveness of CTL immunotherapy such that it may not be evident whether CTLs suppress allergic airway inflammation in the described adoptive transfer model. To this end, $5 \times 10^6$ CD4+ T cells were co-administered with $5 \times 10^6$ CTLs into naïve C57BL/6 recipients, and additionally, two groups received $3 \times 10^6$ CD4+ T cells (with $5 \times 10^6$ CTLs), hypothesised to generate stronger inflammatory infiltrate than that previously seen when $2 \times 10^6$ CD4+ T cells were given, but possibly less than that induced by $5 \times 10^6$ cells, due to the lower number of effector cells immediately responding to allergen challenge (Fig 4.3 A). The resulting BAL on day 3 revealed that the number of infiltrating total cells, eosinophils, lymphocytes and neutrophils were higher in OVA-treated mice that received $3 \times 10^6$ CD4+ T cells or $5 \times 10^6$ CD4+ T cells, compared to mice that received $5 \times 10^6$ CD4+ T cells without OVA challenge; though this was not statistically significant for neutrophils (Fig 4.3 B). These cell populations showed a trend towards additional increase in mice that received $5 \times 10^6$ CD4+ T cells compared to those that received $3 \times 10^6$ CD4+ T cells; a statistically significant increase in eosinophilia was evident. DCs showed a similar trend in terms of increased numbers in $3 \times 10^6$ and $5 \times 10^6$ CD4+ T cell treated groups compared to naïve controls, although statistical significance was not
Figure 4.3
Figure 4.3: The induction and suppression of airway inflammation by adoptively transferred T cells

(A) 3 x 10^6 or 5 x 10^6 CD4+ T cells were injected with or without 5 x 10^6 CTLs into naïve mice and a single airway allergen challenge was carried out one day later. BAL was performed 3 days after intranasal challenge. (B) Inflammatory cell infiltrate was assessed by flow cytometry as described in Fig 3.1 to enumerate eosinophils, neutrophils, DCs and alveolar macrophages in BAL. Mean cell number in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group. *p <0.05, ***p <0.001, ****p <0.0001, ns= not significant as assessed by one way ANOVA with Bonferroni’s post test.

reached. Alveolar macrophages were not significantly altered in number between any of the groups. Interestingly, when 5 x 10^6 CTLs were administered, CTLs suppressed the inflammatory infiltration of all cell populations, regardless of whether it was 3 x 10^6 or 5 x 10^6 CD4+ T cells that had been co-injected; this was statistically significant in terms of suppression of eosinophilia and lymphocytosis. These results reveal that BAL inflammatory infiltrates in an adoptive transfer model of strong allergic airway inflammation induced by 5 x 10^6 CD4+ T cells may be effectively ameliorated by immunotherapy with 5 x 10^6 CTLs.

4.3.3 CTLs suppress inflammation in an adoptive transfer model of allergic airway disease in a manner dependent on perforin

Having established the conditions of an adoptive transfer model where substantial BAL inflammatory infiltrate was inducible, and additionally effectively abrogated by CTL immunotherapy, the effects of CTLs on multiple disease parameters of allergic airway inflammation were then examined within this model.

The in vivo sensitisation model described in Chapter 3, in accordance with published work (226), demonstrated the ability of CTLs to suppress allergic airway inflammation in a manner that was dependent on the expression of perforin. As the adoptive transfer model employs in vitro-generated CD4+ T cells, it was not known whether this system would also require the cytotoxic function of CTLs for suppression of airway inflammation. To investigate this question, mice were exposed to the adoptive transfer model depicted in Fig 4.3 A, with 5 x 10^6 CD4+ T cells alone, or with 5 x 10^6 WT or perforin knock out (PKO) CTLs
co-administered. A titration of the dose of OVA for the intranasal challenge was additionally performed, with mice receiving either 1, 10 or 100μg of OVA i.n., to determine whether changes occur in disease parameters that may not be evident in the presence of excess allergen.

As shown in Figure 4.4, in mice injected with CD4+ T cells and no CTL, the number of total cells and eosinophils was only marginally increased after challenge with 1μg OVA, further increased with 10μg OVA challenge, and was significantly elevated in mice challenged with 100μg OVA. In contrast, in mice co-injected with CD4+ T cells and WT CTLs, there was no increase in the number of total cells and eosinophils at any of the OVA doses used, suggesting that 10μg OVA was sufficient to elicit the suppressive activity of CTL. Co-injection of PKO CTLs had no effect on airway eosinophilia induced by CD4+ T cells. T cells, alveolar macrophages and neutrophils were not significantly different in number between groups or OVA concentrations. B cells showed a trend towards a marked decrease in number in all CTL and PKO CTL treated groups, compared to all CD4 only groups, however overall their numbers were potentially too low to be confident of the data, as they are near background levels. While there was little difference in DC numbers between 1μg OVA groups, both 10μg and 100μg treatments showed DCs were significantly decreased in WT CTL-treated mice compared to CD4 only. DC numbers were also partially reduced in 100μg OVA and PKO CTL treated mice, compared to 100μg OVA and CD4 treated mice, however, they remained significantly elevated in comparison to DCs in mice treated with WT CTL in the 100μg OVA group. Together, these data indicate that CTLs must be able to mediate cytotoxic function in order to effectively suppress airway inflammation in the adoptive transfer model. The titration of OVA i.n. showed that 100μg OVA induced the clearest separation between cell numbers in mice treated with or without CTLs.

To establish whether Th2 cytokine production was affected by CTLs, a similar adoptive transfer experiment was carried out where mice were injected with CD4+ T cells, with or
Figure 4.4
Figure 4.4: CTLs suppress inflammatory infiltrates in an adoptive transfer model of allergic airway inflammation in a manner dependent on perforin

Mice were treated as depicted in Fig 4.3 A; 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT or PKO CTLs. 1, 10 or 100μg of OVA was administered i.n one day later and inflammatory cell populations measured in BAL 3 days after airway challenge. Mean cell number in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 3-5 mice per group. *p <0.05, **p <0.01, ***p <0.001, as assessed by two-tailed, unpaired t test.

without WT CTLs, and BAL was performed 1 day after intranasal challenge, while lungs were harvested for histological examination after 3 days (Fig 4.5 A). The concentration of the characteristic Th2 cytokines IL-4, IL-5 and IL-13 were measured, all of which were elevated in BAL in the OVA-treated CD4 only group, but not in the other groups tested (Fig 4.5 B). CTL transfer significantly inhibited the production of IL-4, IL-5 and IL-13 to similar levels to those observed in PBS control mice and mice that did not receive CD4+ T cells. IFN-γ was only detected in CTL-treated, OVA-challenged mice; surprisingly IL-10 production was also induced in this group. No IL-17 response was detectable. Consistent with these observations, the lungs of mice adoptively transferred with CD4+ T cells displayed characteristic allergic histopathology with an increase in immune cell infiltrate (Fig 4.5 C, upper panel) and concomitant goblet cell hyperplasia and mucus production (Fig 4.5 C, lower panel). This response required CD4+ T cell transfer as no immune cell infiltration and no mucus production were observed in mice that received no CD4+ T cells before intranasal challenge with OVA. In addition, reduced immune cell infiltrate and no increase in mucus production were observed in mice injected with WT CTL together with CD4+ T cells. Therefore, this adoptive transfer model generates the hallmarks of allergic airway disease, similar to an in vivo sensitisation model, and can be suppressed by CTLs.

4.3.4 Time course of the infiltration of adoptively transferred CD4+ and CD8+ T cells into the lung and BAL

The adoptive transfer model provided a platform for investigating effects on, and effects mediated by, disease-driving CD4+ T cells. In this experiment, CD4 T cells were generated
Figure 4.5
Figure 4.5: Adoptive transfer of OTII cells recapitulates in vivo sensitization models of allergic airway inflammation and the inhibitory effect of allergen-specific CTL

(A) 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT CTLs. OVA or PBS were administered intranasally one day later. (B) Cytokines were measured in day 1 BAL samples by Bio-Plex. Bar graphs show mean + SEM amount of each cytokine in pg/ml, from one representative experiment of two that gave similar results, each with 5 mice per group. **p < 0.01, ***p < 0.001, as determined by one way ANOVA with Bonferroni’s post test. (C) Day 3 lung histology shows cellular infiltrate by H&E stain (upper panel) and mucus producing goblet cells by ABPAS staining (lower panel). Scale bars represent 200μm (upper panel) and 100μm (lower panel). Stained sections are from one experiment that shows representative histopathology of three experiments, each with 5 mice per group.

from OTII x B6SJ lymph node cells and CTL were generated from OTI x B6SJ cells. Both CD4+ T cells and CTLs were injected into C57BL/6 hosts and the transferred cells could be distinguished by CD45.1+ staining. As described in Fig 4.5 A, analysis of the cellular composition of the lung was carried out 3 days after intranasal allergen exposure. Representative flow cytometric staining for the identification of host and adoptively transferred CD4+ and CD8+ T cells in the lung is shown in Fig 4.6 A.

Adoptively transferred CD4+ T cells did not migrate to the lung in significant numbers when PBS was administered intranasally, but OVA allergen challenge induced markedly elevated numbers of transferred CD4+ T cells in the lung (Fig 4.6 B). The co-administration of CTLs resulted in a significant decline in adoptively transferred CD4+ T cell numbers in the lung. Interestingly, the same pattern was evident for host CD4+ and CD8+ T cells; infiltration increased in OVA-treated CD4 only groups, and this increase was significantly diminished in the presence of allergen-specific CTLs. Lung infiltrates in PBS treated groups were unaffected by cell transfer. A similar experiment showed that the suppression of infiltrating adoptively transferred CD4+ T cells in the lung was not achieved by PKO CTLs (Fig 4.6 C). Therefore, disease-mediating CD4+ T cells are present in significantly lower numbers in the lungs of mice treated with CTLs, and this suppression occurs in a perforin-dependent manner.

As the infiltration of CD4+ T cells was strongly decreased in the lungs of CTL-treated mice, the question was raised as to whether the ability of these cells to migrate and infiltrate into the lungs had been affected, and whether this dynamic was altered over time. To address
Figure 4.6
Figure 4.6: CTLs suppress the infiltration of adoptively transferred and host CD4+ and CD8+ T cells
Mice were exposed to the adoptive transfer model depicted in Fig 4.3 A. 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT CTLs. OVA or PBS were administered i.n one day later. (A) Lungs were harvested on day 3, processed to a single cell suspension and stained for the identification of adoptively transferred and host CD4+ and CD8+ T cells by flow cytometry, as depicted by representative flow panels. Panels in shaded area (far right) shows background staining in the absence of adoptively transferred T cells. (B) Mean cell number in lungs + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group. (C) 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT or PKO CTLs. Control mice received no cell transfer. OVA was administered i.n to all groups one day later and adoptively transferred CD4+ T cells identified in lungs on day 3. Mean cell number in lungs + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group. **p < 0.01, ****p < 0.0001, ns = not significant as determined by one way ANOVA with Bonferroni’s post test.

In this, a time-course was carried out to determine whether CD4+ T cells preferentially relocated to alternate tissues; BAL, lung-draining mediastinal lymph nodes (MLN), spleen and peripheral blood, and non-draining lymph nodes (brachial, axial, inguinal, cervical). An adoptive transfer experiment was carried out as previously described (Fig 4.5 A), with end points on days 1, 2, 3 and 6 after intranasal challenge for the harvesting of tissues.

The kinetics of the infiltration of CD4+ T cells into BAL resembled that of the lung. In CD4 only-treated mice, CD4+ T cells were present in low numbers on days 1 and 2, peaked at day 3, and then dropped greatly by day 6; CTL treated mice experienced only a minor increase in CD4+ T cells over time, significantly lower at the day 3 peak compared to CD4 only mice (Fig 4.7 A). Cell numbers in the lung-draining MLN were similar in both CD4 only and CD4 + CTL groups, and were raised in number during the early stage, days 1 and 2, before decreasing on day 3. Spleen tissue had the highest number of CD4+ T cells, steadily increasing from days 1 – 6 in both CD4 and CD4 + CTL treated groups. Adoptively transferred CD4+ or CD8+ T cells were not able to be detected in peripheral blood or non-draining lymph nodes at any of the time points tested (therefore, data not shown). In all tissues examined as depicted, the general trend showed CD4+ T cell numbers were lower in CTL-treated mice compared to the CD4 only group, significantly so in the lung and BAL on
Figure 4.7

A

CD4+ T cells in Lung

Cell number (x10^6)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CD4 only
CD4 + CTL

****

CD4+ T cells in BAL

Cell number (x10^3)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CD4 only
CD4 + CTL

***

CD4+ T cells in MLN

Cell number (x10^7)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CD4 only
CD4 + CTL

CD4+ T cells in Spleen

Cell number (x10^9)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CD4 only
CD4 + CTL

B

CD4+ T cells and CTLs in Lung

Cell number (x10^9)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CTLS
CD4 T cells

**

CD4+ T cells and CTLs in BAL

Cell number (x10^9)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CTLS
CD4 T cells

CD4+ T cells and CTLs in MLN

Cell number (x10^9)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CTLS
CD4 T cells

CD4+ T cells and CTLs in Spleen

Cell number (x10^9)

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

CTLS
CD4 T cells

***
**Figure 4.7: Tissue infiltration kinetics of adoptively transferred CD4+ and CD8+ T cells in vivo**

Mice were exposed to the adoptive transfer model depicted in Fig 4.5 A. 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT CTLs. OVA was administered in one day later. Lungs, BAL, MLN and spleens were harvested on days 1, 2, 3 and 6, processed to a single cell suspension and stained for the identification of adoptively transferred CD4+ T cells (A) or both CD4+ T cells and CD8+CTLs (B) by flow cytometry. (A) The effects of CTLs on the number of CD4+ T cells in tissue over time is shown; mice were treated with CD4+ T cells only, or with CD4+ and CTLs. (B) A comparison of the number of CD4+ T cells versus the number of CTLs within each tissue is shown; all mice were treated with both CD4+ T cells and CTLs. Mean cell number in lungs + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 3 mice per group. **p < 0.01, ***p < 0.001, ****p < 0.0001, as determined by two way ANOVA with Bonferroni’s post test.

day 3; therefore, CD4+ T cells do not appear to be migrating differently in the presence of CTL immunotherapy, but rather are decreased in number overall. The number of adoptively transferred CTLs infiltrating each tissue was also examined and compared to CD4+ T cell numbers within the same mice. In CD4 + CTL treated mice, the number of CTLs in the lung and BAL showed a trend of being consistently higher than the number of CD4+ T cells at the time points measured; significantly, on the day 3 peak of inflammation in the lung, CTLs outnumbered CD4+ T cells three-fold, a result not mirrored in the BAL (Fig 4.7 B). MLN and spleen did not show significant differences between cell numbers throughout the time-course, with the exception of day 1 spleen tissue where CTLs were present in higher proportion than CD4+ T cells. These data show that in CTL-treated mice, immunosuppressive CTLs are more frequent in the airways than disease-mediating CD4+ T cells; the significance of this finding is unclear. It is also not known whether this is a feature of the adoptive transfer model that may not be the case in mice exposed to in vivo sensitisation.
4.3.5 CTL immunotherapy partially reduces the proliferation of disease mediating CD4+ T cells in a model of allergic airway inflammation

As CTL immunotherapy results in a decrease in disease-mediating CD4+ T cell numbers in the airway without apparent redirection of these cells to the spleen, MLN and non-draining lymph nodes, it was investigated whether the decrease in CD4+ T cells was instead due to reduced proliferation. In vitro cultured CD4+ T cells were labelled with CellTrace Violet™ (CTV) before adoptive transfer into naïve C57BL/6 hosts, with allergen challenge carried out as per Fig 4.5 A. Three days after intranasal challenge, adoptively transferred cells were identified in lungs by flow cytometry as previously described (Fig 4.6 A) and proliferation was examined by evaluating the decrease in CTV labelling. A decrease in the strength of CTV signal could be observed in cells that had undergone proliferation, however proliferation peak analysis was not able to be carried out by analysis software as clearly separated proliferation bands were not evident in all samples, likely due to the variation in CTV retention in pre-activated cells. As such, cells at all stages of proliferation were grouped as “CTV low” and those that had undergone little or no proliferation were “CTV high” (Example of staining, Fig 4.8 A).

As there was no allergen administered intranasally in the PBS-challenged CD4 only mice, no direct stimulus for proliferation of CD4+ T cells was present in this group; therefore, the cell populations that appear CTV low comprise cells that have either undergone homeostatic proliferation upon transfer, or continued residual proliferation as a result of in vitro stimulation, as well as cells in which the CTV stain has faded or leaked out within the course of the experiment. This sets the background for comparison with all OVA-challenged groups where allergen was administered to induce proliferation. The CTV high population, on the other hand, more reliably reports cells in which no proliferation, or little proliferation, has occurred – shown in Fig 4.8 B. The proportion of CTV high CD4+ T cells in OVA-challenged CD4 only mice was reduced to a little over half (55%) that of PBS-challenged CD4 only mice, indicating that increased proliferation had occurred in the presence of OVA allergen, although this did not reach statistical significance. OVA-challenged CD4 + WT CTL-treated
In vitro cultured CD4+ T cells were stained with CellTrace Violet (CTV) before adoptive transfer into naïve mice. Allergen challenge with OVA, or PBS control, and harvesting of lungs was carried out as per Fig 4.5 A. Adoptively transferred cells were identified in lungs by flow cytometry as previously described (Fig 4.6 A). (A) Proliferation was examined by CTV staining as depicted by a representative flow panel. (B) Percentage of CTV high CD4+ T cells reveals cells that have undergone little or no proliferation. (C) CTV MFI of adoptively transferred CD4+ T cell population, comprising both CTV high and CTV low cells. Mean cell number in lungs + SEM are shown. Data are from one experiment of two that gave similar results, each with 5 mice per group. *p <0.05, **p <0.01, ns= not significant, as determined by two-tailed, unpaired t test.

mice had a proportion of CTV high cells that was double that seen in OVA-challenged CD4 only mice; this result revealed that less proliferation was occurring in WT CTL-treated mice, however, this result was again not statistically significant. Contrarily, OVA-challenged CD4 + PKO CTL-treated mice displayed a similar proportion of CTV high cells as OVA-challenged CD4 only-treated mice, showing that comparable proliferation had occurred in these groups, while WT CTLs suppressed proliferation significantly more than PKO CTL.

To account for differences in the fluorescence within groups, cells at all stages of proliferation (including both “CTV high” and “CTV low”) were grouped together and the mean fluorescent intensity of CTV was determined; the higher the MFI, the less proliferation that had occurred. Results showed that the MFI of CTV was highest in PBS-challenged mice, indicating that CD4+ T cells in this group had undergone the least proliferation (Fig 4.8 C). The MFI of CTV in OVA-challenged CD4 + WT CTL-treated mice,
while not statistically significant, was lower than PBS-challenged CD4 only-treated mice revealing that, in fact, some proliferation had occurred in CTL-treated mice. This proliferation was greater, however, in both OVA-challenged CD4 only-treated mice as well as OVA-challenged CD4 + PKO CTL-treated mice, as these groups showed significantly decreased mean fluorescent intensity of CTV compared to the PBS-challenged control, although a statistically significant increase in proliferation (depicted as decreased MFI) was not evident between WT CTL-treated mice and those that received PKO CTL or no CTL. Collectively, these data suggest that WT CTLs may be capable of partially suppressing the proliferation of CD4+ T cells in the adoptive transfer model of allergy, in a manner dependent on perforin; however, as these results were not statistically significant, this was not conclusively demonstrated.

4.3.6 The expression of activation markers on CD4+ T cells is not affected differentially by wild type CTLs, compared to perforin knock-out CTLs

It was then investigated whether CTLs had an effect on the phenotype of CD4+ T cells in vivo. The expression of key activation markers CD44, CD69, ICOS and PD-1 on adoptively transferred CD4+ T cells were examined.

As the expression of these markers is upregulated on CD4+ T cells through the process of activation during in vitro culture with DCs, initial experiments were carried out to determine the time in which these markers are decreased in expression after adoptive transfer into naïve hosts, without in vivo antigenic stimulation. A sample of cultured CD4+ T cells were analysed by flow cytometry before i.v. injection into naïve hosts, and then mice were tail bled on days 2, 4 and 7 after cells were injected (as depicted in Fig 4.9 A). Adoptively transferred CD4+ T cells were identified in blood specimens by flow cytometry, and their expression of CD44, CD69, ICOS and PD-1 was compared to that of the pre-i.v. sample. Flow analysis showed that CD44, CD69 and ICOS were all decreased by day 2 after injection, while PD-1 remained elevated throughout the 7 day time-course (Fig 4.9 B).
Figure 4.9: The expression of activation markers on adoptively transferred CD4+ T cells in the absence of intranasal allergen exposure

(A) *In vitro* cultured CD4+ T cells were adoptively transferred into naïve mice and recovered from peripheral blood 2, 4 and 7 days later; cells were identified as previously described (Fig 4.6 A). (B) The expression of CD44, CD69, ICOS and PD-1 was compared on CD4+ T cells from each time point by flow cytometric analysis. Data are from one experiment of two that gave similar results, each with 5 mice per group.

These results agree with those published by others that show activated CD4+ T cells quickly reduce their expression of these markers after the removal of antigen stimulus (203, 285).
The effects of CTLs on the activation of CD4+ T cells *in vivo* were then examined in a model similar to that depicted in Fig 4.5 A; 5 x 10^6 CD4+ T cells alone, or with 5 x 10^6 WT or perforin knock out (PKO) CTLs were co-administered to mice. After activation markers on CD4+ T cells had decreased, 2 days after i.v. transfer as described in Fig 4.9 B, a single allergen challenge of 1, 10 or 100μg of OVA was delivered intranasally; a titration carried out to determine whether activation markers were differentially affected by the dose of allergen exposure. Three days after allergen challenge, lungs were removed and the expression of CD44, CD69, ICOS and PD-1 on adoptively transferred CD4+ T cells was analysed. Results showed that both CD44 and CD69 expression were similar between all treatment groups, with no statistically significant differences between mice that received CD4 only, CD4 + WT CTLs or CD4 + PKO CTLs (Fig 4.10 A). The same was true for ICOS and PD-1 expression, with no significant difference in expression between treatment groups. Within treatment groups, however, a dose-dependent trend towards increased expression was observed with increasing allergen dose. This response, however, was no different between WT and PKO CTL-treated groups, suggesting that it may be a result of CTL cytokine production. In a similar experiment, the expression of CD49d was examined on adoptively transferred CD4+ T cells in the lung, as this integrin has been shown to mediate the migration of T cells to extravascular spaces (such as the lung). This marker additionally was found to show no difference in expression between CD4 only, CD4 + WT CTL and CD4 + PKO CTL treated groups (Fig 4.10 B). These results show that the expression of the activation markers CD44, CD69, ICOS, PD-1 and integrin CD49d on disease-mediating CD4+ T cells was not affected by WT CTL immunotherapy differentially from the effects mediated by PKO CTLs.
Figure 4.10: The effects of CTL immunotherapy on the activation status of CD4+ T cells in vivo

Mice were exposed to the adoptive transfer model depicted in Fig 4.5 A. 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT or PKO CTLs. OVA was administered i.n two days later; titrated dose (A) or standard 100ug dose (B) was used. Lungs were harvested on day 3 and processed to a single cell suspension and stained for the identification of adoptively transferred CD4+ T cells by flow cytometry. The expression of CD44, CD69, ICOS, PD-1 and CD49d was then examined and compared between treatment groups. Data are from one experiment of two that gave similar results, each with 3-5 mice per group. *p < 0.05, ***p < 0.001 as determined by one way ANOVA with Bonferroni’s post test.
4.3.7 Number of total and Th2 cytokine-producing CD4+ T cells in lung is significantly reduced by CTL immunotherapy

As CTL treatment resulted in reduced Th2 cytokines in BAL, it was investigated whether the decrease in BAL cytokine levels was due to reduced secretion by CD4+ T cells. To assess this, 4C13R-OTII CD4+ T cells were used, where the fluorescent markers AmCyan and dsRed report expression of IL-4 and IL-13, respectively. The bacterial artificial chromosome used to generate 4C13R mice (660) is depicted in Fig 4.11 A.

4C13R CD4+ T cells were adoptively transferred as described in figure 4.11 B, with or without CTLs. BAL was obtained on day 1 and day 3 after airway allergen challenge, to determine whether the 4C13R-OTII cells replicated the eosinophilia and Th2 cytokine production induced by non-reporting OT-II cells. It was found that eosinophils infiltrated in high numbers in 4C13R-OTII treated mice; this was significantly ameliorated in mice also treated with CTLs (Fig 4.11 C). Cytokine production reflected that of the OTII-induced inflammation previously reported (Fig 4.5), and a trend towards a decrease was seen in IL-4, as well as significant abrogation of IL-5 and IL-13, in CTL-treated groups (Fig 4.11 D). These results show that 4C13R-OTII cells may be used in an adoptive transfer model to generate measurable allergic inflammation in the airway, which can be suppressed by CTLs.

Cytokine-reporting CD4+ T cells in the lung were then enumerated in an adoptive transfer experiment as described in Fig 4.11 B. The fluorescent reporting of IL-4 and IL-13 was examined one day after intranasal allergen exposure, when peak cytokine production may be measured, as well as on the day 3 peak of cellular infiltration in the lung. Adoptively transferred 4C13R cells were identified, after gating on live cells, as depicted in the flow panels shown in Fig 4.12 – classified as live, SSC-A low, FSC-H low-to-intermediate, CD3+, CD4+Vα2+ cells. Representative panels showing the number of cells reporting IL-4, IL-13 and both cytokines are depicted for lung samples from day 1 (Fig 4.12 A, upper panel) and day 3 (Fig 4.12 A, lower panel).

On day 1, the number of infiltrating CD4+ T cells in the lung was not significantly different between CD4 + OVA-treated, CD4 + CTL + OVA-treated, and CD4 + PBS-treated groups, and
Figure 4.11: CTLs suppress airway inflammation induced by adoptively transferred 4C13R CD4+ T cells

(A) Bacterial artificial chromosome clone used to generate 4C13R mice (William Paul Lab); LCR - Th2 locus control region, Kif3a - gene encoding kinesin-related protein 3. dsRed reports IL-13 production and AmCyan reports IL-4 production. (B) Adoptive transfer model was carried out with 4C13R OTII CD4+ T cells injected with or without WT CTLs. (C) Eosinophils were enumerated in day 3 BAL samples by flow cytometry. (D) Cytokines were measured in day 1 BAL samples by Bio-Plex analysis. Mean cell number in BAL or mean cytokine concentration in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 5 mice per group. *p < 0.05, **p < 0.01, as determined by one way ANOVA with Bonferroni’s post test or two-tailed unpaired t test.

few of these cells were reporting cytokine production (Fig 4.12 B). Nonetheless, the number of CD4+ T cells that were reporting IL-4 and IL-13 was lower in CTL-treated mice, compared to CD4 + OVA-treated mice; a reduction in IL-4+IL-13-, IL-4-IL-13+ and IL-4+IL13+ cells was observed at this time point (Fig 4.12 B), although the decrease in IL-4-IL-
13+ cells did not quite reach statistical significance (p=0.052). At 3 days post intranasal challenge, consistent with the peak of lung cellular infiltration previously described, 4C13R Th2 cells had infiltrated the lungs in higher numbers (Fig 4.12 C) in CD4 + OVA-treated groups, compared to both CD4 + CTL and CD4 + PBS-treated groups; many of those cells were reporting IL-4 and IL-13 production. In mice where CTLs had been administered, there was a significant decrease in the number of CD4+ T cells reporting IL-4, IL-13 and those reporting production of both cytokines, compared to mice that received CD4+ T cells + OVA. These data suggest that, additional to reducing CD4+ T cells numbers in the lung, CTL immunotherapy may affect CD4+ T cells by decreasing their production of key Th2 cytokines.

**4.3.8 T regulatory cell numbers in the lung are not affected by CTL treatment at the day 3 peak of lung inflammatory cell infiltrate**

Additional to the decreased Th2 cytokines, an increase in IL-10 in BAL was measured in CTL-treated mice (Fig 4.5 B). As the production of IL-10 can stimulate CD4+CD25+FoxP3+ T regulatory (Treg) cells that may lead to the skewing of the allergic environment to a tolerogenic environment (497, 714-716), it was investigated whether there was in fact an increase in Treg cells in the lung infiltrates of CTL-treated mice. An adoptive transfer model (described in Fig 4.5A) was utilised, and lungs were harvested 3 days after intranasal allergen challenge. Lung cells were stained intracellularly with a FoxP3 antibody, or isotype control, as well as surface staining for the markers CD4 and CD25. Flow cytometric analysis revealed no difference in the proportion of Tregs in the lungs of mice treated with CD4 only, CD4 + CTL or CD4 + PKO CTL (Fig 4.13 B). Therefore, IL-10 production in CTL-treated mice does not appear to be accompanied by an increase in Tregs in the lungs at this time point; the peak of inflammatory cellular infiltrates.

The source of IL-10 was also investigated. As CTLs are themselves capable of producing IL-10 depending on environmental stimuli (717, 718), it was examined whether in vitro generated CTLs in these experiments had adopted an IL-10 producing phenotype. PKO CTL cultures were additionally tested for IL-10 production. Cells cultured for 6 days under
Figure 4.12: The number of total and cytokine-reporting CD4+ T cells in the lung is significantly reduced by CTL immunotherapy

Adoptive transfer of 4C13R CD4+ T cells with or without WT CTLs was carried out as described in Fig 4.11 B, with OVA or PBS administered intranasally 1 day later. Lungs were harvested on days 1 and 3 and processed to a single cell suspension for the identification of adoptively transferred cells. (A) Fluorescent reporting of IL-4 and IL-13 production by adoptively transferred 4C13R CD4+ T cells was analysed by flow cytometry on days 1 and 3 as depicted. The total number of transferred CD4+Vα2+ T cells in lungs and the number reporting IL-4 and IL-13 production were enumerated on day 1 (B) and day 3 (C) after intranasal challenge. Bar graphs show mean number of CD4+ T cells ± SEM. Data are from one of two experiments that gave similar results, each with 5 mice per group. *p, 0.05, **p, 0.01. ns, not significant as determined by two-tailed, unpaired t test.
conditions previously described (Chapter 2) were restimulated with OVA$_{257-264}$ antigen (CTL + Ag), or no antigen (CTL no Ag), for 4 hours or 24 hours. Culture supernatant was then sampled and IL-10 measured by Bio-Plex analysis. It was found that both CTLs and PKO CTLs were driven to produce IL-10 after 4 hours and 24 hours of *in vitro* OVA$_{257-264}$ stimulation, while cultured cells that were not restimulated with antigen did not show measureable IL-10 production (Fig 4.13 C). Therefore, the IL-10 measured in the BAL of OVA-challenged, CTL-treated mice could be produced by the CTLs themselves. However, this IL-10 does not result in an increase in Treg cells in lung inflammatory cell infiltrates, and may also be produced *in vivo* by PKO CTLs that do not inhibit allergic airway inflammation. These data suggest that IL-10 production by CTLs is unlikely to have important functional consequences in the suppression of allergy in this model.
Figure 4.13: T regulatory cell numbers in the lung are not affected by CTL treatment at the day 3 peak of lung inflammatory cell infiltrate
An adoptive transfer model (described in Fig 4.5A) was utilised, and lungs were harvested 3 days after intranasal allergen challenge. Lung cells were stained intracellularly with a FoxP3 antibody, or isotype control, as well as surface staining for CD4 and CD25. (A) Representative gating shown for determining CD25+FoxP3+ population. Doublets and dead cells removed prior to this gating, as depicted in figure 3.1. (B) Bar graphs show mean percentage of CD25+FoxP3+ cells of CD4+ T cells in the lung + SEM. (C) WT and PKO CTLs cultured for 6 days under conditions previously described (Chapter 2) were restimulated with OVA257-264 antigen (CTL + Ag), or no antigen (CTL no Ag), for 4 hours or 24 hours. Culture supernatant was then sampled and IL-10 measured by Bio-Plex analysis. Bar graphs show mean concentration of IL-10 in supernatant + SEM. Data are from one of two experiments that gave similar results, each with 3 supernatant samples per group. ***p < 0.001, ****p < 0.0001, ns= not significant as determined by one way ANOVA with Bonferroni’s post test.
4.4 Discussion

**CTLs suppress inflammation in an adoptive transfer model of allergic airway disease**

In this chapter, an experimental model of allergy was described where OTII CD4+ T cells were cultured under Th2 conditions and injected into naïve hosts, generating airway inflammation upon allergen exposure. This inflammatory response was characterised by a robust eosinophilia in BAL, the production of IL-4, IL-5 and IL-13 and lung histopathology including cell infiltrates and increased mucus production. Therefore, similar to the observations of others, (650, 651) this adoptive transfer model replicates many of the features of allergic disease that are generated by *in vivo* sensitisation protocols.

There are, however, some differences in the immunology of the allergic response in the adoptive transfer model compared to *in vivo* sensitisation. Primarily, this is due to the fact that there is no systemic sensitisation in the adoptive transfer model. Thus, the activity of transferred CD4+ T cells is the primary driving factor for the pathology observed in this model. As demonstrated in an *in vivo* sensitisation model of allergic airway inflammation (321), host APCs, importantly DCs, are also required for allergen presentation to adoptively transferred CD4+ T cells to initiate the subsequent allergic response (Daniels, Mucosal Immunology, 2015; *In press*). The allergic disease induced in this model, then, in the absence of host sensitisation, does not rely on IgE or the activation of mast cells. Indeed, IgE was not detected in serum of OTII-transferred, OVA-exposed mice, neither was mastocytosis induced in the lung tissue in the current study. As discussed in Chapter 3, IgE has an important role only where strong eosinophilia is not present (540), and mast cells do not have a critical role in the induction of allergic airway inflammation (444); as such, the absence of measurable IgE or mast cell activation in the adoptive transfer model should not have had a significant impact during the course of the experiments described.

Another important difference between the adoptive transfer and *in vivo* sensitisation models is that the number of allergen-specific CD4+ T cells injected in the adoptive transfer model may exceed that induced by classical prime/challenge protocols. The number of
allergen-specific cells used in published adoptive transfer models varies greatly depending on the purpose of the experiments; as few as $1 \times 10^6$ total cultured splenocytes (including $<1\%$ CD4$^+$CD62L$^{lo}$ cells) have been shown to generate measurable eosinophilic lung inflammation (651). Other authors implemented higher numbers ($5 \times 10^6$) as a means of comparing Th2 versus Th1-induced airway inflammation; experiments requiring a strong inflammatory infiltrate to effectively tease apart these responses (719). While an allergic response may be elicited by low numbers of CD4$^+$ T cells, the recovery and detection of allergen-specific cells from tissue may be difficult. As the present study aimed to track and investigate changes in the CD4$^+$ T cell population in response to CTL immunotherapy, it was important that a significant number of cells be recovered from tissue in order to derive meaningful differences between cells that had been transferred with or without CTLs. The titration performed in this chapter indicated the use of $5 \times 10^6$ adoptively transferred CD4$^+$ T cells; the ensuing airway inflammation being robust, reproducible, and resulting in a distinct population of allergen-specific cells being reliably recovered from multiple tissues for analysis.

CTL immunotherapy effectively prevented the induction of allergic airway inflammation in mice given CD4$^+$ Th2 cells; there was a reduction in lung eosinophilia, IL-4, IL-5 and IL-13 production, mucus hypersecretion and inflammatory infiltrates in CTL-treated mice. Therefore, the adoptive transfer model recapitulates many features of allergic airway inflammation induced by in vivo sensitisation models, as well as the associated inhibitory effect of allergen-specific CTLs.

Effects of CTLs on the number of allergy-driving CD4$^+$ T cells in the lung and airway

Associated with the suppression of allergic airway inflammation, a reduction in the number of disease-mediating CD4$^+$ T cells in the lung was consistently induced by CTL immunotherapy. This finding prompted an investigation into whether the migration, proliferation, activation or cytokine production of CD4$^+$ T cells were being affected by the treatment.
As CD4 +/- CTL-treated mice that received PBS intranasally did not show an increase in adoptively transferred T cells, this indicated that adoptively transferred cells entered the lungs by an active process, induced by allergen exposure. It is interesting, then, that there was a reduction in the infiltration of adoptively transferred CD4+ T cells as well as host CD4+ and CD8+ T cells in the lungs of CTL-treated mice.

Some studies have suggested that co-stimulatory factors play a role in the regulation of Th2-mediated airway inflammation by controlling the migration, and subsequent tissue numbers, of CD4+ T cells in the lung. CD4+ T cells that lack ICOS expression were previously found to have increased lymph node-homing receptors CCR7 and CD62L compared to WT CD4+ T cells; these cells preferentially migrate to non-draining lymph nodes rather than the lung or MLN (720, 721). In the current study, however, the decrease in the number of adoptively transferred CD4+ T cells in the lung and BAL of CTL-treated mice did not appear to be associated with an increase in these cells in the alternative tissues examined; spleen, peripheral blood, and non-lung-draining lymph nodes showed no significant difference in the number of cells recovered throughout the course of inflammation in CTL treated versus untreated groups. It was interesting that despite the elevated CD4+ T cell numbers in the lungs of mice that did not receive CTL therapy, no additional increase in CD4+ T cells in the lung-draining MLN was evident in these mice during the time course. While the migration of OVA-loaded lung APCs and the trafficking of adoptively transferred OTII cells to LNs does occur (486, 722) this may not be necessary for the stimulation of pre-activated cells. Indeed, the ability of these cells to migrate to lymph nodes depends on their expression of the lymph node homing molecules CD62L and CCR7 (274, 723, 724) and previously activated T<sub>CM</sub> and T<sub>EM</sub> cells express these molecules differently, CCR7<sup>-</sup>CD62L<sup>hi</sup> and CCR7<sup>-</sup>CD62L<sup>lo</sup> respectively (274). The majority of in vitro generated OTII cells in the present study were CD62L<sup>lo</sup> upon transfer (Fig 4.1), potentially explaining the absence of significant migration to the MLN. Rather, this trait is in common with T<sub>EM</sub> cells, which have been demonstrated to rapidly respond to antigen exposure in non-lymphoid tissues (274, 275). Harris and colleagues used an adoptive transfer model to examine the fate of in vitro-activated, adoptively transferred CD4+ T cells of differing CD62L expression, upon airway allergen challenge. Expectedly, CD62L<sup>hi</sup> cells migrated to lymph nodes, while CD62L<sup>med-lo</sup> cells
localised to non-lymphoid tissues (486). Indeed, it was demonstrated that in this model, the CD62Lmed-lo cells were capable of rapid effector cytokine production upon allergen exposure in the lung, despite being unable to traffic to the MLN.

Some studies have also shown that antigen-loaded APCs may be preferentially retained within the lung tissue and local antigen presentation to T cells may occur in situ (114, 115). Distinct differences have also been found between the interaction of T cells with lung parenchymal DCs and alveolar DCs; while parenchymal DCs are capable of activating both naïve and antigen-primed T cells, alveolar DCs are only capable of activating antigen-primed (but not naïve) T cells (725). Collectively, these studies reveal the presence of lung DCs specialised in the activation of antigen-primed T cells, and the potential for in situ antigen presentation. It is likely, then, that the pre-activated allergen-experienced adoptively transferred CD4+ T cells described in the present study may not need to be licenced in the MLN.

The decrease in CD4+ T cells in the lungs of CTL-treated mice was found to be dependent on the CTLs being capable of perforin expression. While the direct targeting and killing of CD4+ T cells is a known mechanism for the control of virally-infected cells, especially during HIV infection (726-728), it is unlikely that direct killing would occur in the case of allergen-reactive CD4+ Th2 cells. Unlike HIV infection where viral components are presented on CD4+ T cell MHC-I molecules and recognised by CD8+ T cells through their TCR, allergen-specific Th2 cells have not been reported to display allergens on their surface, or to be capable of cross-presenting allergens, so cannot be recognised for CTL-mediated killing. This result, then, suggests that a cell upstream of the CD4+ T cell response (ie, an APC) is the more likely target of CTL-mediated killing. This hypothesis is examined in Chapter 5.

While the requirement for, and contrasting effects of, CD4+ and CD8+ T cells in allergic airway inflammation have been investigated (222, 707, 729), little has been reported on the direct or consequential effects of CD8+ T cells on CD4+ T cells. One important finding in asthmatic patients, as well as experimental models, was that an increase in CD4+ T cells directly correlates with the severity of AHR; interestingly, an inverse correlation exists with CD8+ T cells (711, 730). It has also been reported, however, that both CD4+ and CD8+ T cell
populations increase in number as chronic disease progresses (731). These findings may not be conflicting, but rather implicate the involvement of temporal factors whereby the balance of CD4+:CD8+ T cells is crucial to preventing the progression of early allergic responses to fully established chronic disease. Indeed, it has been reported that blocking CD8+ T cell responses increases CD4+ T cell responsiveness to antigens, enhancing protection against pathogens (732); however, increased responsiveness may be damaging in an allergic Th2 environment. Additionally, in an experimental model where robust IgE production and a high CD4+:CD8+ T cell ratio exists, like in an allergic asthmatic patient, depletion of CD8+ T cells has been shown to worsen AHR and eosinophilic inflammation (733).

**Effects of CTLs on the proliferation and activation of CD4+ T cells**

While not statistically significant, it appeared that the proliferation of CD4+ T cells may be, in part, affected by CTLs. It has been shown that CD62L\(^{lo}\) adoptively transferred T cells that migrate to the lung, while capable of effector cytokine production, are unable to divide *in situ* (486). It is possible, then, that the moderate effect on CD4+ T cell proliferation observed in the present study reflects the effects of CTLs on the small proportion of CD62L\(^{hi}\) cells within the adoptively transferred population that would have been stimulated to divide in the lymph nodes before trafficking to the lungs. It is known that IFN-\(\gamma\) can mediate the suppression of CD4+ Th2 cell proliferation (734), however in the present study, IFN-\(\gamma\)-sufficient, perforin-deficient CTLs did not induce a similar decrease in proliferation. This, in accordance with the effects on CD4+ T cell numbers, also suggests an indirect mechanism by which CD4+ T cells are affected by CTLs.

Minimal effects were observed in the expression of activation and costimulatory receptors CD44, CD69, ICOS, PD-1 and CD49d on CD4+ T cells in mice treated with wild type CTLs, compared to those treated with perforin deficient CTLs. This implied that the activation of CD4+ T cells was not being interfered with as a mechanism of CTL-mediated suppression of airway inflammation. It was interesting however that PD-1, upregulated after *in vitro* exposure to OVA, remained elevated on adoptively transferred CD4+ T cells in the absence
of *in vivo* allergen stimulation, throughout the 7 day course of testing. PD-1 is rapidly upregulated on T cells after stimulation of the TCR (735, 736) and, consequently, upon ligation with PD-L1 or PD-L2 on APCs, T cell proliferation and effector cytokine production is inhibited (737, 738). This has been proposed to have an important role in modulating and restraining immune responses to avoid autoimmunity and pathology and maintain tolerance (739-742). In allergy, PD-1:PD-L1/2 ligation can either lead to increased or decreased airway hyperreactivity, and effects on the proliferation and cytokine production of CD4+ T cells can be influenced by the amount of antigen present as well as the cytokine environment (738, 743-745). In the presence of high antigen concentrations, the inhibitory effect on proliferation is no longer significant, neither is cytokine production diminished—especially, IL-4 production does not appear significantly restrained (738). In the present study, it is clear that adoptively transferred CD4+ T cells maintain the ability to carry out strong effector function, despite their upregulation of PD-1. This may suggest that the amount of OVA administered intranasally was strong enough to overcome the inhibitory effects elicited by PD-1:PD-L1/2 ligation.

While a dose-dependent trend in all treatment groups was observed in the titration data for PD-1 expression described in this chapter, a significant increase was not present in mice that received CD4+ T cells only. In CTL-treated groups, however, higher doses of OVA induced higher PD-1 expression on CD4+ T cells. As this occurred in both mice treated with WT CTLs and PKO CTLs, it is possibly an effect of higher allergen doses eliciting increased production of CD8+ T cell effector cytokines and molecules. One such cytokine, IFN-γ, has been shown to induce PD-L1 and PD-L2 expression on APCs (737, 746). The similarly high PD-1 expression in these two groups did not reflect the associated CD4+ T cell-mediated inflammatory response; PKO CTL-treated mice had robust Th2 allergic airway inflammation while WT CTL-treated mice showed suppression. This suggests that effects on PD-1 are not important for the amelioration of allergic airway inflammation by allergen-specific perforin-sufficient CTLs.
Effects of CTLs on Th2 cytokine-producing CD4+ T cells in the lung

The ability of CD4+ Th2 cells to mediate allergic airway disease relies heavily on their ability to produce key effector cytokines, IL-4, IL-5 and IL-13 (381, 525, 747-751); these cytokines were found to be decreased in the BAL of CTL-treated mice. In this chapter, the effects of CTL immunotherapy on the IL-4 and IL-13 production of adoptively transferred CD4+ T cells was examined. Results showed that at the day one peak of cytokine production, the number of total CD4+ T cells in the lung were similar whether mice were treated with CTLs or not; however the number of those cells that were reporting IL-4, IL-13 or both cytokines, was significantly lower in CTL-treated mice (Fig 4.12). This finding suggested that the decrease in BAL cytokine production (Fig 4.11) was not simply a consequence of a reduced number of infiltrating CD4+ T cells, but rather that the cytokine production of the cells within the lung may be suppressed. The diminution of the early cytokine response is significant as IL-4 is critical for the recruitment of Th2 cells to the lung (650), while IL-13 has an essential role in generating airway resistance and mucus production (525, 680, 681). This reduction in CD4+ T cell recruitment would prevent the amplification of Th2-mediated pathology, thus restraining the allergic response. Indeed, by day three, the number of total and cytokine-reporting CD4+ T cells in the lung were, additionally, effectively reduced by CTL immunotherapy. Together these findings suggest that the early stimulation of CD4+ T cells may have been insufficient to drive significant allergic airway inflammation, perhaps implying inadequate interaction with antigen presenting cells. This hypothesis will be examined in Chapter 5.

Conclusions

In this chapter, an adoptive transfer model of allergic airway inflammation was established and characterised; key features of pathology induced by in vivo sensitisation were replicated within this model and allergy-driving CD4+ T cells were tracked. Investigation of the effects of CTL immunotherapy on allergy-driving CD4+ T cells revealed that effective abrogation of allergic airway inflammation was associated with a decrease in CD4+ T cell numbers in the lung. This appeared to be predominantly due to decreased Th2 cytokine production during the early stage of the response to allergen exposure, and in part by a
reduction in CD4+ T cell proliferation. These results suggest that in the presence of CTL immunotherapy, CD4+ T cells may receive insufficient stimulation by antigen presenting cells, rendering them incapable of effectively driving allergic airway inflammation.
Chapter 5

Allergen-specific CTLs target CD103+ and CD11b+ lung DCs to suppress allergic airway inflammation
5.1 Introduction

The role of CD8+ T cells in allergic asthma is not well understood, and has been a point of some controversy, with studies reporting both exaggerative (227-229) and suppressive (220-226) activity on airway inflammation, as discussed in Chapter 1. Previous work in our lab highlighted the critical requirement for perforin in successful amelioration of acute disease (226). Further to the work of Enomoto et al, Chapter 3 of this thesis describes long term preventative and therapeutic effects of allergen-specific CTLs in a multiple allergen exposure model of allergic airway inflammation; these effects were also dependent on perforin. It was thus apparent that CTL-mediated killing is critical for the suppression of airway disease, however, further investigation was required to identify the cellular targets.

The localisation of CD8+ T cells in the allergy-inflamed lung has not been reported, nor has the interaction of CTLs with lung DCs during active disease been elucidated. Critical to the induction of the allergic response, dendritic cells stimulate Th2 responses in the lung that culminate in airway inflammation (321). While DCs may elude being killed by CTLs to enable continued antigen presentation (752-754), CTL-mediated killing of DCs in vivo has also been demonstrated (755-757). Additionally, a decrease in DC numbers in the lung-draining MLN of CTL-treated mice has been reported (226), a finding that proposes the possibility that allergen-specific CTLs kill allergen-presenting DCs. This may result in the suppression of CD4+ Th2-driven inflammation.

Recent advances in inflammatory and conventional DC subset-distinguishing markers, lung imaging technology, and the development of transgenic mice that display fluorescence in DC populations have provided new insights into the localisation and functional activities of DCs in the lung and MLN. Critical to the induction of secondary Th2 responses to aeroallergens (493), the depletion of CD11c+ DC during allergen challenge of sensitised mice ameliorates inflammation (321), revealing their necessity in the effector response. CD11b+ cDCs have recently been examined through the use of CD11c<sup>eYFP</sup>-expressing mice (652), counterstained with CD11b-APC, to reveal their distribution within the lung. Following allergen challenge of sensitised mice, CD11b+ DCs increase in number and localise within the vicinity of allergic airways (494). Conversely, little is known about the
localisation of the CD103+ lung DC population in allergic disease, this subset being specialised in cross-presentation to CD8+ T cells (123, 126). It has been reported that these DCs are primarily found in the vicinity of the airway mucosa and arteriole walls (320), however, further study is required to elucidate their redistribution, function and interactions with T cells during the course of allergic airway inflammation. Expression of XCR1 identifies this CD103+ DC population (655, 758), and the recent generation of XCR1venus-expressing (653) and novel XCR1GFP-expressing reporter mice (Generated by Okada, Kaisho, et al; Daniels, Mucosal Immunology, 2015 In Press) provide invaluable tools for the examination of these DC in the lung.

Dendritic cell vaccines that elicit endogenous antigen-specific CTL-mediated immune responses have been widely proven effective in anti-tumour immunity (602-605), additionally, immunisation with allergen-pulsed DCs has been shown to elicit an immune response that mediates suppression of allergic airway disease in a similar manner to the direct adoptive transfer of CTLs (226). The present study elucidated the preventative and therapeutic effects of allergen-specific CTLs on allergic airway inflammation, in accordance with effects previously reported (220, 222-224, 226), suggesting a CTL-inducing vaccine may be effective at mediating the suppression of allergy. To this end, a self-adjuvanting glycolipid-peptide conjugate vaccine was recently generated through collaboration between the Malaghan Institute of Medical Research and the Ferrier Research Institute, as recently described (668). This vaccine consists of the OT-I CD8+ T cell-specific peptide OVA257-264 conjugated to α-galactosylceramide (α-GalCer) by a linker that incorporates cleavage sites. Upon i.v. administration, the conjugated vaccine remains inactive until the linker is cleaved; the specific defined enzymatic cleavage sequences were designed to preferentially release the active components of the vaccine within APCs. Upon release, both the OVA257-264 peptide and α-GalCer may be processed for presentation by APCs. The α-GalCer binds CD1d to trigger activation of group 1 NKT cells (759), recruiting their ‘help’ for the activation of APCs such as DC. An endogenous CD8+ T cell response may then be initiated upon presentation of the OVA257-264 peptide. Therefore, this vaccine may potentially have therapeutic effects similar to those mediated by adoptively transferred CTLs. The effects of this vaccine on allergic airway inflammation are investigated in this chapter.
5.2 Aims

The purpose of this chapter was to investigate the mechanism of action of perforin-dependent allergen-specific CTL immunotherapy of allergic airway disease, with a specific focus on determining the cellular targets of CTL-mediated killing. Additionally, the localisation and interaction of CD4+ T cells and CTLs with lung DC subsets in the inflamed lung was examined, to investigate killer-target interactions. Furthermore, it was purposed to determine whether a peptide conjugate vaccine- initially designed to elicit an endogenous CTL-mediated anti-tumour response- may be effective in the suppression of allergy. As the expression of perforin is critical to the effective suppression of disease, and the depletion of lung CD11c+ DC results in amelioration of asthma, the hypothesis was that CTL-mediated killing of allergy-driving DC may be occurring.

The specific aims were:

• To determine the targets of CTL-mediated killing
• To elucidate the localisation of cross-presenting CD103+ DCs in the lung during allergic airway inflammation
• To examine the interaction of CD4+ T cells and CTLs with DC populations within the lung
• To assess the capability of an allergen-specific peptide conjugate vaccine to ameliorate allergic airway inflammation
5.3 Results

5.3.1 CTL therapy increases the expression of pro-apoptotic caspases in DCs

To determine the potential cellular targets of CTL-mediated killing in OVA-challenged mice, the fluorogenic substrate FLIVO was used to directly identify pre-apoptotic cells expressing active caspases in vivo. An in vivo sensitisation model of allergic airway inflammation was used (Fig 5.1 A) so that all induced cell populations may be examined. Lung and mediastinal lymph nodes (MLN) were extracted and the cellular composition determined by flow cytometry. The proportion of MLN cells displaying active caspases revealed that these were increased in the CTL-treated group compared to the OVA/Alum and PKO CTL-treated groups (Fig 5.1 B), indicating that the increase in apoptotic cells was dependent on the administration of cytolytic perforin-sufficient CTLs. In lymph nodes, cells undergoing apoptosis displayed increased fluorescence (Fig 5.1 C, upper panel), distinguishable from samples without FLIVO staining (Fig 5.1 C, lower panel), as depicted in representative flow panels. Unfortunately, the presence of autofluorescent cells made the identification of apoptotic cells in lung tissue difficult, therefore this tissue could not be further examined. Upon further analysis of the FLIVO+ cells in CTL-treated mice, it was discovered that the majority of these cells showed the FSC/SSC and CD11c+MHCII+ characteristics typical of dendritic cells (Fig 5.1 D, upper panel); this population was highly overrepresented in the FLIVO+ population, making up 66.1% of the cells compared to the total lymph node cells in which DCs only account for 3.4% (Fig 5.1 D, lower panel). Additionally, about 63% of FLIVO+ DCs were MHCIIhi, characteristic of migratory DCs, compared to 45% in the total DC population (Fig 5.1 E, upper and lower panels). These results suggest that targeted cell killing occurs in WT CTL-treated mice, and a migratory DC population in the MLN is preferentially affected.
Figure 5.1: Increased activation of pro-apoptotic caspases in DCs after CTL therapy

(A) Mice were sensitized by i.p. injection of 2μg OVA in alum adjuvant and challenged with OVA by intranasal instillation according to the indicated schedule. OVA-specific CTL were given 1 day before OVA challenge. One day after OVA challenge, mice were treated with FLIVO to identify apoptotic cells, and were sacrificed 3 h later. (B) The proportion of apoptotic cells in the mediastinal lymph node was determined by FLIVO staining as shown in (C). The bar graph shows the mean proportion apoptotic cells of live cells in the MLN for 3-5 mice/group, + SEM. Data are from one of two experiments that gave similar results. ***p<0.001 as determined using one way ANOVA with Bonferroni correction. (C) Identification of apoptotic cells by flow cytometric analysis of mediastinal lymph nodes.
from FLIVO-treated (upper panel) and untreated mice (lower panel). DAPI-high cells were gated out before the apoptotic cells were identified and gated. (D) Characterization of the apoptotic population by flow cytometry. FLIVO+ cells gated as in (C) were examined for expression of CD11c and MHCII/3JP (upper panel). Expression of CD11c and MHCII in the total LN population is shown as a comparison (lower panel). (E) The proportion of MHCII-high cells (lung-derived DC) in the FLIVO+CD11c+MHCII+ population was determined (upper panel); the enrichment of this population was compared to the total MHCII-high cells in the LN (lower panel). Data are from one of two experiments, each with 3-5 mice per group, that gave similar results.

5.3.2 CTLs elicit a perforin-dependent decrease in lung DCs

To determine whether CTLs also target DCs in the lung, an adoptive transfer model of allergic airway inflammation was carried out as described in Fig 5.2 A, where mice received 5 x 10⁶ CD4+ Th2 cells, with or without 5 x 10⁶ WT or PKO CTLs. A single cell suspension was generated from lungs one day after intranasal challenge and the numbers of DCs were examined. Steady state CD11b+CD64- and CD103+ DCs, as well as inflammatory CD11b+CD64+ monocyte-derived DCs were identified by flow cytometry as depicted (Fig 5.2 B). Mice treated with WT CTLs, but not PKO CTLs, showed a decrease in all three DC subsets compared to the CD4 only group (Fig 5.2 C). These data suggest that CTL therapy causes a perforin-dependent decrease in DC numbers in the lung.

The expression of CD86 and ICOS-L on lung DC subsets was additionally examined in experiments utilising the adoptive transfer model (described in Fig 5.2 A), with the dose of OVA titrated to elucidate changes occurring in the presence of low to high allergen concentration. An antigen-dependent increase in CD86 expression was apparent on both steady state populations, the CD103+ and CD11b+CD64- DCs, in all treatment groups, but did not appear to increase in CD11b+CD64+ DC (Fig 5.3). In mice that were not treated with T cells, but received 100μg OVA intranasally, the expression of CD86 on CD11b+CD64- and CD11b+CD64+ DC populations appeared similar to that of T cell-treated mice that received 100μg OVA. The increase in CD86 expression was therefore dependent on the concentration of antigen administered to the airway. The rapid upregulation of CD86 in response to antigen and increasing cytokine stimulation by IL-4 and GM-CSF has been
Figure 5.2: CTLs elicit a perforin-dependent decrease in lung DCs

(A) C57BL/6 mice were injected with activated CD4+ T cells and CTLs as depicted, and challenged with OVA by i.n. instillation 1d later. Lung DC were examined 1d after i.n. OVA challenge. (B) Representative gating of lung DC by CD11c and MHCII staining, and identification of CD11c+CD103+, CD11c+CD11b+CD64- and CD11c+CD11b+CD64+ DC subsets by flow cytometry. FMO, Fluorescence Minus One (Lower right panel) for APC staining shows distinction between CD64+ and CD64- cells. (C) Numbers of CD103+, CD11b+CD64- and CD11b+CD64+ DC per lung were determined as shown in (B). Bar graphs show mean DC numbers ± SEM in the indicated experimental groups. Data are from one of two experiments, each with 3-5 mice per group, that gave similar results. **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant, as determined using one way ANOVA with Bonferroni correction.
reported by others (760-762) additionally, histamine can exert similar effects (763). The expression of CD86 on the CD103+ DC population in mice not treated with T cells, however, did not increase above that which was evident in T cell-treated mice that had been given 1μg OVA. This result may suggest that CD103+ DC, that localise near to the airway wall, have increased antigen uptake in response to the presence of inflammatory cells in the airway, more so than CD11b+ DC.

While not statistically significant, ICOS-L showed an opposite trend to the expression of CD86; ICOS-L decreased in expression with increasing allergen exposure in all three DC subsets, across all treatment groups. In general, the MFI of ICOS-L expression was similar in CD103+ and CD11b+CD64- cDC subsets, while CD11b+CD64+ DC expressed higher levels; this was in contrast to the MFI of CD86 expression, which was highest overall in CD103+ DCs, while CD11b+CD64- and CD11b+CD64+ populations displayed similar levels.

In terms of the expression of ICOS-L and CD86 in relation to T cell administration, there were no significant differences detected in the expression of these molecules at this time point for any of the DC subsets, when comparing mice treated with CD4 only, CD4 + CTL or CD4 + PKO CTL. These data suggest that, while some DC subpopulation and antigen concentration-dependent differences exist, co-treatment with WT or PKO CTLs did not appear to affect the expression of ICOS-L and CD86, compared to treatment with CD4+ T cells only.

5.3.3 CTL therapy targets CD11c+CD11b+CD64- conventional lung dendritic cells

As shown in Fig 5.2, DC numbers increase in the lung during allergic airway inflammation, a phenomenon that may be suppressed in response to treatment with CTLs. To determine whether the decrease in DC numbers in CTL-treated mice was related to an overall decrease in lung inflammation, or whether specific deletion of DCs was occurring, an experimental model was devised where DC viability could be compared among mice where airway
**Figure 5.3: The effects of CTL immunotherapy on the activation status of DC populations in vivo**

Mice were exposed to the adoptive transfer model depicted in Fig 5.2 A. 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT CTL or PKO CTLs. OVA was administered i.n 1d later, titrated in dose. Lungs were harvested 1d after i.n, processed to a single cell suspension and stained for the identification of DC subsets by flow cytometry as described in Fig 5.2 B. The expression of ICOS-L and CD86 were then examined and compared between treatment groups. Data are from one experiment of two that gave similar results, each with 3-5 mice per group. *p < 0.05, **p < 0.01 as determined by one way ANOVA with Bonferroni’s post test.
inflammation would be expected to be similar. For this purpose, mixed bone marrow chimeras were generated using bone marrow cells from wild type H2-Kb (WT CD45.1+) mice mixed with bone marrow from H2-Kbm1 (bm-1 CD45.2+) mice. Naïve recipient B6-SJ mice were depleted of CD4+ and CD8+ T cells as well as NK cells by intraperitoneal injection of anti-GK1.5, anti-CD8 and anti-NK1.1 one day before bone marrow transfer, as well as one day after bone marrow transfer. This was to avoid the development of T cell or NK cell-mediated attacks between donor bone marrow and the host. DCs that express the mutated H2-Kbm1 MHC-I molecule are unable to interact with OVA-specific CTLs, but express wild type MHC-II, thus retaining the ability to present antigen to CD4+ T cells (Fig 5.4 A). The success of chimerism was examined six weeks after the bone marrow mix was transferred into irradiated recipient mice. A peripheral blood sample was taken and examined by flow cytometry for the presence of WT CD45.1+ and bm-1 CD45.2+ cells, as well as B220+, CD11b+, CD4+, and CD8+ subsets. At this time, both CD45.1+ and CD45.2+ populations in the blood predominantly consisted of B220+ cells (Fig 5.4 B), as well as CD11b+ cells. CD4+ and CD8+ cells were detected in much fewer numbers. As the B220+ cells were the most prevalent population at this time point, these cells were gated and examined to determine the degree of chimerism in individual mice. In the majority of mice, WT and bm-1 cells were approximately equal in proportion (Fig 5.4 C). Where the proportion of cells from one bone marrow to the other was greater than 60% to 40%, those mice were excluded from further experimentation due to inequivalent reconstitution; that is, mouse numbers 6, 7, 8 and 14 in the depicted experiment (Fig 5.4 C).

To investigate the effects of CTLs on DC populations, chimeric mice were injected with activated CD4+ T cells alone or co-transferred with CTLs as described in Fig 5.5 A and sacrificed 1 day after allergen challenge. The ratio of WT to bm-1 DCs in the lungs and mediastinal LN was determined by flow cytometry. To account for potential variations in the degree of chimerism in individual mice, this ratio was normalised to the CD45.1+/CD45.2+ ratio of total CD11c+ cells in the spleen of each mouse, where inflammation was not expected to significantly alter cell proportions. CTL treatment resulted in decreased ratios of WT to bm-1 DCs in the CD103+ as well as CD11b+CD64- populations in the lung, but was only statistically
Mixed bone marrow chimeras were generated by injecting a mixture of bone marrow cells from wild type B6-SJ (WT CD45.1+) and H2-Kbm-1 (bm-1 CD45.2+) mice into irradiated B6-SJ hosts. (A) WT DCs interact with both CD4+ T cells and CTLs, however, DCs that express the mutated H2-Kbm-1 MHC-I molecule are unable to interact with OVA-specific CTLs, but express wild type MHC-II, thus retaining the ability to present antigen to CD4+ T cells. Airway inflammation is thus maintained in chimeric mice. (B) The success of chimerism was examined six weeks after the bone marrow mix was transferred into irradiated recipient mice. Peripheral blood samples were examined by flow cytometry for the presence of WT CD45.1+ and bm-1 CD45.2+ cells, delineated into B220+, CD11b+, CD4+, and CD8+ subsets. Graphs show the proportion of cells of each subset present in individual mice. (C) The most prevalent cells at this time point, B220+, were gated and examined to determine the proportion of each bone marrow type that reconstituted individual mice. Mice numbers 6, 7, 8 and 14 were excluded from further experimentation due to inequivalent reconstitution of WT to bm-1 cells. Data are from one experiment of two that gave similar results at this time point, with 20-60 mice tested in each experiment.
Figure 5.5: CTL immunotherapy targets CD11c+CD11b+CD64- conventional lung dendritic cells

Wild type B6-SJ and bm-1 mixed bone marrow chimeras were generated and tested for chimerism as described in Fig 5.4. (A) Chimeric mice were injected with activated CD4+ T cells and CTLs and challenged with OVA i.n. as depicted. DC populations in lung and MLN were examined by flow cytometry 1d later. (B) Normalized ratio of WT (CD45.1+) to bm1 (CD45.2+) DC in the lung of chimeric mice. DC subsets were identified by flow cytometry as in Figure 5.2; ratios were normalized to the ratio of CD45.1/CD45.2 DC in spleen to account for any differences in the take of BM cells in individual mice. Bar graphs show mean ratio ± SEM; each symbol represents one mouse. (C) As in (B), except that normalized ratios of DC in MLN are shown. *p<0.05, **p<0.01, ****p<0.0001 as determined using the one way ANOVA assay with Bonferroni's correction.
significant in the CD11b+CD64- DCs (Fig 5.5 B). No difference was apparent in the CD11b+CD64+ inflammatory DC population. Similar changes were observed for each of the DC populations in the MLN, where significant decreases in both CD103+ and CD11b+CD64- cDC were evident in CTL-treated compared to CD4 only-treated mice; again no difference was observed in the CD11b+CD64+ DC population (Fig 5.5 C). These results suggest that CD103+ and CD11b+CD64- cDCs are specifically targeted by CTL treatment.

5.3.4 DCs form clusters with CTLs near small airways in the lung during allergic airway inflammation

Data in Figure 5.5 suggest that CTL can target both CD103+ and CD11b+ DC subsets in the lung. To obtain further evidence of this, the interaction of CTL populations with DC subsets in lung tissue was investigated. To elucidate the localisation and interaction of CTLs and DCs, XCR1<sup>GFP</sup> mice were primed with OVA/Alum or Alum only; the XCR1<sup>GFP</sup> marker identifying CD103+ DC (758). In a modified in vivo sensitisation model, naive XCR1<sup>GFP</sup> mice were intraperitoneally primed with a single 100μg dose of OVA in Alum adjuvant, followed 7 days later by airway allergen challenge with 100μg OVA (depicted Fig 5.6 A). Tandem-dimer Tomato (TdT) fluorescent CTLs were administered to one group of mice, the day before airway allergen exposure. One day after intranasal challenge, lungs and MLN were harvested to elucidate the localisation of DC and T cells in tissue. The examination of fresh lung tissue slices revealed that in OVA/Alum primed mice, 18 hours after airway challenge, XCR1<sup>GFP+</sup> DC had redistributed to form large clusters; TdT CTLs appeared to preferentially localise to the same areas (Fig 5.6 B). The migratory XCR1<sup>GFP+</sup> DCs were also visualised in the MLN and could be seen interacting with CTLs at this site (Fig 5.6 C). To confirm allergic airway inflammation was present in the airway using this experimental procedure, BAL was performed 3 days after intranasal allergen exposure and inflammatory cell infiltrates examined. The resulting inflammatory pattern was similar to that expected of the two-prime in vivo sensitisation model used throughout this thesis. Total cellularity was increased in CD4+ T cell treated mice, which was lower in CTL treated mice, although not statistically significantly so, while eosinophils and CD4+ T cells in BAL were significantly reduced by CTL therapy (Fig 5.6 D).
**Figure 5.6: XCR1\textsuperscript{GFP}+ DCs redistribute to form clusters in the allergic lung and MLN**

(A) XCR1\textsuperscript{GFP} mice were primed with OVA/Alum or Alum only. Tandem-dimer Tomato (TdT) fluorescent CTLs were then adoptively transferred and OVA intranasal challenge carried out 1d later. After 18 hours, lungs and MLN were harvested. (B) Fresh lung tissue was sliced and examined for localisation of GFP+ DCs and TdT+ CTLs. (C) MLN were frozen in OCT media, 8μm cryosections were mounted onto glass slides and counterstained with anti-GFP AF488 and DAPI for examination. Cell populations are identified by the colours noted on each image. Scale bars (white) represent 200μm. (D) A small separate cohort of mice were used to check inflammatory cell infiltrates in BAL on day 3, after being treated with CD4+ T cells with or without CTL as previously described (Fig 5.2 A). Mean cell number in BAL + SEM are shown for 1-3 mice per group.

To examine the distribution of DCs and CTLs within Airways, frozen lung tissue was sectioned and additionally stained with CD11c-APC for the identification of CD11c+XCR1-DC, which primarily consist of CD11b+ lung DC. Analysis revealed that there was little or no DC clustering around the larger Airways; XCR1\textsuperscript{GFP} DC preferentially lined the airway wall,
while CD11c+XCR1- DC were distributed more diffusely (Fig 5.7 A). CTLs did not frequently appear to be in close association with DCs around the large airways. Higher magnification of the small airways, however, showed that both XCR1\textsuperscript{GFP}+ and CD11c+XCR1- DC clustered at these sites; additionally, TdT CTLs were found in the same clusters (Fig 5.7 B). These results suggest that allergic airway inflammation induces DC clustering in the vicinity of small airways in the lung, and that CTLs preferentially migrate to these DC-abundant sites.

### 5.3.5 CTLs and CD4+ T cells co-localise with both XCR1\textsuperscript{GFP}+ and CD11c+XCR1- DCs in the lung

CD103+ DC are specialised at cross-presentation to CTLs (123), however, less is known about the importance of CD11b+ DC:CTL interactions in the allergic lung. Data from chimera experiments revealed that the CD11b+ cDC population, critical mediators of allergic airway disease (323), are in fact decreased in CTL-treated mice (Fig 5.5). To determine whether CTLs were directly interacting with CD11b+ DC, CD103+ DC, or both, mice were primed and challenged with OVA/Alum as previously described (Fig 5.6 A) and lung sections were analysed for co-localisation. Throughout the lung tissue, TdT CTLs were found in direct association either XCR1\textsuperscript{GFP}+ DC (Fig 5.8 A), or CD11c+XCR1- DC (Fig 5.8 B), suggesting that in fact both DC subsets were interacting with CTLs.

To investigate the location of disease-mediated CD4+ Th2 cells in the lung, non-fluorescent CD4+ T cells were injected either alone, or co-transferred with TdT-expressing CTLs into XCR1\textsuperscript{GFP} x CD11c\textsuperscript{YFP} mice as depicted in Fig 5.9 A. These double-positive XCR1\textsuperscript{GFP} x CD11c\textsuperscript{YFP} mice dually reveal both XCR1+ (CD103+) and other CD11c+ (CD11b+) DC populations. Intranasal OVA was administered and, 18 hours later, fresh lung tissue slices were stained with anti-CD45.1 and examined by confocal microscopy; the CD45.1 staining identifies adoptively transferred non-fluorescent CD4+ T cells, and partially stains TdT CTLs. It was discovered that CD4+ T cells also localised to the areas of DC clustering (Fig 5.9 B, CD4 only...
Figure 5.7: CTLs are located sparsely around large airways in the lung; more predominantly clustering with XCR1\(^{\text{GFP}^+}\) and CD11c\(^+\)XCR1\(^-\) DC around small airways

*In vivo* sensitisation and challenge of XCR1\(^{\text{GFP}^+}\) mice as well as administration of Tdt-expressing CTLs was carried out as described in Fig 5.6 A. Lungs were frozen in OCT media, 8\(\mu\)m cryosections were mounted onto glass slides and stained with anti-GFP AF488, CD11c-APC and DAPI for examination. (A) Tissue sections from OVA-challenged mice show that XCR1\(^{\text{GFP}^+}\) DC localize around the epithelia of large airways, whereas CD11c+XCR1\(^-\) DC are spread throughout the lung parenchyma. (B) High magnification images of tissue sections from OVA-challenged mice show the clustering of XCR1\(^{\text{GFP}^+}\) DC, CD11c+XCR1\(^-\) DC and CTL to the inflamed areas around the small airways. Cell populations are identified by the colours noted on each image. Scale bars (white) represent 200\(\mu\)m (A) and 100\(\mu\)m (B).
panel), similar to CTLs (Fig 5.9 B, CD4 + CTL panel, and Figs 5.6 and 5.7). Upon higher magnification of the DC clusters in mice treated with both CD4+ T cells and CTLs, it was apparent that both CTLs and CD4+ T cells co-localised with XCR1\textsuperscript{GFP+} DCs as well as CD11c+XCR1- DCs (Fig 5.9 C); panels in Fig 5.9 C show Tandem tomato CTLs (left), CD45.1+ CD4+ T cells and some staining of CTLs (middle), and the merged image (right) reveals CD4+ T cells as solely yellow stained, while CTLs appear red-yellow. BAL was performed on a small number of separate mice, 1-3 from each group, on day 3 after intranasal challenge, to check whether robust airway inflammatory infiltrates were induced in these experiments. Unfortunately, and potentially due to differing lab and experimental conditions (as discussed in Chapter 5.4 Discussion), BAL inflammation was not established comparable to that previously seen using the adoptive transfer model. Mice treated with CD4+ T cells only had very low BAL cellularity, with few eosinophils or CD4+ T cells (Fig 5.9 D), while mice treated with CD4+ T cells and CTLs did experience some inflammation including an increase in total cells, eosinophils and CD4+ T cells in BAL, although this was still much lower than expected. While the BAL data appears to suggest that the adoptively transferred CD4+ T cells were not effective at establishing allergic airway inflammation, it is evident from the airway histology that the CD4+ T cells were capable of migrating to the lung and interacting with DCs.

Together, these results support the findings reported in Figures 5.2 and 5.5: CTLs can target both CD103+ and CD11b+ lung DC subsets. This occurs in zones of abundant DC gatherings, where antigen presentation to CD4+ T cells may also be taking place.
**Figure 5.8: CTLs interact with both XCR1\(^{GFP}\) DC and CD11c\(^{+}\)XCR1\(^{-}\) DC**

*In vivo* sensitisation and challenge of XCR1\(^{GFP}\) mice as well as administration of Tdt-expressing CTLs was carried out as described in Fig 5.6 A. Lungs were frozen in OCT media, 8\(\mu\)m cryosections were mounted onto glass slides and stained with anti-GFP AF488 and CD11c-APC for examination. **(A)** Tissue section showing Tdt-CTL mainly co-localizing with XCR1\(^{GFP}\) DC. **(B)** Tissue section showing Tdt-CTL mainly co-localizing with CD11c\(^{+}\)XCR1\(^{-}\) DC. Cell populations are identified by the colours noted on each image. Scale bars (white) represent 100\(\mu\)m (left panels) and 50\(\mu\)m (right enlarged panel).
**Figure 5.9: CD4+ T cells also localise within DC/CTL clusters**

(A) Non-fluorescent CD4+ T cells were injected either alone, or co-transferred with Tandem-Tomato expressing CTLs into XCR1GFP x CD11cYFP mice as depicted. Intranasal OVA was administered and lungs harvested 18h later. Fresh lung sections were stained with anti-CD45.1 Cy5 and examined by confocal microscopy. The CD45.1 staining identifies adoptively transferred non-fluorescent CD4+ T cells, and partially stains Tandem Tomato CTLs. (B) Three separate lung sections are depicted, one from each group as noted. CD45.1+ CD4+ T cells cluster with DCs (CD4 only panel) similarly to CTLs clustering with DCs (CD4 + CTL panel). (C) A single lung section is depicted, from a CD4 + CTL treated mouse. Panels show the presence of Tdt-expressing CTLs (left, red), and CD45.1-stained CD4+ T cells (centre, yellow) interacting with XCR1GFP+CD11cYFP+ DCs (turquoise) and XCR1GFP- CD11cYFP+ DCs (dark blue). The image on the right shows an overlay of the CTL and CD45.1 images. CTLs show dual red (centre) and yellow (surface) staining, while CD4+ T cells appear yellow only. Scale bars (white) represent 100μm (B) and 25μm (C). (D) A small separate cohort of mice were used to check inflammatory cell infiltrates in BAL on day 3. Mean cell number in BAL + SEM are shown for 1-3 mice per group.

### 5.3.6 OVA-specific CTLs abrogate allergic airway inflammation in OVA/HDM dually sensitised mice

Many individuals, both children and adults, who suffer from allergy are sensitised to multiple allergens (22, 578, 579). As CTLs target allergen-presenting DCs, this may diminish the response to other allergens, particularly as the DC population critical to driving allergic responses, CD11b+ cDC, are affected. To investigate this, the effect of OVA-specific CTLs in mice sensitised to both OVA and house dust mite (HDM) was examined.

A common cause of allergic airway inflammation in humans, HDM may be used in experimental models of allergic disease and causes sensitisation directly through airway exposure without the requirement for exogenous adjuvants or systemic priming (373, 619). Therefore, this model allergen was chosen as a contributory agent for allergic airway inflammation, which might be expected to present as a cause of allergy in polysensitised individuals. As C57BL/6 mice are not often used in HDM models of airway allergy, due to their reduced propensity for the development of allergic disease as compared to BALB/c hosts (628), it was necessary to establish an HDM model of allergy in C57BL/6 mice that generates sufficient airway inflammation in which to test the effects of OVA-specific CTLs.
To this end, mice were sensitised by intranasal instillation of 100μg HDM in 50μL PBS on day 0, followed by intranasal challenges with 25μg HDM in 50μL PBS that were administered either once, on day 14, or three times, on days 14, 15 and 16 (Fig 5.10 A). Control mice received PBS alone. Mice were sacrificed 24 hours after the last challenge, on day 15 or 17, and BAL was performed. The total cellularity in BAL was higher in mice that had three HDM challenges than those that received one HDM challenge, although this did not reach statistical significance, while PBS treated groups showed no difference in cellularity when administered either one or three PBS challenges (Fig 5.10 B). Importantly, eosinophilia was increased 10-fold in mice given three HDM challenges, compared to the PBS control, while those that received a single HDM challenge had a 3-fold increase in eosinophilia, which was not statistically significant. This finding suggests that carrying out three intranasal exposures to HDM, rather than one, results in allergic inflammation that is more distinguishable from PBS controls.

Interestingly, additional HDM challenges did not appear to have a cumulative effect on neutrophil infiltration; a robust neutrophilia was induced by a single HDM challenge and was not significantly different between HDM-challenged groups. As neutrophil infiltration in this model is likely due, at least in part, to the endotoxin content of the HDM preparation, it is possible that the infiltrating neutrophils are an acute phase response to the endotoxin challenge, which does not result in active accumulation of additional neutrophils as the HDM-induced allergic response progresses. DC numbers, while low overall, were significantly higher in mice exposed to three HDM challenges compared to those that received one HDM challenge, and a strong increase in B cells was observed in the 3 x HDM group alone. The numbers of alveolar macrophages and T cells were similar between HDM-challenged groups. These results suggest that the three-challenge model is better for testing the effects of CTL immunotherapy as this protocol generated the greatest disparity in eosinophilia between HDM and PBS treated groups, with increases also observed in DCs and B cells.

To investigate the effects of OVA-specific CTLs on allergic airway inflammation in dually sensitised mice, mice were primed with HDM alone or both OVA and HDM; all mice were given three HDM challenges as well as a single OVA intranasal challenge (Fig 5.11 A). One
Figure 5.10: HDM-induced allergic airway inflammation in C57BL/6 mice

(A) Mice were sensitised with HDM i.n. on day 0 and challenged i.n. on day 14 alone, or on days 14, 15 and 16; control mice were given PBS. Black arrows refer to the single PBS/HDM challenge and end point, while blue arrows refer to the triple PBS/HDM challenges and end point. Bar graphs are correspondingly coloured. (B) Total cells, eosinophils, neutrophils, DCs, B cells, alveolar macrophages and T cells were enumerated in BAL on day 15 for mice challenged once, and day 17 for mice challenged thrice, by flow cytometry. Mean cell number in BAL + SEM are shown. ns = not significant, *p < 0.05, **p < 0.01, ****p <0.0001 as assessed by one way ANOVA with Bonferroni’s post test. Data are from one representative experiment of two that gave similar results, each with 5 mice per group.
Figure 5.11: OVA-specific CTLs abrogate allergic airway inflammation in OVA/HDM dually sensitised mice

(A) Mice were primed with HDM alone or both OVA and HDM. One group were administered $5 \times 10^6$ CTLs intravenously before the first challenge, on day 13. All mice were then challenged three times with HDM as well as a single OVA intranasal challenge. (B) Inflammatory cell populations measured in BAL on day 17. Mean cell number in BAL + SEM are shown. Data are from one representative experiment of two that gave similar results, each with 3-5 mice per group. *p < 0.05, **p < 0.01 as assessed by one way ANOVA with Bonferroni’s post test.
group were administered $5 \times 10^6$ CTLs intravenously before the first challenge, on day 13. It was found that the total cell number and eosinophilia in BAL were increased in challenged mice that were sensitised to both OVA and HDM, compared to those sensitised with HDM only (Fig 5.11 B). Dually sensitised mice that were treated with CTLs showed a marked suppression of BAL cellularity and eosinophils. A similar trend of CTL-induced suppression of increasing cell numbers in OVA/HDM-primed mice was seen in other BAL populations: neutrophils, B cells, macrophages and T cells, although these results were not statistically significant. DCs were detected in fewer number than previous experiments (i.e., compared to Fig 5.10), and could not be differentiated between treatment groups, possibly due to their low numbers in these BAL samples. These data show that the allergic response is exaggerated when dually sensitised mice are exposed to both HDM and OVA allergen challenge, compared to HDM-induced inflammation alone, and that OVA-specific CTLs can ameliorate the lung cellular inflammatory infiltrate. Therefore, it is possible that CTL-mediated targeting of allergy-driving DCs (as reported in Figs 5.2, 5.5, 5.8, 5.9) may in fact lead to suppression of allergic airway inflammation even in polysensitised individuals where inflammation may be generated to multiple allergens.

5.3.7 A novel peptide conjugate vaccine elicits an allergen-specific CTL response that inhibits allergic airway inflammation

To investigate the potential for inducing allergen-specific CTL-mediated suppression of allergic airway inflammation by means of immunisation, a novel self-adjuvancing peptide conjugate vaccine (668) was tested in an in vivo sensitisation model. The OVA$_{257-264}$-conjugated vaccine (chemical and functional structure depicted in Fig 5.12 A) was administered intravenously to OVA/Alum primed mice 7 days before intranasal OVA challenge (Fig 5.12 B), as cytotoxic activity peaks after 7 days (668). Treatment with an LCMV$_{gp33}$-conjugate and unconjugated αGalCer + OVA$_{257-264}$ were included to ascertain allergen specificity of the response and benefit of the vaccine conjugate structure, respectively. Additionally, allergen-specific CTLs were administered to compare the effects of the vaccine with CTL therapy.
It was found that the OVA\textsubscript{257-264}-conjugate (CN-OVA\textsubscript{257-264}) vaccine suppressed allergic airway inflammation as effectively as treatment with CTLs; total infiltrating cells and eosinophils in the BAL were significantly reduced compared to untreated OVA/Alum groups (Fig 5.12 C). Conversely, the irrelevant peptide conjugate (CN-gp33) and unconjugated αGalCer + OVA\textsubscript{257-264} did not mediate effective suppression of cell infiltrates or eosinophils compared to CN-OVA\textsubscript{257-264}-treated mice, though some reduction in eosinophilia was observed in the CN-gp33 treated mice. All OVA/Alum primed mice generated similar levels of total and OVA-specific IgE, and this was not affected by administration of any of the vaccines, nor CTL immunotherapy (Fig 5.12 D). These results suggest that CN-OVA\textsubscript{257-264} suppresses airway inflammation in a predominantly allergen-specific manner, and that the efficacy relied on the conjugate design of the vaccine.

Under the same experimental conditions, histological examination of the lung was carried out, revealing infiltration of immune cells in OVA/Alum treated mice that was not present in PBS-challenged mice, and clearly reduced in CTL-treated mice (Fig 5.13 A, upper panels). Treatment with CN-OVA\textsubscript{257-264} similarly protected mice from lung infiltrates, while CN-gp33 and unconjugated αGalCer + OVA\textsubscript{257-264} did not (Fig 5.13 A, lower panels). This trend was paralleled in the effect on mucus production induced by the various treatments; untreated OVA/Alum mice as well as those treated with CN-gp33 and unconjugated αGalCer + OVA\textsubscript{257-264} displayed extensive mucus production in the lung, and this was distinctly diminished in mice treated with CTLs or CN-OVA\textsubscript{257-264} (Fig 5.13 B). Collectively, these data reveal that the peptide conjugate vaccine CN-OVA\textsubscript{257-264} is capable of mediating suppression of allergic airway inflammation in a similar manner to CTL immunotherapy.
Figure 5.12
Figure 5.12: A novel peptide conjugate vaccine inhibits allergic airway inflammation

(A) Chemical structure of OVA$_{257-264}$-conjugated vaccine, annotated with simplified functional structure. (B) Mice were sensitised and challenged with OVA as depicted. Vaccines were administered 7 days before intranasal challenge: either vaccine conjugated to OVA$_{257-264}$ or unrelated peptide gp33, or unconjugated αGal + OVA$_{257-264}$. A CTL-treated group was also included for comparison. (C) Day 3 BAL samples were analysed by flow cytometry to enumerate total cell number and eosinophils. (D) Serum IgE was measured by ELISA. Total serum IgE is presented in ng/mL and OVA-specific IgE from a 1:2 serum dilution is reported as the OD at 450nm. Bar graphs show mean cell number, concentration, or OD + SEM, individual symbols represent each mouse. *p < 0.05, ***p < 0.001, ns = not significant, as determined by one way ANOVA with Tukey’s post test.
Mice were sensitised and challenged with OVA/PBS, and treated with vaccines or CTLs as described in Fig 5.12. Day 3 lung histology shows cellular infiltrate by H&E stain (A) and mucus producing goblet cells by ABPAS staining (B). Scale bars represent 200μm (A) and 100μm (B). Stained sections are from one experiment that shows representative histopathology of two experiments, each with 5 mice per group.

**Figure 5.13: A novel peptide conjugate vaccine prevents airway histopathology**


5.4 Discussion

The inhibitory effects of allergen-specific CTLs on allergic airway inflammation are dependent on the expression of perforin, suggesting CTL-mediated killing of critical allergy-driving cells as a mechanism of suppressing airway disease. This chapter aimed to determine the identity and localisation of the targets of CTL-mediated killing, and investigate the efficacy of eliciting an endogenous CTL response by use of a novel vaccine.

Allergen-specific CTLs target dendritic cell populations

Successful CTL-mediated suppression of allergic airway inflammation has been demonstrated, in this chapter, to result in a decrease in the number of DCs in the lung and MLN of treated mice. This suggested that it was possible that allergen-specific CTLs may be targeting allergen-presenting DCs as a mechanism of suppression. Indeed, the phenomenon of direct CTL-mediated killing of DCs in vivo has been demonstrated in different experimental contexts (755, 756). This occurrence would require the direct engagement of allergen-MHC-I complexes on target DC with the CTL TCR, implying the affected DC population must be able to either cross-present allergen to CTLs, or acquire allergen-MHC complexes from other DCs. Of the migratory lung DC populations, CD103+ cDCs have been clearly demonstrated to be the most efficient at cross-presenting lung-acquired antigens in the lymph nodes (73, 123, 126). Conversely, the cross-presenting function of CD11b+ cDCs is not well established (764). Intriguingly, in the present study, it was not only CD103+ DC that were affected by CTL therapy; a decrease in the CD11b+ cDC population was also evident in the lungs and MLN of normal and chimeric CTL-treated mice. The interaction of CTLs with CD11b+ cDC, as implicated in these experiments, had not been reported in this context and was rather unexpected. Additionally, as CD11b+ cDCs are the primary DC subset involved in priming airway allergy (306, 323), this finding could elucidate the mechanism by which CTL therapy suppresses allergic airway inflammation.

To investigate the contacts of CTLs in vivo, examination of lung tissue was carried out using the newly developed XCR1GFP mice and previously described CD11cYFP mice (652), allowing
for the visualisation of interactions between adoptively transferred CTLs with endogenous DC populations. Fascinatingly, both CD103+ DC and CD11b+ DC (CD11c<sup>GFP</sup>+) redistributed after airway allergen challenge to form clusters around small airways. CTLs were also preferentially located in these areas and, within clusters, both DC subsets could be observed interacting with CTLs. CD4+ T cells were also clustered with DCs and CTLs, however, in terms of the interaction of these cells with CD4+ T cells, the experiments carried out as presented in Fig 5.9 did not show the expected BAL inflammation. These experiments were carried out in a different research institute under conditions not entirely similar to that of the adoptive transfer experiments reported in the rest of this thesis. The results showed that, while the clustering phenomenon in the histology appeared to be similar to that of in vivo sensitised mice (Figs 5.6, 5.7, 5.8), the BAL of CD4 only-treated mice did not reveal significant inflammatory cell infiltrates, whereas the CD4 + CTL-treated mice did experience some BAL cellularity. There were numerous potentially confounding factors involved such that it was difficult to conclude why effective airway inflammation was not established by CD4 T cells, and why the co-injection of CTLs did, in fact, result in some inflammatory infiltrates. Nevertheless, the clustering of CD11b+ DC with CD4+ T cells was recently reported in the lungs (494) and, despite the low airway inflammation in the Fig 5.9 experiments described herein, the clustering phenomenon in the lung appeared to be occurring in a similar manner. Therefore, this may be a true representation of the interaction of CD4+ T cells with lung DC and as such the previously unreported interaction of CD4+ T cells with CD103+ DC, and in association with CTLs, may also be valid observations. Thornton et al proposed DC:T cell clustering to be a function of facilitating antigen presentation, however the critical elements involved in the formation and biologically significant functions of these clusters remains to be elucidated. Other authors have described DC clustering in dermal perivascular areas of the skin, during contact hypersensitivity reactions, that enabled the activation of T cells (765). This similar pattern of cluster formation could suggest the importance of this phenomenon in the generation of local immune responses across multiple tissue sites.

The association of XCR1<sup>GFP</sup>- CD11c<sup>YFP</sup>+ DCs with CTLs provided interesting insight into the mechanism of action of CTL-mediated immunotherapy. Both conventional CD11c+CD11b+C64- DCs and monocyte-derived CD11b+CD64+ DCs, upon upregulation of
CD11c, contribute to the total CD11c<sup>YFP</sup> + cell population during airway inflammation. It is worth noting the possibility that alveolar macrophages, due to their expression of CD11c, may additionally be revealed by CD11c<sup>YFP</sup> + fluorescence in the lung sections examined in this study, however, the majority of these cells would have been removed from the airway by BAL prior to imaging. Additionally, the CD11c<sup>YFP</sup> + cells visualised appeared morphologically more similar to DCs than alveolar macrophages; although dendrites were not easily distinguished in some CD11c-stained sections, close-up visualization of lungs from the inherently fluorescent CD11c<sup>YFP</sup> mice by confocal microscopy revealed the distinct DC-like morphology of these cells (Fig 5.9). Further analysis of tissue sections could include counter-staining with CD11b antibodies to conclusively identify this subset as the predominant constituent of the CD11c<sup>YFP</sup> + cell population, however, other authors have reported success in distinguishing alveolar macrophages from DCs in a similar manner; based on their morphological roundness, rather than dendritic appearance (494). The identification of CD11b+ cDC versus moDC is not as straight forward, however, their spatial characteristics in lung tissue may allow for discrimination of these DCs.

While monocyte-derived DCs have been demonstrated to effectively stimulate CD8+ T cell responses through cross-presentation (766-769), imaging of moDC infiltrating the lung tissue upon allergen challenge revealed they were primarily confined to the parenchymal tissue rather than the airway mucosa (770). Therefore, moDCs may not make up a significant proportion of the CD11c<sup>YFP</sup> + DCs observed clustering in association with CTLs around small airways in the present study, and may preclude them from being the major targets of CTL-mediated killing. This postulation is in accordance with the finding that chimeric CTL-treated mice did not display a significant decrease in the CD11b+CD64+ moDC population.

Conversely, the localisation of CD11b+ cDC around allergic airways has been demonstrated (494). The co-localisation of CTLs with CD11b+ cDC does not conclusively imply that effective cross-presentation is occurring, a function that has not been widely recognised for this DC subset, however, it appears that interactions between these populations exist in the allergic lung that may not have been realised. If direct cross-presentation of allergens by CD11b+ cDCs was occurring, and resulted in CTL-mediated killing of those DCs, this may
effectively prevent allergic airway inflammation as evidenced in this thesis, and in accordance with the proposed physiological role of CTL-mediated killing: to reduce the magnitude of antigen presentation and prevent undesirable immune responses from proceeding (756, 771-773).

Alternatively to the direct processing and cross-presentation of allergens by CD11b+ cDC, the formation of DC clusters during allergic airway inflammation may facilitate the cross-dressing of CD11b+ cDCs, potentially acquiring allergen-MHC-I complexes from nearby specialised cross-presenting CD103+ DC. This phenomenon has not been investigated in migratory CD11b+ lung cDC specifically, however, cross-dressing has been implicated as the major mechanism by which poor cross-presenting APCs are enabled to stimulate effective naïve and memory CD8+ T cell responses (76, 128-132). Additionally, bm-1 DCs that lack the ability to cross-present OVA peptides have also been demonstrated as capable of stimulating OT-I CD8+ T cells through their acquisition of peptide-MHC complexes from other cross-presenting DCs (131). Cross-dressing has been described to occur through the acquisition of peptide-MHC complexes by at least two distinct mechanisms: either through the transfer of exosomes from surrounding cells (76, 129, 774-776), or by direct cell:cell interaction during which intact peptide-MHC complexes may be obtained (131). The method of exosome uptake, however, will result in successful presentation of acquired peptides only in cells capable of reloading these peptides onto their own MHC-I molecules for cross-presentation. APCs that can not carry out cross-presentation on their own, such as bm-1 DCs, may only present intact peptide-MHC complexes obtained through direct cell:cell contact with cross-presenting APCs (131). In the microscopic examination of lung tissue sections, there were numerous fields of view, exemplified in Figure 5.8, where CTLs were associated with XCR1\textsuperscript{GFP}- CD11c+ DC in apparent isolation from close contact with other DC subsets. This could rather support the proposal that direct cross-presentation is occurring, however, potentially, these DC acquired allergen-MHC complexes elsewhere in tissue and subsequently migrated to the vicinity of CTLs. The results of mixed bone marrow chimera experiments provided insight into these queries. As mixed bone marrow chimeras showed a specific decrease in the ratio of WT DCs to bm-1 DCs in CTL-treated mice (Figure 5.5), this suggests the dependence of DC-targeting on the DC’s ability to cross-present the allergen of
interest; an equivalent decrease in both populations may be expected if bm-1 DC were effectively acquiring and presenting peptide-MHC-I complexes.

While CD103+ and, presumably CD11b+, CD11c+ DCs were identified interacting with CTLs in the present study, it is possible that there may be other targets of CTL-mediated killing. Mast cells and basophils have been demonstrated to carry out cross-presentation of antigens (777, 778), potentially rendering them targets to the same fate as cross-presenting DCs. However, these cell populations are not critical to the priming and differentiation of Th2 cells (435, 444, 685, 779), as discussed in Chapter 3, implying they are less significant targets than CD11c+ DCs, the depletion of which effectively abrogates airway inflammation (321). Additionally, the CTL-mediated killing of these populations in vivo has not been reported. It was also shown in Chapter 4 that CTLs successfully mediated the suppression of allergic airway inflammation in the adoptive transfer model, where the elicitation of mast cells and basophils was not significantly involved. Collectively, these evidences suggest that mast cells and basophils may not be critical targets of CTL-mediated killing in the suppression of allergic airway inflammation.

The CTL-mediated killing of DCs could occur upon cross-presentation of allergens in the lung, or after the migration of DCs to the MLN. Experiments with the fluorogenic substrate FLIVO revealed an increase in pre-apoptotic cells in the MLN of CTL-treated mice that predominantly phenotypically resembled migratory DCs; thus suggesting that killing was occurring in the lungs, though perhaps not exclusively so. Antigen presentation in the lungs has been shown to be important for facilitating local immune responses, and significant numbers of antigen-loaded APCs are retained within the lung (114, 115). As such, the targeting of allergen-presenting DCs in the lung may halt the induction of an allergic response at the local site; this would reduce the migration of DCs to draining LN where many T cell contacts, as well as inter-DC antigen transfer, may subsequently amplify the T cell response (74). Indeed, the finding that DCs are reduced in number in the lungs of both normal and mixed bone marrow chimeric mice in the experiments described in this thesis, supports the notion of local targeting. Therefore, it has recurrently been indicated in this study that CTLs target allergen-presenting DCs in the lung.
Effects of CTLs in airway inflammation driven by an alternative allergen specificity

The targeted killing of DCs revealed a means by which allergy-driving APCs may be depleted in the lung; the effect of this phenomenon on the initiation of unrelated allergic responses was thus investigated. In a model of dual sensitisation, OVA-specific CTLs appeared to be capable of effectively preventing HDM-and-OVA-induced airway inflammation, the mechanism of which was not fully elucidated.

It is possible that the auto-adjuvanting properties of HDM may contribute to CD8+ T cell activation in the lungs. The major HDM allergen, Der p 2, displays similar structural and functional homology to MD-2: the protein associated with TLR4 for the recognition of LPS (596-598). Therefore, the combination of HDM endotoxin and Der p 2 mimicry of MD-2 could initiate TLR4 signalling and lead to subsequent activation of CTLs. This bystander CTL effect has been reported in the context of viral infections, the biological importance of which has been questioned; some evidence suggests that effective immune responses may be carried out by CTLs of alternative antigen specificities where the total induced and activated cell number reaches a relatively high threshold level (224, 599-601). Additionally, it has been reported that bystander effector/memory CD8+ T cells in the airway can mediate suppression of allergic responses in the lung, in an IFN-γ dependent manner (221). However, these experiments were carried out in the presence of endotoxin, unlike the systems generated in this thesis, and reported by others (226), where low endotoxin conditions resulted in the suppression of allergic airway inflammation that was critically dependent on the expression of perforin, while no requirement for IFN-γ was determined. Additionally, while TLR signalling may lead to the activation of bystander CTLs, the lack of TCR engagement would preclude CTL-mediated killing.

An alternative mechanism may be proposed, then, whereby both OVA and HDM are presented predominantly on the same APCs concurrently, either via cross-presentation or cross-dressing. As such, the effective killing of OVA-and-HDM-cross-presenting DCs by OVA-specific CTLs may reduce the presentation of both OVA and HDM to CD4+ T cells, leading to the suppression of inflammation associated with either allergen (as depicted in Fig 5.14).
Figure 5.14: Proposed mechanism of OVA-specific CTL-mediated suppression of OVA- and HDM-induced airway inflammation

Suppression of allergic airway inflammation induced by both OVA and HDM was mediated by OVA-specific CTLs alone. As OVA-specific CTL may target OVA-presenting (A), but not HDM-presenting (B) DC, this finding suggested DCs may be predominantly presenting both OVA and HDM simultaneously (C); these DC may be targeted by OVA-specific CTL, resulting in a decrease in both OVA and HDM presentation to CD4+ T cells, and subsequent suppression of allergic airway inflammation.

Kedl and colleagues describe a phenomenon whereby the simultaneous presentation of two different antigens on the same DC led to competitive exclusion of T cell responses of the sub-dominant antigen specificity, due in part to spatial interference (780). It could thus be argued that in the dual-sensitisation system, the high-affinity of OVA-specific T cells for
OVA-and-HDM-presenting DCs could allow the physical exclusion of some HDM-specific T cells, reducing HDM-induced airway inflammation. However, in the experiments described in this thesis, competition between T cells, that is, CD4+ T cells and CTLs, is not implicated as a key mechanism for the suppression of allergic disease, as WT OT-I CTLs abrogate inflammation while PKO OT-I CTLs are unable to do so; spatial exclusion by higher-affinity cells, as a primary mechanism, would result in equivalent suppression by either CTL population, as both populations interact with APCs. In fact, it has been demonstrated that PKO cytotoxic lymphocytes have extended interaction time with target cells due to their inability to effectively kill the cells and subsequently disengage (781). This finding rather implicates, yet again, the CTL-mediated killing of allergen presenting DCs.

For the killing of OVA-and-HDM presenting DCs to be so effective in the suppression of the HDM-induced component of airway inflammation, as reported in Figure 5.11, the majority of HDM-presenting DCs would also need to be presenting OVA. It is possible that this phenomenon could have occurred due to the surplus concentration of OVA allergen compared to HDM used in these experiments, such that the majority of allergen-exposed DCs in the lung had picked up OVA with or without HDM. Alternatively, it is possible that specific DCs are more likely to acquire and present these allergens, and as such, the same population(s) of DCs are more frequently presenting both allergens. Thornton and colleagues describe CD11b+ DC as being better at soluble antigen uptake than other lung DCs, however it was postulated that this may be due to their localisation within tissue and the higher frequency of these DC (494); the uptake of multiple antigens was not assessed. It would be interesting to carry out subsequent OVA/HDM experiments where the concentrations of OVA and HDM were limiting and more equivalent, such that the co-presentation of OVA or HDM on DCs would be expected to occur less frequently, unless a bias towards specific DCs was indeed a factor.

As the majority of individuals affected by allergy are polysensitised (22, 578, 579), potential benefits exist for therapeutic interventions that may confer cross-protection to multiple allergens, while targeting specific allergen-presenting DCs.
**Immunotherapy of allergic airway inflammation by a novel peptide conjugate vaccine that elicits an allergen-specific CTL response**

The efficacy of utilising tumour-derived (782, 783) or genetically engineered T cells (784) for the purpose of anti-tumour immunotherapy has been demonstrated. While the identification of antigen-specific CD8+ T cells from peripheral blood samples is extremely difficult, due to their low proportion of total circulating lymphocytes (785), analogous T cell engineering could in theory provide a CD8+ T cell-based vaccine for allergic asthma. Similarly, treatment with autologous tumour-antigen-presenting DCs has also proven beneficial in the induction of CTL-mediated anti-tumour responses (786-788). The generation of cell-based vaccines, however, can be arduous; leukapheresis to obtain patient-specific haematopoietic precursor cells is a somewhat invasive and time-consuming procedure that may have variable success in the recovery of adequate numbers of precursor cells. Additionally, the processing involved in the subsequent antigen-pulsing and enrichment procedures, or engineering of cells may affect cell viability, the final cellular preparation for immunisation thus being potentially variable in efficacy. Furthermore, these processes render the generation of cell-based vaccines an expensive endeavour for individualised medicine.

For the clinical treatment of allergic asthma, a cell-free vaccine that induces an endogenous response may be preferable and more feasible than the use of cellular immunisation. A novel conjugate vaccine has herein shown similar efficacy in the suppression of allergic airway inflammation to that reported using the adoptive transfer of allergen-specific CTLs; its effects generated by the elicitation of an endogenous CTL-mediated response (Figure 5.12 and 5.13, and (668)). This vaccine may have several benefits over cellular immunotherapy as well as conventional CTL-eliciting vaccines. Firstly, there is no requirement for the recovery of cells from patients, additionally, the vaccine may be designed to conjugate common allergens that affect numerous individuals so that personalised treatment is not required in every case. Finally, many existing cell-free vaccines are designed to recruit CD4+ T cells as their helper functions have been shown to play an essential role in the propagation of successful anti-tumour immune responses (789-792), however, in the context of allergy the stimulation of CD4+ T cells may potentially
exacerbate disease, rendering this approach undesirable for the treatment of allergic airway inflammation. For this purpose, the conjugate vaccine rather employs the assistance of NKT cells by presentation of αGalCer on DC CD1d molecules.

The therapeutic potential for the conjugate vaccine in the context of established airway inflammation was not examined in this chapter, neither was long-term efficacy determined. The vaccine, however, induces CTLs of a similar phenotype to those generated in vitro for adoptive transfer; IFN-y producing, and no detectable production of the Tc2-type cytokine IL-4 (668). Additionally, vaccine treatment suppresses disease similarly to the adoptive transfer of CTLs. Therefore, it could be hypothesised that the vaccine may also abrogate existing disease in the same way.

Conclusions

In this chapter, allergen-specific CTLs were demonstrated to abrogate airway inflammation by targeting allergen-loaded DCs within the lungs, importantly, by inducing apoptosis of the critical CD11b+ cDC population. This required the expression of perforin and the recognition of cross-presented allergen on DC MHC-I. Additionally, novel insights were gained into the localisation of DC subsets in the allergic lung; visualisation of lungs revealed a redistribution of DCs, after allergen challenge, to form clusters around small airways within which CTLs were interacting with both CD103+ DC and, unexpectedly, CD11b+ DCs. This targeting of allergy-driving DCs appeared to have cross-protective effects that may be beneficial to individuals suffering from reactions to multiple allergens. Finally, a novel peptide conjugate vaccine was described that is capable of eliciting effective endogenous CTL-mediated suppression of airway inflammation. Collectively, these findings reveal the exciting potential for effective immunotherapy of allergies through the targeting of allergy-driving airway DCs.
Chapter 6

General Discussion
6.1 The main findings of this thesis

This thesis aimed to determine the therapeutic potential for allergen-specific CTLs in the treatment of established allergic airway inflammation, and to elucidate the mechanism by which CTLs mediated suppression of disease. It was hypothesised that CTLs may target allergy-driving APCs, reducing the presentation of allergen to CD4+ T cells and subsequent Th2-polarised inflammation. Key findings are highlighted in this chapter, and some limitations are also discussed herein.

The main findings of this thesis may be summarised as follows:

- Immunotherapy with allergen-specific CTL has long-term preventative and therapeutic effects on established airway inflammation, in a manner dependent on perforin;
- CTL-mediated suppression of allergic inflammation is associated with reduced number and cytokine production of disease-mediating CD4+ Th2 cells in the airway;
- Lung CD11b+ and CD103+ DC form clusters with CTL and CD4+ T cells during allergic inflammation, where killing may be facilitated;
- Allergen-specific CTLs target and kill lung CD11b+ and CD103+ dendritic cell populations in a manner that is dependent on MHC-I engagement;
- Both the classical cross-presenting CD103+ DC as well as critical allergy-driving CD11b+ DC present allergens to CTLs;
- Allergic airway inflammation can be suppressed using a CTL vaccine

6.1.1 CTL-mediated suppression of allergic disease is associated with effects on disease-mediating CD4+ Th2 cells

Despite being critical to the development of allergic asthma, the role and activity of CD4+ T cells is not fully resolved; indeed, the role of CD4+ T cells is being revised due to the recent discovery of ILCs and innate mediators that may posses overlapping or complementary roles in the development of Th2 immunity. The direct or consequential effects of CD8+ T
cells on CD4+ T cells in allergic airway inflammation also remains to be fully elucidated. In this study, an adoptive transfer model was developed that replicated the characteristic features of allergic airway inflammation and allowed for the identification of allergen-specific CD4+ T cells. This model provides a platform for others to carry out additional investigations. Within this model, CTLs suppressed allergic disease and it became apparent that lower numbers of CD4+ T cells in the lung tissue and reduced Th2 cytokine production were associated with CTL-mediated amelioration of disease, thus suggesting that CTLs were affecting the stimulation of pathogenic CD4+ T cells.

The absence of host sensitisation in this model, however, subverts the elicitation of a number of cell populations that would normally participate in the induction of a Th2 response. Additionally, allergen-specific IgE is not generated. Importantly, epithelial cells and ILCs would not have been activated prior to allergen challenge. It could be argued that the main crucial outcome of the co-operation of these cells in the development of allergic disease is the stimulation of CD4+ Th2 cells, long recognised as critical mediators of allergy (707-709, 793, 794). In this regard, adoptively transferred CD4+ Th2 cells are sufficient to orchestrate the late phase asthmatic reactions that replicate the characteristic features of allergic disease (650, 651, 795, 796). Indeed, it was found in this study that significant eosinophilia, mucus production and histological pathology were induced by the transferred cells, without the requirement for host sensitisation.

It is additionally true, however, that the in vivo sensitisation model utilised in this study may not elicit the recruitment of innate cells for the initiation of the Th2 response, either. Indeed, a criticism of the i.p. sensitisation protocol is the failure to stimulate local airway cells, including ILCs and epithelial cells, that would naturally be induced during the sensitisation phase of models employing the intranasal administration of allergens (274, 611, 612). Consequently, it has been demonstrated that while ILC2 play a crucial role in the initiation of Th2 responses that are generated through local sensitisation, like that driven by the repeated inhalation of allergens such as HDM, an important initiating role has not been described for ILC2 where sensitisation is carried out by allergens administered i.p. (467, 797, 798). Therefore, although the characteristic features of allergic airway inflammation were generated using both the adoptive transfer and i.p. sensitisation models
in this study, these models may not involve the recruitment and involvement of a co-ordinated response between CD4+ T cells and Th2-driving innate cells.

The importance of innate cells such as epithelial cells and ILCs, and their associated cytokines, has recently become increasingly apparent, as multiple roles in allergic airway inflammation have been described. A recent report by Christianson and colleagues described multiple feedback loops involving these cells that are important for the persistence of chronic asthmatic disease. The stimulation of epithelial cells resulted in IL-33 production that led to the recruitment of ILC2s; subsequent ILC2 production of IL-13 prompted the upregulation of IL-33 receptors and facilitated the autoinduction of IL-33 (674, 799, 800). Chronic asthma was resolved upon elimination of any of these components. These findings therefore reveal a previously unappreciated role for epithelial cells and ILC2 in the maintenance of chronicity through multiple feedback loops. This study also reported that while T cells contributed to the severity of chronic asthma, they were not critically required for persisting AHR and airway remodelling; this result was in accordance with that previously published by another group in a study of chronic disease (799, 801), adding to the argument that CD4+ T cells are not solely responsible for co-ordinating airway pathology. Previous studies have, however, demonstrated an essential role for CD4+ T cells in the initial development of AHR; when CD4+ T cells are depleted from sensitised mice before allergen challenge, AHR does not develop (475, 476, 709, 747, 802, 803). Nevertheless, significant interplay still exists between innate cells and Th2 cells in chronic asthma (675, 804-806). Together, these findings reveal some important differences in the role and requirement for effector CD4+ T cells in different stages of disease, and implicate the importance of ILCs and epithelial cells in established chronic disease. Therefore while Th2 priming and airway disease may develop without the requirement for these cells, there may be aspects of pathology that are not replicated without their recruitment.

The recruitment of ILCs, in collaboration with innate cytokines IL-33 and TSLP, has also been deemed of clinical importance, as these factors are involved in driving corticosteroid resistance in asthma (807-811). These studies recently showed that, while Th2 cells and eosinophils may be predominantly sensitive to corticosteroid therapy, ILC2s may evade cell death in response to innate cytokines. The induction of TSLP in an IL-33/OVA model of
allergic airway inflammation was shown to cause ILC2 to become resistant to steroid-mediated cell death through a STAT5-dependent pathway (807, 808), thus enabling ongoing inflammation. IL-33 has also been implicated in patients with severe therapy-resistant asthma, found in high concentration in the inflamed airway (811, 812). Strikingly, it was demonstrated experimentally that corticosteroid sensitivity could be restored through blockade of the TSLP/STAT5 signalling pathway (808, 813). Therefore, IL-33 and TSLP represent potential therapeutic targets in ILC2-driven corticosteroid-resistant asthma, and it would be useful for studies further to the work described in this thesis to address the effects of CTL immunotherapy on ILC2s.

The involvement of epithelial cells in allergic disease, interestingly, differs between the commonly used strains of mice. While a critical role has been demonstrated for epithelial cells in OVA-induced allergic airway inflammation in BALB/c mice, this requirement does not apply in C57BL/6 models (635, 814). As experiments in this study were carried out in C57BL/6 mice, it stands to reason that epithelial cell involvement may not have been critical in these models, regardless of whether adoptive transfer or in vivo sensitisation was performed; although, they would have of course contributed to pathology. As more extensive genetic heterogeneity exists in humans, it is possible that these cells also contribute differently in the perpetuation of human asthmatic disease, between individuals.

In summary, it is evident that while the generation of allergic airway inflammation is reliant on a robust Th2 response, like that induced in the adoptive transfer and in vivo sensitisation models, it is possible that the absence of local airway cell sensitisation results in allergic pathology that may differ in some aspects to naturally acquired allergic airway inflammation. Specifically, the late-phase asthmatic response is generated, but early-phase response is not. Nevertheless, it was found that CTL immunotherapy inhibited airway inflammation both in the adoptive transfer and in vivo models, suggesting that the mechanism of suppression is likely to be conserved across models.
6.1.2 Allergen-specific CTLs counteract Th2 immune responses to suppress allergic airway inflammation

This thesis shows that allergen-specific CTLs target lung DCs, resulting in amelioration of airway inflammation. Effective targeting was dependent on perforin expression and apoptosis was likely initiated in the lung. The interaction of CTLs with DC MHC-I was crucial to the killing response, suggesting that cross-presenting DCs were targeted. While CD103+ cDC, classically recognised as the most efficient cross-presenting migratory DC population in the lung, were targeted, critical allergy-driving CD11b+ cDC were also found to be affected. Indeed, fluorescent imaging of the lung showed both subsets closely interacting with CTLs; allergen challenged mice fascinatingly revealed the formation of DC-CTL-CD4+ T cell clusters within which each of these cell types appeared to interact with each other. Together, these findings describe the mechanism by which CTLs mediate effective suppression of allergic disease. A novel conjugate vaccine was also described that elicits endogenous CTLs to suppress airway inflammation, providing the potential for translation of these findings into the treatment of human disease.

The potent efficacy of CTL-mediated DC killing as a central mechanism in the suppression of allergic airway disease begs the question of how this may relate to physiological interactions normally occurring between DCs and CTLs in human health and disease. It has been proposed that CTL-mediated killing, in addition to the clearance of virally-infected or tumorous cells, functions to reduce excessive presentation of antigens which may drive superfluous or inappropriate immune responses (73, 123, 126, 322, 324, 755, 756, 771-773, 815). Indeed, Ma and colleagues report that under particular experimental conditions, CTL-mediated killing of DCs may result in reduced proliferation of naïve CD4+ T cells in lymph nodes (764, 816). For the purpose of ensuring that effective immunity is developed, however, dendritic cells may possess mechanisms by which they can avoid being killed by CTLs during antigen presentation. Numerous mechanisms have been examined in in vitro studies; DCs may express anti-apoptotic molecules to protect themselves from FasL-mediated killing (817-824) and in the defence of perforin/granzyme-mediated killing, DCs may express serine protease inhibitors (serpins) that neutralise granzyme B: serpin B9 in humans (formerly known as PI-9), or the murine homologue, SPI-6 (226, 752). Interaction
with memory CD8+ T cells was also reported to protect DCs from being killed (320, 754). In vivo, however, these factors were not shown to protect DCs from CTL-mediated killing (494, 757, 825, 826). While a universally protective mechanism does not appear to have been clearly demonstrated in vivo, it may be that DCs possess these features for the avoidance of CTL-mediated killing. Alternatively, DCs may remain susceptible to CTL-mediated killing as an immune regulatory function.

In the current study, allergen-presenting DC did appear to be susceptible to CTL-mediated killing. It would be interesting to investigate whether allergen-presenting lung DCs in humans that suffer from allergic asthma differ in their susceptibility to CTL-mediated killing to those from non-allergic individuals. This could occur through defective CTL-mediated killing or enhanced DC protection mechanisms, which may lead to a failure to suppress allergic responses. However, in the case of defective CTLs, this would of course lead to additional serious pathologies. Indeed, in patients suffering from perforinopathies, such as Familial Haemophagocytic Lymphohistiocytosis, CTLs cannot kill effectively which leads to an increase in activated immune cells and excessive cytokine production causing widespread, often fatal, pathology (255, 258, 765). To the best of my knowledge, studies have not yet been performed to investigate the relative presence or absence of CTL-killing avoidance mechanisms between DCs of allergic and non-allergic individuals. It has been shown however, that atopic asthmatic patients have increased numbers of DCs in the airway mucosa and peripheral blood (765, 827-829), and while this may be a consequence of airway inflammation, it has also been shown that excess DC proliferation is promoted in the airways by aeroallergen challenge (755, 757, 770, 825, 826). Interestingly, increased CD8+ T cells in asthmatics has been shown to correlate with decreased severity of AHR (711, 773, 816). However, large numbers of activated CD8+ Tc2 CTLs exacerbate disease and express decreased cytotoxic function (830-836). In a murine model, the depletion of CD8+ T cells prior to the induction of allergen sensitisation and challenge resulted in increased Th2 responses and worsened allergic airway disease (222, 733, 837). The work of Laprise and colleagues (711, 838, 839) shed light on some particularly interesting evidence of a potential role for CD8+ T cells in preventing the progression of asymptomatic AHR to symptomatic asthmatic disease. It is known that individuals who suffer from atopy – the genetic predisposition to mount mucosal IgE responses – often develop asymptomatic
AHR, which later develops into allergic asthma, although it is not clear how or why this progression of disease occurs (6, 840). Laprise and colleagues followed a number of individuals presenting with asymptomatic AHR, and over a 2 year period a number of those subjects had developed asthma symptoms. It was discovered that the progression to asthma was associated with a decrease in bronchial CD8+ T cells, with a concomitant increase in CD4+ T cells and subepithelial fibrosis (542, 711). This finding reveals a potential protective role for CD8+ T cells in the progression of asthmatic disease.

In this thesis, significant numbers of adoptively transferred CTLs were recovered from the lung tissue, and where enumerated, these represented a larger infiltrating population than the co-transferred CD4+ T cells. This correlates with the findings of others that have described a greater CD8:CD4 T cell ratio being associated with decreased severity of disease (711, 730, 733, 841-843). It is possible, however, that the severity of disease may not be fully predicted through the enumeration of these cell populations by the method used in this study. Recent studies have utilised an intravascular staining technique (831, 833, 835, 836, 844) to investigate the localisation of CD8+ T cells in the lung during an antiviral response. Strikingly, it was discovered that a large majority of the cells thought to be located within the lung tissue- which had been perfused and digested- were in fact in the pulmonary vasculature (845-847). It may therefore not be possible to use this measure as an indicator of protective immunity, or in the case of experimental allergic airway inflammation, that higher numbers of CD8+ T cells in digested whole lung tissue may not represent an established lung-resident population. It has not yet been thoroughly investigated, however, whether this massive population of cells in the lung vasculature are in fact significant participants in local immune responses in the lung. Indeed, their proximity to the airway may enable rapid activation and migration into the lung tissue upon sensing of chemotactic signals. The work of others also revealed that lymphocyte isolation carried out by flow cytometric analysis of nonlymphoid tissues may fail to recover a large proportion of the cells actually present in tissue, and particular subsets may be more frequently recovered (848). These revelations suggest the re-evaluation of how cell populations are isolated, identified, and enumerated from tissue samples and what conclusions may therefore be drawn. While these factors were not investigated in the current study, it was recurrently demonstrated herein that the enumeration of CD4+ T cells
in whole digested lung tissue accurately correlated with therapeutic success of CTL immunotherapy.

Together, the evidence from this study and the related work of other authors discussed herein suggest that CD8+ T cells regulate immune responses and may be beneficial to counteracting Th2 immunity. Additionally, that CTLs are capable of suppressing airway inflammation in a perforin-dependent manner, and eliminating DCs in vivo- which are increased in asthmatic airways and elicit CD4+ T cell responses- may implicate therapeutic potential.

6.1.3 Allergen-specific CTLs have therapeutic effects on established airway inflammation

In a multiple allergen challenge model, allergen-specific CTLs were shown to be effective long term in the prevention of allergic airway inflammation throughout repeated allergen exposure. Additionally, the characteristic features of disease were abrogated when CTLs were administered during established airway inflammation. These findings revealed the exciting prospect that CTL immunotherapy may be developed for the treatment of persistent airway inflammation, presenting in the clinic as established disease.

It was not investigated in this study, however, how CTLs may affect immunological allergen-specific memory. Through allergen sensitisation, central and effector memory populations of CD4+ T cells are generated, residing in lymph nodes and peripheral tissues such as the lungs (274-276). In the mechanism of suppression elucidated by this study, CTLs target allergen-presenting DCs to reduce the stimulation of CD4+ Th2 cell responses. This was not only found to be effective during acute primary challenge, but also in a multiple challenge model where established memory T cell populations would be expected to predominate the rapid response to allergen exposure (669). Indeed, as both models involved the treatment of mice that were already sensitised to allergen, memory T cells
were presumably present before CTLs were administered in all of these in vivo sensitisation experiments. The numbers of CD4+ T cells infiltrating the airway as measured by BAL and lung harvesting, or those present in the lung-draining lymph node, were examined in this study after allergen challenge. It was consistently found that CD4+ T cells were fewer in the lungs and BAL fluid of CTL-treated mice. It was not determined, however, the extent to which memory CD4+ T cell populations remained after the contraction of the allergen-induced responses, neither were memory CD8+ T cell populations directly examined in tissue. Elucidation of these factors may be important for the development of therapeutic interventions, as this will give some indication of the longevity of vaccine efficacy. Nevertheless, it was apparent from this study that a single dose of CTLs was effective at suppressing allergic inflammation over multiple allergen challenges, suggesting that the effect of CTLs was long-lived.

It would be interesting to discover whether the long-term efficacy of CTL therapy was due to the persistence of memory CTLs throughout these experiments, or whether their initial effector function was sufficient to prevent further allergic episodes. It is feasible that CTLs generated in response to allergic stimuli may reside in the tissue long-term and upon exposure to allergen at some later time, become reactivated and mediate suppression of allergy. It is known that, following the resolution of viral infection, antigen-specific memory CTL persist in the lung tissue, the memory pool being maintained by two distinct populations: a large minimally-dividing population deposited in the airways during the acute anti-viral response, and a second smaller population maintained in the airways by homeostatic proliferation (846, 849, 850). This second population was initially thought to derive from lymphoid tissue (850), however it was recently reported that specialised niches exist in the lung where memory cells are generated and maintained (851). In the absence of on-going antigenic stimulation, these lung-resident memory CTL down-regulate cytolytic molecule transcription, despite maintaining an activated phenotype (849, 852), and upon exposure to their cognate antigen, clonal expansion is triggered (853). In contrast, CD4+ T cell memory was initially reported to wane in the absence of antigenic stimulation (289), although other authors have since shown that mice exposed to an allergen sensitisation and challenge protocol, and subsequently rested, are capable of mounting allergic responses upon re-exposure to allergen some time later (669). It is not known,
however, whether this is in fact due to the persistence of memory CD4+ T cells, as of course long-lived memory B cells and plasma cells producing allergen-specific IgE contribute to ongoing allergy. In the clinical context, the persistence of allergy is somewhat dependent on the allergen, and severity of allergic responses; up to 85% of infants with milk or egg allergy have been reported to tolerate these allergens by age 3 or 5, respectively (707-709, 793, 794, 848), whereas only 10-20% of peanut allergic children lose their sensitivity over time (274-276, 650, 651, 795, 796). Individuals with severe allergy, especially those who have experienced anaphylaxis, may rigorously avoid exposure to the allergen for many years before suffering another episode some time later. Human CD4+ memory T cells, including CD45RO+CCD7+CD62L+ central memory and CD45RO+CCR7-CD62L+/- effector memory subsets have been identified (274, 611, 612, 669), and Th2 memory cells have additionally been described; expressing the prostaglandin D2 receptor (CRTH2), and of a characteristic phenotype, gene expression profile, cytokine production, and response to allergens (467, 797, 798, 846, 849, 850). Interestingly, the central memory Th2 subset were found to undergo substantial expansion only in response to TSLP-activated DCs, and are thought to be maintained through their association with these DCs (674, 799, 800, 850). While this evidence exists to suggest that Th2 memory may persist in humans, it is difficult to isolate and identify allergen-specific T cells from patients, and it is therefore not known how long these populations may survive and whether their depletion would resolve allergy. If allergy-driving memory CD4+ T cells do in fact decline in number due to lack of antigen presentation, and this is sufficient to diminish the effects of long-lived IgE-producing plasma cells, this could potentially mean that the continued suppressive function of allergen-specific memory CTLs may effectively out-live CD4+ T cell memory, as well as prevent the expansion of CD4+ T cells in response to allergens. Alternatively, as it appears that specific DCs are required for the maintenance of memory Th2 cells (799, 801, 851), it is possible that the CTL-mediated targeting of allergen-presenting DC may therefore diminish the stimuli required for the persistence of the memory Th2 population.

In the experiments described in Chapters 3 and 5 of this thesis, allergen-specific IgE was generated in response to i.p. sensitisation alone, and was detected in both CTL-treated and -untreated mice at the experimental end point, despite resolution of the characteristic features of allergic airway inflammation in mice that received CTLs or peptide conjugate
drugs. During the sensitisation process, IgE-producing allergen-specific B cells are generated, through CD40/CD40L interactions with CD4+ T cells and in the presence of IL-4 (475, 476, 709, 747, 802, 803, 849, 852). It has also been reported that the majority of secondary IgE responses require IL-4 stimulation, although this is not always the case (675, 804-806, 853). After CTL treatment, these stimuli for IgE production are diminished, as DC-targeting leads to a reduction in CD4+ Th2 cell numbers and cytokine production. As such, it is unclear why IgE persisted throughout experiments.

Some recent papers have focussed on determining the factors that drive IgE+ B cell generation, and how these cells may derive from both T cell-dependent and independent pathways (289, 807-811). Indeed, human patients that are deficient in CD40L do in fact produce IgE+ B cells (669, 807, 808), although it is not known whether the pathway by which these cells are generated may be normally activated in immunocompetent individuals. Other authors have found that the blocking of inducible T cell tyrosine kinase (ITK), a critical regulator of Th2 cytokine production, was sufficient to suppress allergic airway inflammation without affecting the generation of IgE (811, 812). Further evidence has revealed that ILC2 are able to enhance IgE production from B cells despite not being able to produce IL-4 (808, 813). The mechanism of this is unclear, although Magri and colleagues determined that this may involve the promotion of CD40/CD40L interactions between lymph node marginal reticular cells and B cells (635, 814). While it is evident that, in the experiments in this thesis, the inflammatory manifestations of disease in the allergic airway are diminished by CTL immunotherapy regardless of the presence of allergen-specific IgE, it would be important to determine whether the generation of IgE was persistent despite CTL treatment, which may have implications for on-going success of the clinical treatment of allergic disease. This question could be addressed by carrying out a long-term experiment where sensitised/challenged mice are rested before the re-measurement of IgE titres and further airway allergen challenges are carried out.
6.2 Importance of findings in the understanding of allergy

CD11b+ DC present allergens to CD8+ T cells
An interesting discovery in the study conducted herein was that of the interaction of the major lung DC populations with CD4+ and CD8+ T cells. There is widespread agreement that CD11b+ DC are specialised in the presentation of antigen to CD4+ T cells, while CD103+ DC specialise in cross-presentation, effectively stimulating CD8+ T cell responses (73, 123, 126, 322, 324, 755, 756, 771-773, 815). While this is not untrue, the findings of this thesis gave weight to the importance of CD11b+ DC cross-presentation in the lung; an interaction that can result in these critical allergy-driving DCs being eliminated by CTLs. Desch and colleagues have very recently shown that antigen-specific CTLs may be induced by CD11b+ DC upon TLR7 ligation (764, 816); the natural ligands of which are single-stranded RNAs, from viruses such as influenza, Sendai and Coxsackie virus (817-824). Together these findings have expanded our understanding of the ability of specific DC subsets to stimulate T cell responses. It would be interesting, however, to investigate whether there is any effect of the concentration or nature of the antigen administered on the occurrence of CD11b+ DC cross-presentation, as these factors were not determined in the present study.

The same DCs present allergen to both CD4+ and CD8+ T cells
If DC specialisation translates to strict antigen presentation patterns this logically extends to the assumption that different DC are likely to be presenting allergens to CD4+ T cells from those cross-presenting to CD8+ T cells. However, the implication of the findings in the present study is rather that the same DCs must predominantly be presenting antigen to both CD4+ and CD8+ T cells, as the CTL-mediated targeting of DCs resulted in apparent reduced antigen presentation to CD4+ T cells. This also appeared to be confirmed by fluorescent visualisation of DC-T cell interactions in the lung. These findings agree with the postulations of Enomoto and colleagues (226, 752).
CD103+ DC form clusters with DCs and T cells during allergic inflammation

Recognised for their role in the cross-presentation of antigens and migratory transport of antigens from the lung to the draining lymph nodes, surprisingly little is known about the localisation of CD103+ DCs in the lung. One group reported that these DCs predominantly reside within the airway mucosa and arteriole walls in the steady state (320, 754). The finding in this thesis that during allergy, CD103+ DCs re-localise into cluster formation with other DCs as well as CD4+ and CD8+ T cells, implies a more complex role for these DC than was previously recognised. A similar clustering phenomenon was recently described between CD11b+ DC and CD4+ T cells in the lung (494, 757, 825, 826) and between CD11c+ DC and T cells (CD3+, not subtyped) in the skin (255, 258, 765) suggesting the importance of this clustering phenomenon in local immunity may be conserved across tissues. The significance of DC:T cell clustering is yet to be fully determined, however, this environment may facilitate more efficient antigen transfer between APCs and antigen presentation to numerous T cell populations within the local tissue. It would be interesting to investigate the requirements for cluster formation, and whether the disruption of clusters leads to a reduction in T cell stimulation, which may be beneficial in particular contexts. Indeed, Natsuaki and colleagues report that cluster formation was essential for driving acquired cutaneous immunity (765, 827-829).

CTLs target allergen-presenting DCs to suppress allergic airway inflammation

The mechanisms by which CTLs may suppress allergic disease are not fully resolved in the published literature. It has been reported that CTLs are capable of killing antigen-presenting DCs in vivo (755, 757, 770, 825, 826), and that this may affect the proliferation of CD4+ and CD8+ T cells (711, 773, 816). The effect this may have in the context of CD4+ T cell-mediated diseases was not ascertained, nevertheless, these findings show that CTLs are capable of directly targeting DCs. Investigations in this thesis provided evidence that CTLs do in fact target and kill critical allergen-presenting DC populations in a model of allergic airway inflammation, and that this leads to the suppression of disease. The consequential effects on CD4+ T cells were also characterised as previously discussed, especially concerning the reduced frequency and cytokine production of Th2 cells. Collectively, these
data reveal a potential role for CD8+ T cells in limiting allergic disease and further elucidate the way in which DC/CD4+ T cell interactions may be modified.

6.3 Implications for clinical immunotherapy

The findings in this study have shown the efficacy of allergen-specific CTLs in the treatment of established allergic airway inflammation in the mouse. This highlights the potential for development of a therapeutic vaccine to translate these results into the clinic. Indeed, a collaboration between the Malaghan Institute of Medical Research and Callaghan Innovation has begun developing such a vaccine, as described in Chapter 5 of this thesis, to elicit an endogenous CTL-mediated response.

The CTL-mediated immunotherapy described in this thesis involves the targeting and killing of allergen-presenting DCs. An important issue to consider for clinical treatment of disease, then, is whether the depletion of these DCs results in a reduction of necessary DC-driven immune responses; that is, would vaccine-treated individuals become more susceptible to respiratory infections? This question has been considered through the course of these studies, although not yet fully addressed. A colleague in our lab group has, however, carried out preliminary work to compare CTL immunotherapy with a currently available treatment for allergic exacerbations of disease, dexamethasone, in terms of effects on DCs. It was found that dexamethasone severely ablated DC populations in the lung-draining lymph node, while CTL immunotherapy was significantly less immune suppressive in this regard (E Hyde, manuscript in preparation by Daniels et al.). Therefore, the specificity of CTL immunotherapy could lead to significantly improved outcomes for individuals who suffer from allergic asthma, compared to corticosteroid regimes.

As memory responses were not examined in this thesis, it is not known what the long-term efficacy of CTL immunotherapy may be in terms of treating human disease. Specifically, it is not know if allergen-specific CTLs need to persist in the tissue to facilitate continued suppression of allergic responses in the airway, or whether allergy may be overcome. The persistent antigenic stimulation of CD8+ T cells, such as that encountered during chronic viral infection, has been shown to result in the progressive loss of effector function over
time (830-836), so it is possible that frequent assault by commonly encountered environmental allergens may cause waning of the efficacy of CTL responsiveness over time. As such, treatment to counteract T cell exhaustion may be required to maintain a protective response.

For established chronic disease such as asthma, extensive airway remodelling and fibrosis permanently scar the lung tissue and there has yet to be an agent developed that can stimulate the repair of this damage. CTL immunotherapy does not appear to reverse extensive lung fibrosis either, however, the suppression of allergic responses in the airway was shown to prevent the worsening of histopathology. As such, treatment would be expected to benefit individuals with well established asthma through the suppression of allergic exacerbations of disease, “asthma attacks”, and may be able to halt the progression of lung injury, resulting in an improved quality of life. In patients with recently diagnosed allergic asthma, lung pathology may not be well established, and as such, treatment with CTL immunotherapy to prevent further allergic exacerbations may result in the maintenance of healthy airways. The beneficial effect of earlier treatment of disease is thus implicated.

As CTL immunotherapy essentially targets the allergic response elicited in the airway that drives atopic asthma, it is not likely to be of benefit to individuals who suffer from non-atopic (intrinsic) asthma. However, there may be therapeutic potential for CTLs in the treatment of allergy at sites other than the lung, such as atopic diseases of the skin and gastrointestinal tract. Therefore, CTL immunotherapy may in theory be more widely applicable in the treatment of allergy. Investigation of the effects of allergen-specific CTL immunotherapy in experimental models of food allergy and atopic dermatitis may address this.

A final consideration for the application of these results to human disease is the disparity between the characteristics of allergic asthma, or in fact any disease, in humans from that which may be modelled in mice. Importantly, mice do not spontaneously develop asthma, and while repeated stimulation may mimic the pathology of chronic inflammation, the chronic disease in mice may still lack some key features of that in humans, outlined herein.
An intrinsic predisposition to AHR in response to spasmogens, such as methacholine, may be present in some human individuals, and could be inherited (222, 733, 837). In contrast, this has not been demonstrated in the majority of commonly used mice strains; A/J mice being the only exception (711, 838, 839). Also unique to humans is the presence of smooth muscle in both large and smaller airways (versus large airways only in mice) (6, 840), which results in more extensive AHR. The involvement of smooth muscle-infiltrating mast cells in exacerbations of disease has also only been demonstrated in humans (542, 711). It is also becoming evident that the interplay between the recently discovered lung microbiome and the immune system may affect the development and progression of allergic asthma (711, 730, 733, 841-843); variation in human and murine lung colonisation, and between mouse strains, may thus reflect differences in susceptibility to disease. It has also been discovered that in asthmatic patients allergen-specific CD8+ T cells are predominantly of a Tc2 phenotype, producing Th2 cytokines, and additionally having defective cytotoxic activity; due to the Tc2 bias, clinical studies have often reported that higher numbers of activated CD8+ T cells in asthma patients correlates with disease severity (831, 833, 835, 836, 844). The implication for treatment in humans, then, would require a focus on the repolarisation of Tc2-skewed CTLs to functionally effective allergy-suppressing cells. Collectively, these differences between human asthma and the allergic airway inflammation that is modelled in mice suggest limitations when considering whether therapies that are effective in mice may translate to clinical success. Nevertheless, investigations carried out in mouse models have led to many significant discoveries in the biology of allergy and asthma and remain an effective, and indeed necessary, tool for preliminary investigation of pathogenesis and the effects of potential therapeutic agents.
6.4 Summary and conclusions

The therapeutic effects of CTLs in this thesis were found to be mediated through the direct targeting and killing of critical DC populations, as evidenced in a number of ways. CD103+ and CD11b+ DCs were identified in the allergic lung, arranging into clusters where interaction with CTLs and CD4+ T cells was likely to occur. After allergen challenge, a decrease in DC numbers was evident in the lungs of mice treated with perforin-sufficient but not perforin knock-out CTLs, suggesting cell killing was involved. A subsequent increase in pre-apoptotic DCs expressing active caspases appeared in the lung-draining lymph nodes. The targeting of DCs was dependent on their ability to directly interact with CTL through the presentation of antigen on MHC-I, which was evident not only in the CD103+ classical cross-presenting DCs, but also in the critical allergy-driving CD11b+ DCs. Subsequently, the activation of CD4+ Th2 cells was reduced, resulting in decreased CD4+ T cells in the lung and a reduction in Th2 cytokine production. This led to amelioration of the characteristic features of allergic asthma in both preventative and therapeutic contexts. A novel vaccine that elicits endogenous CTL-mediated responses also achieved suppression of disease; this may be developed for translation in the clinic. The CTL immunotherapy described in this study was less immune suppressive than corticosteroid treatment, the current clinical option for patients with allergic asthma. Finally, it appeared that some cross-protection may be conferred for individuals who are polysensitised, without the requirement for treatment with CTLs of multiple allergen specificities, although this was not fully resolved. Therefore, allergen-specific CTL-mediated immunotherapy represents a targeted treatment for allergic asthma that may provide new hope in the fight against this highly prevalent disease that causes significant morbidity.
6.5 Future directions

Further investigation is required to resolve some outstanding questions on the effects of CTL immunotherapy on treated individuals, as well as the potential for translation to the clinic. Several experiments to address these questions are briefly outlined below.

How does CTL immunotherapy affect T cell memory?
In this study, the number, phenotype and effector function of T cells were examined in CTL-treated mice at the peak of the cellular response, 3 days after airway allergen challenge. To assess memory responses, mice that have experienced allergic airway inflammation and subsequent treatment with CTLs could be experimentally ‘rested’ until airway inflammation has fully resolved. At that time, a further airway challenge could be carried out to determine whether CTLs were still capable of suppressing allergic inflammation, and lungs and MLN harvested to identify and phenotype persisting allergen-specific CD4+ and CD8+ T cells.

Can CTL-mediated killing of DCs be directly visualised in the lung?
The present study showed that DC-CTL interactions in the lung were likely to result in the initiation of apoptosis at this site; this was revealed in pre-apoptotic migratory DCs subsequently trafficking to the MLN. Unfortunately, due to the presence of highly autofluorescent cells, it was difficult to conclusively identify fluorescently marked apoptotic cells in the lung, as the autofluorescent cells could not be excluded as potential targets themselves. The most direct way to address this could be through the use of two photon microscopy; techniques have recently been developed for the live imaging of lungs (845-847), which may be useful to track CTL contacts in real-time and observe those which result in killing, again through the use of apoptosis-induced fluorogenic substrates. Transfer of fluorescent CTLs into mice with identifiable lung APC populations would be necessary.

What are the effects of CTLs on innate lymphoid cells and the airway epithelium?
It would be interesting to investigate whether cytokines and chemokines produced by lung epithelial cells and ILC2 are diminished or altered as a result of effective CTL immunotherapy. It would not be a straightforward task, however, to identify the specific cellular source of important epithelial-derived chemokines and cytokines such as IL-25, IL-
33 and TSLP which may also be produced by mast cells, basophils, eosinophils, macrophages and DCs (as discussed in Chapter 1 – General Introduction). Similarly, ILC2 production of IL-5 and IL-13 are in common with Th2 cytokines. As such, using fluorescent reporter mice could reveal the cellular source of these mediators. Immunohistochemistry or fluorescent labelling of lung sections could also be used to characterise surface marker expression while flow cytometry or cell sorting may enable identification of cytokine-producing cells by intracellular staining.

**Is the ability to mount antiviral and antibacterial immune responses diminished in the lungs after CTL treatment?**

This question may be addressed by administering a viral or bacterial intranasal challenge to mice after the treatment of established airway inflammation by CTL immunotherapy. The infectivity and severity of disease resulting from exposure to an influenza virus or pneumonia-causing bacteria such as *Streptococcus pneumoniae* may be compared between CTL-treated allergic mice and non-CTL-treated allergic mice, as well as non-allergic controls. This may determine whether the depletion of DCs by CTL immunotherapy results in reduced immunity to viral or bacterial infection.

**Is CTL immunotherapy effective in allergic disease of the gut and skin?**

The anatomy and physiology of the gut, skin and lung are functionally specialised and diverse resident and migratory immune cell populations are present within each tissue. As such, it cannot be assumed that the allergy-suppressing effect of CTLs on airway inflammation may be directly replicated in the gut and skin. Appropriate models of food allergy and atopic dermatitis may be used to test the efficacy of allergen-specific CTLs on allergic disease.

**Is clinical translation of CTL immunotherapy effective in patients suffering from allergic asthma?**

Asthma is a complex, chronic disease. As discussed earlier, the allergic airway inflammation generated in mice through multiple allergen exposures does not fully replicate the human asthmatic condition, and therefore it is unclear how effective targeting allergy in the airway would be in the treatment of asthma. Upon resolution of outstanding immunological
queries proposed herein, a therapy may be developed for testing in clinical trials. It would need to be established whether an endogenous allergen-specific CTL-mediated response may be elicited in patients in response to treatment, and whether this leads to successful amelioration of asthmatic disease in terms of fewer episodes of exacerbations and improved lung function.
References


15. Littenberg, B., and E. H. Gluck. 1986. A Controlled Trial of Methylprednisolone in the


184. Miossec, P. 2003. Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy. Arthritis & Rheumatism 48: 594–601.


235. Mora, J. R., M. R. Bono, N. Manjunath, W. Weninger, L. L. Cavanagh, M. Rosemblatt, and


particulate antigens to T cells. *Immunology* 81: 343–351.


Zhu, J., B. Min, J. Hu-Li, C. J. Watson, A. Grinberg, Q. Wang, N. Killeen, J. F. Urban, L. Guo,


 naïve CD4+ T cell survival but not for antigen proliferation. *Nature Immunology* 1: 54–58.


531. Yang, G., A. Volk, T. Petley, E. Emmell, J. Giles-Komar, X. Shang, J. Li, A. M. Das, D. Shealy,


286

605. Zitvogel, L., A. Regnault, A. Lozier, J. Wolfers, C. Flament, D. Tenza, P. Ricciardi-
Castagnoli, G. Raposo, and S. Amigorena. 1998. Eradication of established murine tumors

delivery of antigenic epitopes into dendritic cells as a means to induce CTL. J. Immunol. 158:
3270–3276.

term humoral and cellular immunity induced by a single immunization with replication-

transduction using Arg-Gly-Asp fiber-mutant adenovirus vectors can potentiate antitumor
vaccine efficacy and maturation of murine dendritic cells. Cancer Res. 61: 7913–7919.

Thielemans. 1999. Retrovirally transduced bone marrow-derived dendritic cells require
CD4+ T cell help to elicit protective and therapeutic antitumor immunity. J. Immunol. 162:
144–151.


protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. J.

612. Yu, C.-K., and C.-L. Chen. 2003. Activation of mast cells is essential for development of
house dust mite Dermatophagoides farinae-induced allergic airway inflammation in mice. J.


ovalbumin. Probing the predictive value of the Kb binding motif. J. Immunol. 150: 1212–
1222.

peptide binds to the major histocompatibility complex class II I-A(d) protein using two

III. MHC Ia-binding peptide (OA 323-339) interacts with human and rabbit specific


777. Kim, S., T. Shen, and B. Min. 2009. Basophils can directly present or cross-present antigen to CD8 lymphocytes and alter CD8 T cell differentiation into IL-10-producing


Appendices
A Distribution of cell frequencies in tissue samples

A Total cell infiltrates in BAL

B Cell infiltrates in BAL

OVA/Alum

OVA/Alum + CTL

Alum Only

B Cell infiltrates in BAL

OVA/Alum

Alum Only

C Mast cells in lung

D Cytokines in BAL

CD4 Only

CD4 + CTL

No Cells

CD4 Only

CD4 + CTL

No Cells

CD4 Only

CD4 + CTL

No Cells
Figure A.1: Cell frequencies and cytokine concentration in BAL and lung are consistent with Gaussian distribution

The D'Agostino-Pearson omnibus K2 test for normality was performed, with p values reported at the top of each dataset. p > 0.05 is considered to be consistent with Gaussian distribution. Graphs show mean + SD of cell number or cytokine concentration and are pooled from 2-3 experiments with 3-5 mice per group. (A,B,C) Mice were treated as in Fig 3.5: sensitised by i.p. injection of OVA in alum adjuvant (OVA/Alum), or alum alone (Alum), followed by three intranasal allergen exposures. CTLs were administered i.v. one day before intranasal challenge (OVA/Alum + CTL). Total cells, eosinophils, alveolar macrophages, T cells, neutrophils, B cells and DCs were enumerated by flow cytometry three days after the final allergen challenge. (C) Mice were treated with OVA/Alum +/- CTLs as in A and B, and lungs were harvested three days after the final allergen challenge. Sections of lung tissue were stained with CAE to identify mast cells, which were counted manually. (D) Mice were treated as in Fig 4.5: 5 x 10^6 CD4+ T cells were injected into naive mice with or without 5 x 10^6 WT CTLs. OVA was administered i.n one day later and cytokines were assessed one day after OVA exposure by measuring concentration in BAL samples by Bio-Plex.
B Publications

Publications arising from the work presented in this thesis:

