CELLULAR AND HUMORAL MECHANISMS OF ALLERGIC DISEASE

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Abstract

CD154 is a T cell activation marker, transiently expressed following ligation of the T cell receptor, therefore providing direct access to an antigen-specific T cell population. Using peripheral blood samples taken from allergic individuals and healthy non-atopic controls, this project identified and phenotyped CD154⁺ T helper cells following ex vivo stimulation with native allergen extracts (birch pollen, cat dander, grass pollen). Peripheral blood mononuclear cells were stimulated with allergen extract for 16 hours in the presence of Brefeldin A. Responding CD154⁺ T cells were identified and phenotyped using multiparametric flow cytometry. Activated CD154⁺ T⁺, T⁺ and T⁺⁺-like cells, that co-expressed IFNγ, IL4 and IL10 respectively, were identified in allergic and non-allergic participants. A close correlation was observed between T⁺, T⁺ and T⁺⁻-like cell frequency in non-allergic participants, such that the three parameters increased together to maintain a low T⁺:T⁺⁺ ratio. The relationship between T⁺, T⁺ and T⁺⁻-like responses was dysregulated in allergic individuals, with abrogation of the IL10 response and a higher T⁺:T⁺⁺ ratio. A close correlation was observed between Th2 cell frequency and the absolute concentration of birch-specific IgE. This work confirms previous reports of a more differentiated T cell phenotype in allergic subjects with regard to seasonal allergens. The detection of CD154⁺ T cells after short-term antigen stimulation may be a useful method for the detection of T cell responses to allergens when cost, speed and convenience are priorities.

The CD154 assay was also used to investigate allergen-specific T cells in two situations: [1] during allergoid immunotherapy and [2] at peak pollen season compared to out of season. This preliminary data suggests an increased frequency of T⁺ cells in allergic individuals during the birch pollen season compared to healthy non-allergic controls, and a decrease in the T⁺:T⁺⁺ ratio following successful immunotherapy.

A proliferation assay utilising the cell surface PKH dye was also optimised to investigate proliferative responses to native allergen extracts in allergic and non-allergic subjects.
Colonisation with superantigen-producing staphylococci is common in atopic diseases and may contribute to the initiation and maintenance of allergic sensitisation. However, little is known regarding T cell responses to superantigens in atopic individuals. This project sought to investigate T helper cell responses to the superantigen Staphylococcal Enterotoxin B (SEB) from *Staphylococcus aureus* in non-atopic individuals and highly atopic polysensitised individuals. Peripheral blood mononuclear cells were stimulated with SEB for 16 hours in the presence of Brefeldin A. Responding T<sub>H1</sub>, T<sub>H2</sub>, T<sub>H17</sub>, T<sub>R1</sub>-like and naturally occurring regulatory T cells were identified using multiparametric flow cytometry. This preliminary study identified a downregulated T<sub>H17</sub> response to the superantigen SEB in highly atopic individuals that does not relate to T<sub>Reg</sub> cell frequency or TCRVβ3 expression.

The Pollen-Food Syndrome (PFS) is caused by sensitisation to homologous panallergens within aeroallergens and food proteins. Information regarding the sensitisation profiles of individuals with PFS in the UK is limited and investigations into causative panallergens are not routinely performed. In a small study, patients with symptoms suggestive of PFS were recruited from the Allergy Clinic at Brighton and Sussex University Hospital NHS trust. A standardised food allergy questionnaire was completed and serum analysed by component-resolved diagnosis using ImmunoCAP ISAC technology. This study cohort conformed to the Northern European pattern of birch-pollen associated PFS with cross-reactivity between the major birch pollen allergen Bet v 1 and homologues in food proteins. Sensitisation to LTPs and profilins was also noted, but the clinical relevance of these remains to be elucidated. Profilin sensitisation was associated with reactions to a significantly larger number of foods compared to PR-10 monosensitised individuals.
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I would like to thank my family, and in particular my parents, for the support they have provided so I could go to university. I could not have completed this PhD without them. I would also like to thank James Bockhart for his support and encouragement.
Author’s Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. This thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

Date:
Table of Contents

ABSTRACT .......................................................................................................................................... I
ACKNOWLEDGEMENTS .................................................................................................................. III
AUTHOR’S DECLARATION ............................................................................................................. V
TABLE OF CONTENTS ................................................................................................................... VI
LIST OF TABLES .............................................................................................................................. XI
LIST OF FIGURES ............................................................................................................................ XII
ABBREVIATIONS ............................................................................................................................ XVI

CHAPTER 1: INTRODUCTION .................................................................................................................. I

1.1 AN OVERVIEW OF ALLERGIC DISEASE .......................................................................................... 1
  1.1.1 Epidemiology of Allergy ............................................................................................................. 2
    1.1.1.1 Environmental Factors ........................................................................................................... 3
    1.1.1.1.1 Geographical Variations ........................................................................................................ 3
    1.1.1.1.2 The Hygiene Hypothesis ...................................................................................................... 3
    1.1.1.1.3 Lifestyle and Perinatal Factors .............................................................................................. 5
    1.1.1.2 Genetic Factors ....................................................................................................................... 6

1.2 CELLULAR AND HUMORAL RESPONSES ......................................................................................... 6
  1.2.1 The Role of IgE ............................................................................................................................. 6
  1.2.2 Innate Cells in Allergic Disease .................................................................................................. 7
    1.2.2.1 Mast Cells ............................................................................................................................... 7
    1.2.2.2 Eosinophils ............................................................................................................................. 9
    1.2.2.3 Neutrophils ........................................................................................................................... 10
  1.2.3 Mechanisms of Allergic Disease – The Early and Late Phase Response ....................................... 10
    1.2.3.1 The Sensitisation Phase ........................................................................................................... 10
    1.2.3.2 The Early Phase Response ...................................................................................................... 12
    1.2.3.3 The Late Phase Response ....................................................................................................... 12

1.3 THE ROLE OF T CELLS IN ALLERGY ............................................................................................... 12
  1.3.1 T Cell Development .................................................................................................................... 12
  1.3.2 Antigen Presentation and T Cell Activation ................................................................................ 13
  1.3.3 The T_{H}1/T_{H}2 Paradigm ........................................................................................................... 20
  1.3.4 The Role of T_{H}1 Cells and T_{H}1 Cytokines in Allergy ................................................................. 20
  1.3.5 The Role of T_{H}17 Cells and T_{H}17 Cytokines in Allergy ............................................................ 23
  1.3.6 Regulatory T Cells ..................................................................................................................... 24
  1.3.7 The Role of Regulatory T Cells in Allergy .................................................................................. 25
  1.3.8 T Cells in Allergy – A Summary ................................................................................................. 27

1.4 ALLERGENS ..................................................................................................................................... 27
  1.4.1 Characteristics of Allergens ........................................................................................................ 27
  1.4.2 Pollens ........................................................................................................................................ 28
  1.4.3 Animal Allergens ........................................................................................................................ 28
  1.4.4 Food Allergens ........................................................................................................................... 29
    1.4.4.1 Pollen-Food Syndrome ......................................................................................................... 29
    1.4.4.1.1 PR-10 Protein Family ......................................................................................................... 32
    1.4.4.1.2 Lipid Transfer Proteins ...................................................................................................... 32
    1.4.4.1.3 Profilin ............................................................................................................................... 32

1.5 TREATMENT OF ALLERGIC DISEASE ......................................................................................... 33
  1.5.1 Allergen Avoidance ..................................................................................................................... 33
  1.5.2 Antihistamines ............................................................................................................................ 33
CHAPTER 2: MATERIALS AND METHODS

2.1 STATEMENT OF CONTRIBUTION .................................................................. 52
2.2 ETHICAL APPROVAL .................................................................................. 52
2.3 PARTICIPANT RECkrUITMENT .................................................................... 52
2.4 MATERIALS ............................................................................................... 53
2.4.1 Cell Culture Media ............................................................................... 53
2.4.2 Antigens .............................................................................................. 53
2.4.3 Cell Culture Reagents ......................................................................... 54
2.4.4 Buffers and Reagents .......................................................................... 54
2.4.5 Antibodies ........................................................................................... 55
2.4.6 ImmunoCAP ISAC Allergen Microarray Technology Kit ...................... 58
2.4.7 TNFα ELISA Kit .................................................................................. 58
2.5 CELL CULTURE PROTOCOLS .................................................................. 59
2.5.1 Serum Sample Preparation .................................................................. 59
2.5.2 PBMC Isolation ................................................................................... 60
2.5.2.1 Overnight Allergen Stimulation .................................................... 60
2.5.2.2 Seven-Day PKH T Cell Proliferation Assays ................................. 60
2.5.3 PKH Staining of PBMCs .................................................................... 61
2.5.4 PBMC Stimulation .............................................................................. 62
2.5.4.1 Overnight Antigenic Stimulation .................................................. 62
2.5.4.2 Seven-Day PKH T Cell Proliferation Assays ................................. 63
2.6 EXPERIMENTAL PROTOCOLS ................................................................ 63
2.6.1 Surface and Intracellular Staining of PBMCs ....................................... 63
2.6.2 FOXP3 Intracellular Staining of PBMCs ............................................. 64
2.6.3 Compensation Controls ....................................................................... 66
2.6.3.1 PKH Compensation .................................................................... 66
2.6.3.2 Aqvid Compensation .................................................................. 66
2.6.4 ImmunoCAP ISAC Allergen Microarray Technology ........................... 67
2.6.5 TNFα ELISA ....................................................................................... 68
2.6.6 LAL Test ............................................................................................. 70
2.7 ANALYSIS OF DATA ............................................................................... 70
2.8 STATISTICAL ANALYSIS ........................................................................ 70

CHAPTER 3: ASSAY OPTIMISATION ................................................................... 71
CHAPTER 4:  EX VIVO ANALYSIS OF CD154+ T HELPER CELLS IN ATOPIC BIRCH POLLEN-ALLERGIC AND NON-ALLERGIC INDIVIDUALS ................................................................. 94

4.1 INTRODUCTION .................................................................................................................. 94
4.2 AIMS ........................................................................................................................................ 95
4.3 STUDY PROTOCOL .............................................................................................................. 95
   4.3.1 Laboratory Protocol ....................................................................................................... 95
   4.3.2 Flow Cytometric Analysis ............................................................................................ 96
   4.3.3 Statistical Analysis ....................................................................................................... 96

4.4 PARTICIPANT CHARACTERISTICS ...................................................................................... 96

4.4.1 Detection of CD154, T_{h1}, T_{h2} and T_{h1}-Like Populations ........................................ 98
4.4.1.1 T_{h1}-Like Responses ................................................................................................. 100
4.4.1.1 T_{h1} and T_{h2} Responses ......................................................................................... 102
4.4.1.1 Humoral Responses ................................................................................................ 105
4.4.1.1 A Subset of Non-Allergic Participants Expressing IL4 ............................................ 107
4.4.1.2 A Subset of Birch-Allergic Participants Expressing IL10 ........................................ 107
4.4.1.1 T_{h1}, T_{h2} and T_{h1}-Like Responses Correlate in Health ........................................... 110
4.4.1.1 CD154, T_{h1} and T_{h2} Phenotypes ........................................................................... 112

4.2 DISCUSSION ......................................................................................................................... 114
4.3 CONCLUSIONS .................................................................................................................... 123

CHAPTER 5:  EX VIVO ANALYSIS OF CD154+ T HELPER CELLS IN CAT-ALLERGIC,
GRASS-ALLERGIC AND NON-ALLERGIC INDIVIDUALS .................................................... 125

5.1 INTRODUCTION .................................................................................................................. 125
5.2 AIMS ........................................................................................................................................ 126
5.3 STUDY PROTOCOL .............................................................................................................. 126
   5.3.1 Laboratory Protocol ....................................................................................................... 126
   5.3.2 Flow Cytometric Analysis ............................................................................................ 127
   5.3.3 Statistical Analysis ....................................................................................................... 127

5.4 PARTICIPANT CHARACTERISTICS ...................................................................................... 127

5.1 RESULTS .................................................................................................................................. 127
   5.1.1 Detection of CD154, T_{h1}, T_{h2} and T_{h1}-like Responses ........................................ 128
   5.1.1 T_{h1} and T_{h2} Responses .......................................................................................... 132
   5.1.1 T_{h1}-Like Responses ................................................................................................. 135
   5.1.1 Bimodal Distribution of IL4 in Non-Allergic Participants ........................................... 136
   5.1.1 Bimodal Distribution of IFN_{gamma} in Grass-Allergic Participants ............................. 138
   5.1.1 Bimodal Distribution of IL10 in Allergic Participants ............................................... 140
List of Tables

Chapter 1
Table 1.1. Mediators released from mast cells 8
Table 1.2. Mediators released from eosinophils 9
Table 1.3. Role of T_{H}2 cytokines in allergic diseases 21
Table 1.4. Regulatory T cell populations 24

Chapter 2
Table 2.1. List of antibodies 55
Table 2.2. Volumes of antigen for overnight stimulation of PBMC 62
Table 2.3. Volumes of antigen for 7-day stimulation of PBMC 63

Chapter 4
Table 4.1. Key Characteristics of participant groups 96
Table 4.2. Participant demographics 97

Chapter 5
Table 5.1. Participant demographics 127

Chapter 6
Table 6.1. Participant demographics 161

Chapter 7
Table 7.1. Definitions of T helper cell subsets 179
Table 7.2 Participant demographics 180

Chapter 8
Table 8.1. Frequency of PKH^{Low} T cells 208

Chapter 9
Table 9.1. Participant demographics 218

Chapter 12
Table 12.1. BD Biosciences LSR II flow cytometer settings 237
List of Figures

Chapter 1

Figure 1.1. The early and late phase response in allergy 11
Figure 1.2. Initial interaction between the T cell and APC 15
Figure 1.3. Activation of T cells 17
Figure 1.4. Phosphorylation pathway of T cell activation 18
Figure 1.5. Structures of PR-10 proteins, LTPs and profilins 31
Figure 1.6. Mechanisms of specific immunotherapy 37
Figure 1.7. Layout of a simple flow cytometer 44
Figure 1.8. Spectral overlap of commonly used fluorochromes 45
Figure 1.9. Light scatter dot plots 46
Figure 1.10. Analysis of two cell populations 47

Chapter 3

Figure 3.1. CD154 expression dose response curve 73
Figure 3.2. TNFα concentrations of allergens 75
Figure 3.3. LPS content of allergens 76
Figure 3.4. CD154 and HLA-DR expression 78
Figure 3.5. Flow cytometric data in a representative non-allergic subject 81
Figure 3.6. Allergen-stimulated CD154, TH1 and TH2 cell frequencies 82
Figure 3.7. Phenotypic analysis of CD154+ T cells 83
Figure 3.8. Flow cytometric comparison of antibodies 85
Figure 3.9. Gating strategy for CD154+ T cells 87
Figure 3.10. SEB stimulated cytokine responses 89
Figure 3.11. Gating strategy for SEB-stimulated T cell cytokines 91
Figure 3.12. Gating strategy for SEB-stimulated regulatory T cells 92


Chapter 4

Figure 4.1. Original data in one representative non-allergic subject

Figure 4.2. Birch-induced CD154^IL10^ T cells

Figure 4.3. Expression of regulatory T cell markers in IL10^ T cells

Figure 4.4. T_H2, T_H1, T_H2:T_H1 ratio and ROC curve analysis

Figure 4.5. PHA-induced cytokine expression

Figure 4.6. Correlation of T_H2 cells and birch-specific IgE

Figure 4.7. Birch-specific IgG4 analysis

Figure 4.8. Birch-induced IL4 responders vs. IL4 non-responders

Figure 4.9. Birch-induced IL10 responders vs. IL10 non-responders

Figure 4.10. Correlations of CD154, T_H1, T_H2 and T_R1-like responses

Figure 4.11. Phenotypes of CD154, T_H1 and T_H2 responses

Figure 4.12. Further phenotyping of T_H2 cells

Chapter 5

Figure 5.1. Original data in one representative non-allergic subject

Figure 5.2. Original data in one representative non-allergic subject

Figure 5.3. T_H2, T_H1, T_H2:T_H1 ratio and ROC curve analysis

Figure 5.4. PHA induced cytokine expression

Figure 5.5. CD154^IL10^ T cells

Figure 5.6. Grass-induced IL4 responders vs. IL4 non-responders

Figure 5.7. Grass-induced IFN_γ responders vs. IFN_γ non-responders

Figure 5.8. Cat-induced IL10 responders vs. IL10 non-responders

Figure 5.9. Grass-induced IL10 responders vs. IL10 non-responders

Figure 5.10. Correlations of CD154, T_H1, T_H2 and T_R1-like responses

Figure 5.11. Correlations of CD154, T_H1, T_H2 and T_R1-like responses

Figure 5.12. Phenotypes of CD154, T_H1 and T_H2 responses

Figure 5.13. Atopic non-allergic and sensitised participants
Figure 5.14. Atopic non-allergic and sensitised participants  

**Chapter 6**

Figure 6.1. Seasonal changes of \( T_H2, T_H1 \) and \( T_H2:T_H1 \) ratio  
Figure 6.2. Seasonal changes of IL10 expression  
Figure 6.3. Seasonal changes of CD154\(^+\) T cell phenotype  
Figure 6.4. Seasonal changes of \( T_H1 \) cell phenotype  
Figure 6.5. Seasonal changes of \( T_H2 \) cell phenotype  
Figure 6.6. \( T_H1, T_H2 \) and \( T_R1 \)-like responses following immunotherapy  

**Chapter 7**

Figure 7.1. SEB-induced cytokine expression in CD4 T cells  
Figure 7.2. SEB-induced cytokine expression in CD8 T cells  
Figure 7.3. SEB-induced cytokine expression in CD4 T cells  
Figure 7.4. SEB-induced cytokine expression in CD8 T cells  
Figure 7.5. PHA-induced cytokine expression in CD4 T cells  
Figure 7.6. PHA-induced cytokine expression in CD8 T cells  
Figure 7.7. SEB-induced CD4\(^+\)CD25\(^+\)FOXP3\(^+\) regulatory T cells  
Figure 7.8. SEB-induced CD8\(^+\)CD25\(^+\)FOXP3\(^+\) regulatory T cells  
Figure 7.9. SEB-induced TCRV\(\beta3\) expression  
Figure 7.10. SEB-induced TCRV\(\beta1\) expression  

**Chapter 8**

Figure 8.1. PKH67 dye concentration  
Figure 8.2. RPMI culture media supplementation  
Figure 8.3. Time course of birch-induced PKH proliferation  
Figure 8.4. IL2 supplementation  
Figure 8.5. PKH birch allergen dose response  
Figure 8.6. CD154 expression in PKH\(^{\text{Low}}\) T cells
Chapter 9

Figure 9.1. Foods causing local oro-pharyngeal symptoms 221
Figure 9.2. Non-cross-reactive aeroallergen molecules 223
Figure 9.3. Profilin co-sensitisation 224

Chapter 11

Figure 11.1 FMO controls (OAS antibody panel) 238
Figure 11.2. FMO controls (SEB antibody panel) 244
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPA</td>
<td>Allergic Bronchopulmonary Aspergillosis</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
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<tr>
<td>BPE</td>
<td>Birch Pollen Extract</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CDE</td>
<td>Cat Dander Extract</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl Ester</td>
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<tr>
<td>CLA</td>
<td>Cutaneous Lymphocyte-Associated Antigen</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T Lymphocyte Antigen 4</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence Minus One</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>GPE</td>
<td>Grass Pollen Extract</td>
</tr>
<tr>
<td>hAB</td>
<td>Human AB Serum</td>
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<tr>
<td>HEPA</td>
<td>High Efficiency Particulate Air</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-Gamma</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immunodysregulation Polyendocrinopathy Enteropathy X-Linked</td>
</tr>
<tr>
<td>ISAC(C)</td>
<td>International Society for Analytical Cytology (Cytometry)</td>
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<tr>
<td>ISU</td>
<td>ISAC Standardised Units</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
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<td>LTP</td>
<td>Lipid Transfer Protein</td>
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<td>MACS</td>
<td>Magnetic-Activated Cell Sorting</td>
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<tr>
<td>OAS</td>
<td>Overnight Allergen Stimulation</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKH</td>
<td>Paul Karl Horan</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
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<tr>
<td>PNU</td>
<td>Protein Nitrogen Units</td>
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<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
</tr>
<tr>
<td>PFS</td>
<td>Pollen Food Syndrome</td>
</tr>
<tr>
<td>rBet v 1</td>
<td>Recombinant Bet v 1 (major Birch Pollen Allergen)</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute (Culture Medium)</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
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<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcus Enterotoxin B</td>
</tr>
<tr>
<td>SPT</td>
<td>Skin Prick Test</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα Converting Enzyme</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumour Growth Factor Beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor Alpha</td>
</tr>
<tr>
<td>T_{Reg}</td>
<td>Regulatory T Cells</td>
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Chapter 1: Introduction

1.1 An Overview of Allergic Disease

Von Pirquet (1906) originally defined the term allergy to mean an altered immunological response to antigens in a general sense (1), after he noted adverse reactions to tuberculin anti-sera upon secondary administration. Later, the term allergy became limited to the description of an IgE-mediated inappropriate immune response to environmental allergens (type I hypersensitivity) (2). Examples include pollens, pet allergens, insect venom and food proteins.

Atopy is defined as the genetic predisposition to produce IgE antibodies against environmental allergens (3). Atopic individuals may develop allergic diseases such as rhinitis, asthma and food allergy and have higher eosinophil counts and levels of circulating IgE. The sequential development of allergic conditions during infancy and childhood is known as the “atopic march”, where eczema is usually the first condition to appear, followed by food allergies, asthma and allergic rhinitis (4). With the exception of nut allergy (which often remains persistent), most individuals become tolerant of food allergens by adulthood and may outgrow asthma and rhinitis. Nonetheless, allergies can also develop in adulthood.

Allergic rhinitis is an IgE-mediated inflammatory disease of the nasal mucosa associated with sneezing, itching, nasal discharge and obstruction, affecting approximately 10-20 million individuals worldwide (5). Allergic rhinitis falls into two categories: perennial allergic rhinitis, in which symptoms manifest all year round (house dust mite, cat dander, moulds) and seasonal allergic rhinitis, in which symptoms manifest during the various pollen seasons (tree, grass).

Asthma is a chronic inflammatory disease of the lower respiratory tract. Constriction of bronchial smooth muscle, mucus hypersecretion and airway hyperreactivity contribute to wheezing, airway obstruction and difficulty breathing. Over time, this inflammatory response leads to airway remodelling, which can cause fibrosis and narrowing of the airways causing significantly worsened symptoms. A relationship between asthma, allergens and IgE has been identified (6); however, approximately 30% of asthmatic individuals are non-atopic (7).
Food allergies involve the development of an IgE-mediated immune response to a variety of food proteins. This causes a more generalised or systemic reaction with symptoms such as angioedema, urticaria and respiratory and/or cardiovascular symptoms. In infants and children, the allergens most often implicated include egg, peanut, milk and wheat. By contrast, the most common cause of food allergy in adults is pollen-food syndrome, which results from sensitisation to panallergens common to both pollen and plant-derived foods.

Atopic dermatitis (eczema) is an inflammatory response in the skin, typically manifesting as itchy and/or swollen rashes and lesions. Typical features include severe itching, very dry skin, skin abrasions, epidermal thickening and increased susceptibility to bacterial infection, most commonly seen in the backs of knees and elbows.

Systemic anaphylaxis describes a generalised allergic reaction. Anaphylaxis was first described by Portier and Richet in 1902, who demonstrated a fatal reaction in immunised dogs after secondary injection of sea anemone extract (8). Manifestations include angioedema, urticaria, gastrointestinal symptoms, respiratory symptoms (including wheezing and airway obstruction) and/or a compromised cardiovascular system with increased vascular permeability, vasodilation and a subsequent drop in blood pressure. The most common causes of systemic anaphylaxis are allergic reactions to foods (peanut), drugs (penicillin) and insect stings (bee/wasp venom). Anaphylaxis can also be non-IgE mediated, in which an antigen may directly or indirectly stimulate mast cells through complement pathway interaction or modulation of arachidonic acid metabolism, as seen with some reactions to radio-contrast media.

1.1.1 Epidemiology of Allergy

Allergic diseases affect one third of individuals in the UK (9, 10) costing the NHS approximately £900 million a year and comprising 10% of primary care prescription costs (11). Epidemiological studies have demonstrated the UK has the highest prevalence of allergy in Europe and one of the highest in the world (12). Research suggests the development of allergy is multifactorial and may involve both environmental and genetic characteristics.
1.1.1.1 *Environmental Factors*

1.1.1.1.1 *Geographical Variations*

Allergic diseases are more prevalent in urban populations such as Western Europe, United States and Australia (12). For example, the incidence of allergic disease is higher in mid-Europe compared to Northern or Southern Europe (13), and similar patterns are reported in urban areas of the Gambia compared to rural communities (14). Allergic diseases were less common in East Germany compared to West Germany (15), but the incidence of allergy increased significantly in East Germany following the reunification (16). These geographical variations indicate a “Westernised” lifestyle is an important factor in the prevalence of allergic disease. This may reflect reduced microbial exposure and improved cleanliness described in the hygiene hypothesis.

1.1.1.1.2 *The Hygiene Hypothesis*

The hygiene hypothesis postulates an insufficient transfer from Th2 to Th1 immune responses in infancy caused by reduced microbial exposure. This may result from vaccinations, antibiotic use and increased cleanliness in Westernised populations. Infants with minimal exposure to microorganisms will not undergo immune deviation, therefore maintaining production of Th2 cytokines and eliciting IgE-mediated responses. Publications supporting the hygiene hypothesis are numerous.

Individuals with multiple siblings have a lower incidence of allergic disease compared to children without siblings (17): this is particularly evident in the youngest children (18). This may reflect increased microbial exposure during sibling interaction or through the maternal immune system. Microbial burden may increase with each successive pregnancy (19), therefore increasing the introduction of microorganisms on younger siblings. The burden of common childhood respiratory diseases increases in children attending day care, but the long-term outcome describes protection against the development of asthma and atopic disease (17, 20), suggesting a possible relationship between early microbial exposure and atopy.

Early life infection with Herpes simplex 1, measles or Hepatitis A virus is linked to a lower incidence of allergic disease (21), although it is unknown if a general microbial burden rather than a single infection is more important for regulating
immune responses in infancy (22). The hygiene hypothesis also reports vaccinations may indirectly prevent the development of “natural” $T_H1$ immune responses (23), although large epidemiological studies have failed to identify a significant relationship between childhood vaccinations and the incidence of allergic disease (24, 25).

Children brought up in a farming environment are less likely to develop allergic diseases (26), perhaps related to the presence and exposure of microbial antigens in livestock, barns and unpasteurised milk. Indeed, children who drink unpasteurised milk exhibit lower total serum IgE, decreased eczema severity and increased IFN$\gamma$ responses (27). This may relate to the increased level of endotoxin present in a farming environment. An inverse relationship has been demonstrated between the presence of endotoxin and development of eczema, allergic rhinitis and atopic sensitisation in childhood (28).

Studies investigating microflora of the gastrointestinal tract report IgE sensitisation, food allergy and eczema in individuals with low lactobacilli, *Toxoplasma gondii* and *Helicobacter pylori* colonisation (7). This is also evident with increased clostridia colonisation (29). Helminthic gastrointestinal infection prevented the development of allergic asthma in a murine model (30) and disruption of microflora in murine models is known to induce allergic airway disease (31). The delicate balance of commensal microflora is important for the maintenance of both mucosal and systemic immune function, therefore alterations could potentially affect the reactivity of allergens.

Antibiotics influence and change the commensal flora of the gut and early life antibiotic administration may be a risk factor for childhood asthma (32). In addition, the use of antibiotics by expectant mothers increases the risk for asthma and wheeze of the infant as the commensal flora in the third trimester of pregnancy is reported to influence that of the newborn (33). The timing and exposure of antibiotic use seems to play a critical role in the risk of asthma, with those infants undergoing antibiotic treatment in the first year of life demonstrating a significantly higher prevalence of asthma compared to infants on antibiotics after 1 year (34). However, any link identified between the prevalence of asthma and antibiotic use may be representative of reverse causation associated with increased respiratory infections in asthmatic
individuals. Alongside antibiotics, frequent use of paracetamol has been associated with a higher prevalence of asthma (35) and children are more likely to develop allergic disease if expectant mothers frequently take paracetamol late in the pregnancy (36).

The hygiene hypothesis has become an important theoretical framework for research into the development of allergic diseases. However, allergy is still present in rural populations and alternative causes have also been described.

1.1.1.3  Lifestyle and Perinatal Factors

The prevalence of obesity has simultaneously increased alongside allergic disease, therefore sparking interest in the relationship between these conditions. In asthma, increased symptom severity, higher levels of circulatory IgE and changes in the immune response and mechanical function in the lung have been linked to obesity (37). Research has also focused on the diet of expectant mothers during pregnancy and breastfeeding. Deficiencies of vitamin E and zinc in the diet during pregnancy are associated with increased asthma and wheeze (38). In addition, a high intake of polyunsaturated fatty acids is associated with an increased risk of asthma in infants (39).

Total serum IgE is significantly higher in smokers compared to non-smokers (40) and children of parents or expectant mothers who smoke are more likely to develop allergic diseases compared to children not introduced to passive smoking (41). Individuals with asthma have symptom exacerbations with exposure to smoking; however, this confers a relationship between smoking and symptom severity rather than cause.

Maternal atopy is a significant risk factor for the development of allergic disease and confers greater risk than paternal atopy, particularly in the case of eczema, the first stage of the atopic march. It is hypothesised that components of the atopic maternal or placental immune system, such as the mother’s own increased circulating levels of IgE, may contribute to allergic sensitisation in the infant (7).

Breastfeeding has been inversely correlated with the development of allergic disease. Breastfed babies illustrate a lower prevalence of allergy compared to bottle-feeding (42). This may relate to immunoactive components of breast milk such as soluble
CD14; a critical molecule for modulation of colonisation and adaptive immune responses in the gastrointestinal tract. A decreased level of CD14 in breast milk is associated with development of eczema and allergic sensitisation in the first year of life (43).

1.1.1.2 Genetic Factors

Allergic disease strongly runs in families and a significant number of susceptibility genes and genetic factors for atopy have been described. For example, one mutation on chromosome 11q12-13 encoding the FceRI receptor on mast cells has been associated with asthma and eczema (44). This mutation affects ligation of allergen to FceRI receptor-bound IgE on mast cells and subsequent mast cell degranulation. Chromosome 5q31-33 encodes a cluster of genes for the Th2 cytokines IL3, IL4, IL5, IL9 and IL13, which exhibit important applications in allergy (45). A mutation within this gene cluster, as identified in candidate-gene studies, causes variation in expression of Th2 cytokines. In addition, polymorphisms in this gene can also cause changes in mucus production and bronchial hyperreactivity in susceptible asthmatic individuals. Recently, mutations in the filaggrin gene, encoding the epidermal barrier protein, were reported to be strongly associated with eczema (46, 47) and asthma severity (48). It is possible this structural variant and subsequent skin barrier dysfunction allows allergens to penetrate the skin more readily, initiating Th2 immune responses and leading to development of atopic diseases (49).

1.2 Cellular and Humoral Responses

1.2.1 The Role of IgE

IgE, present at very low concentrations in serum, is normally involved in the immune response to parasitic infections. Typically, allergic individuals produce an IgE-mediated immune response to environmental allergens compared to an IgG response in non-allergic subjects. In addition, atopic individuals have a higher level of circulatory IgE compared to non-atopic individuals and serum total IgE levels are a good predictor for the risk of asthma (50).

IgE production is initiated by the Th2 cytokines IL4 and IL13, by inducing transcription of the epsilon (ε) class of the constant region of IgE with subsequent isotype switching of B cells. IL4 also activates CD4+ T cells ligating B cells through
CD154-CD40 interactions to activate heavy chain rearrangement for IgE production. IgE differs from other immunoglobulin classes residing in tissues, with the majority bound to high affinity FcεRI receptors on mast cells and basophils, where it can remain stable for many weeks. IgE binds to the FcεRI receptor approximately $10^2$-$10^5$ times more strongly than IgG or IgA to their respective receptors (51). The binding of allergen to receptor-bound IgE induces mast cell degranulation and release of important biological mediators and recruitment of eosinophils, basophils and T cells to the site of allergic inflammation. However, the exact role of IgE in allergy is not clear-cut: some individuals may have detectable circulating allergen-specific IgE but no clinical features of allergic disease.

1.2.2 *Innate Cells in Allergic Disease*

1.2.2.1 *Mast Cells*

Mast cells develop from pluripotent stem cells in the bone marrow. These undifferentiated, non-granular cells, migrate into tissues to undergo differentiation and maturation under the influence of stem cell factor (SCF) and T_{H2} cytokines (7). These cells are present universally throughout connective tissue and mucosal surfaces, in particular at regions interacting with the environment, such as the skin, respiratory and gastrointestinal tract. Although normally involved in wound repair and innate responses to bacterial infection, mast cells are also key effector cells in IgE-mediated allergic responses, expressing the high-affinity FcεRI receptor on the cell surface to which IgE binds. The expression of the FcεRI receptor is increased in atopic individuals causing enhanced sensitivity of the mast cell, activation by lower concentrations of specific allergen, and increased release of biological mediators.
<table>
<thead>
<tr>
<th>Mediator</th>
<th>Preformed/Synthesised</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>Preformed</td>
<td>Bronchoconstriction, tissue oedema and mucus hypersecretion</td>
</tr>
<tr>
<td>Heparin</td>
<td>Preformed</td>
<td>Anticoagulant, acts as a storage matrix for mediators and fibroblast activation</td>
</tr>
<tr>
<td>Tryptase</td>
<td>Preformed</td>
<td>Production of bradykinin, increased bronchial hyperreactivity and increasing release of histamine from mast cells</td>
</tr>
<tr>
<td>Chymase</td>
<td>Preformed</td>
<td>Mucus hypersecretion and breakdown of extracellular matrix</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Preformed</td>
<td>Initiates endothelial cell activation, increased vascular permeability and vasodilation</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Preformed</td>
<td>Bronchoconstriction and increased vascular permeability</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>Synthesised</td>
<td>Bronchoconstriction, mucus hypersecretion, tissue oedema, chemotaxis of T\textsubscript{H}2 cells, eosinophils and basophils</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td>Synthesised</td>
<td>Bronchoconstriction, mucus hypersecretion, tissue oedema, production of IL4 from eosinophils, production of IL5 from mast cells, tissue fibrosis</td>
</tr>
</tbody>
</table>

Table 1.1. Mediators released from mast cells and their biological effects

When allergen cross-links FcεRI receptor-bound IgE, mast cell degranulation leads to release of preformed biological mediators, such as histamine, rapidly followed by synthesis and secretion of lipid-derived secondary mediators such as leukotrienes and prostaglandins (Table 1.1). The location of mediator release contribute to typical symptoms of allergy. For example, mast cell degranulation within the airway causes congestion, blockage of airways and wheezing. Mast cell mediators have been identified within the bronchoalveolar lavage (BAL) fluid of asthmatic individuals within 5-10 minutes of local bronchial allergen challenge (52) and the early asthmatic response is significantly exacerbated in the presence of histamine (53). Increased frequencies of mast cells are also noted within the nasal epithelium of individuals with allergic rhinitis (54).

Within the blood vessels, mast cell mediators cause vasodilation and increased vascular permeability, leading to increased fluid, cells and protein within the tissues, causing swelling (oedema). Anaphylaxis is predominantly mediated by systemic mast cell activation leading to cardiovascular and respiratory collapse.
In addition to mediator release, activated mast cells may express MHC class II molecules, acting as antigen-presenting cells at sites of allergic inflammation. Cytokines secreted from mast cells, including IL3, IL4, IL5, IL6, IL10, IL13, GM-CSF and TNFα (45) contribute to cell recruitment to the site of inflammation.

1.2.2.2 Eosinophils

Eosinophils develop from pluripotent stem cells in the bone marrow and exhibit predominant roles in immune responses to bacterial infection, parasites and tumour immunity. The presence of eosinophilic infiltration is an important feature of the allergic immune response. Atopic individuals have a higher frequency of circulating eosinophils, which often correlates with disease severity in populations (55).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Effects</th>
</tr>
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<tbody>
<tr>
<td>Major Basic Protein</td>
<td>Complement activation, increased vasopermeability, stimulates mast cell degranulation, bronchoconstriction, airway hyperresponsiveness</td>
</tr>
<tr>
<td>Eosinophil Peroxidase</td>
<td>Degrades connective tissue, cytotoxicity towards airway epithelium</td>
</tr>
<tr>
<td>Eosinophil Cationic Protein</td>
<td>Mast cell degranulation</td>
</tr>
</tbody>
</table>

**Table 1.2. Mediators released from eosinophils and their biological effects**

Eosinophils secrete biological mediators and cytokines contributing to the typical manifestations of allergic disease (**Table 1.2**). Major Basic Protein (MBP) demonstrates cytotoxic activity responsible for tissue damage of the bronchial mucosa following eosinophil infiltration in asthma (56, 57). TGFβ secreted from eosinophils is implicated in tissue remodelling. Eosinophils also release a small proportion of TH2 cytokines; secretion of IL3, IL5 and GM-CSF is important for the sustained growth, activation and survival of the eosinophil population. Eosinophils also express leukotriene and prostaglandin receptors on the cell surface, including the prostaglandin D2 receptor, CRTH2. These receptors function to co-mediate the recruitment of TH2 cells, eosinophils and basophils to the site of allergic inflammation (58). Expression of these receptors is up-regulated on eosinophils in asthmatic individuals during exacerbations (59).

Due to their significant role in allergy, and particularly asthma, eosinophils remain an attractive target for treatment. Anti-IL5 antibody, known as mepolizumab, has been reported to reduce sputum, blood and airway eosinophilia, but displayed no
effect on bronchoconstriction or airway hyperresponsiveness in mild asthmatics upon allergen challenge (60-62). These findings have questioned the role of eosinophils in airway hyperreactivity, perhaps suggesting IL5 obstruction and eosinophil abrogation alone, is insufficient in treating airway disease.

More recently, the DREAM multi-centre study of Pavord and colleagues illustrated a significant reduction in asthma exacerbations in patients with severe eosinophilic asthma when treated with mepolizumab compared to the placebo control group (63).

1.2.2.3 Neutrophils

Neutrophils, the most abundant type of white blood cell, are short-lived granulocytes originating from myeloid progenitors in the bone marrow, exhibiting important phagocytic functions in immune responses to bacterial and fungal infections. Neutrophils engulf microorganisms and foreign particles via phagocytosis in conjunction with degranulation and release of biological mediators such as neutrophil elastase and defensins. They are highly chemotactic cells and are usually the first cells to appear at sites of inflammation (7). As previously suggested, eosinophils appear insufficient to induce asthma alone (64), and neutrophils have been implicated as an additional contributing factor. There is a strong association between airway neutrophilia, severe asthma (64) and asthma exacerbations (65), and neutrophils have been identified within the sputum and BAL of asthmatic and allergic rhinitis individuals (66).

1.2.3 Mechanisms of Allergic Disease – The Early and Late Phase Response

Allergic reactions in susceptible individuals involve three phases: the sensitisation phase, the early phase response and the late phase response.

1.2.3.1 The Sensitisation Phase

On initial exposure to an allergen, the activation of T\textsubscript{H}2 cells leads to secretion of IL4 and subsequent IgE production by B cells. The secreted IgE binds to the high affinity Fc\varepsilonRI receptors on mast cells and basophils. These cells are sensitised. IgE bound to mast cells and basophils can be stable for weeks.
Figure 1.1. The early and late phase response in allergy; cross-linking of allergen to IgE on mast cells causes degranulation and release of biological mediators causing the acute symptoms of allergy. In the late phase response, inflammatory cells are recruited by release of chemotactic factors and cytokines leading to chronic allergic inflammation.

Allergen cross-linking IgE bound to high affinity FcεRI receptor

**MAST CELL**

Allergen processing and presentation by APCs to T_{h2} cells

APC

**T_{h2} CELL**

**Biological Mediators** (histamine, prostaglandins etc)

**Mediator Effects**

- Bronchoconstriction
- Increased Vascular Permeability
- Mucus Hypersecretion
- Platelet Aggregation

**Acute Allergy Symptoms**

- Wheezing
- Oedema/Erythema
- Airway Obstruction
- Inflammation

**IgE**

**EOSINOPHIL**

**Biological Mediators** (major basic protein, eosinophil peroxidase etc)

**B CELL**

**CHRONIC ALLERGIC INFLAMMATION**
1.2.3.2 The Early Phase Response

Upon re-exposure, allergen cross-links the FcεRI receptor-bound IgE on mast cells leading to degranulation and release of preformed biological mediators (histamine, heparin, bradykinin, serotonin and tryptase), and newly synthesised mediators (leukotrienes and prostaglandins). Each mediator exerts physiological effects within minutes of degranulation, contributing to the typical manifestations of an allergic response, such as rhinitis, conjunctivitis or more generalised anaphylactic reactions (Figure 1.1). This early phase reaction can be identified on sensitised skin a few minutes after allergen challenge (the wheal and flare response). Within an hour, the early phase response is significantly reduced.

1.2.3.3 The Late Phase Response

The late phase response is characterised by bronchoconstriction and mucus hypersecretion in the lung, erythema in the skin and swelling and obstruction in the nose, normally resolving within 48-72 hours. This occurs approximately 8-12 hours after the early phase response (67), with recruitment and migration of cells, notably Th2 cells and eosinophils, to the site of allergic inflammation (Figure 1.1). Antigen presenting cells (APCs) process and present allergen peptides to CD4 T cells causing secretion of Th2 cytokines (IL4, IL5, IL9, IL13) implicated in the initiation and maintenance of allergic disease. These cytokines mediate the second wave of smooth muscle contraction, oedema, tissue remodelling and hyperplasia, associated with the late phase response.

1.3 The Role of T Cells in Allergy

1.3.1 T Cell Development

T cells function in the immune response to pathogens and divide into two main subsets: CD4 T helper (Th) cells and CD8 T cytotoxic (Tc) cells, which bind to MHC class II and MHC class I molecules, respectively. CD4+ Th cells enhance the production of antibodies by B cells and facilitate the cytotoxic activity of CD8+ Tc cells.

T cell development originates in the bone marrow. Pluripotent stem cell progenitors develop into T cell progenitors that migrate to the thymus for maturation through the rearrangement of T cell receptor genes. The thymus contains a network of epithelia,
known as the thymic stroma, from which the T cell precursors receive a number of regulatory signals to undergo differentiation. The significance of the thymus in immune responses is evident in individuals with DiGeorge’s syndrome, in which the thymus is underdeveloped or absent. These individuals produce very few T cells with a resultant increase in infection and autoimmune disease (44).

At first, the T cell precursors entering the thymus do not express any cell surface markers associated with mature T cells (CD3, CD4, CD8). Interactions with the thymic stroma trigger the first stages of differentiation in which the cells develop expression of CD2, but remain CD3-CD4-CD8- “double-negative” thymocytes. These precursor cells produce two distinct lineages of T cells: γδ T cells (5%) and αβ T cells (95%), of which both lineages express different types of the T cell receptor (44).

The “double-negative” thymocytes undergo a series of further differentiation stages in which both CD4 and CD8 co-receptors are expressed. At this stage, the CD4+CD8+ T cell precursors are referred to as “double-positive” thymocytes. The majority of these double positive cells apoptose at this stage due to an intense screening process to determine those able to identify MHC complexes (positive selection) and removing those reacting to self-antigens (negative selection). However, this complex process is not entirely efficient; autoreactive T cells are present in peripheral blood of individuals with autoimmune diseases.

The remaining thymocytes undergo further differentiation to lose either CD4 or CD8 co-receptor expression. The expression of the T cell receptor is simultaneously increased. This results in a population of single positive CD4 or CD8 T cells. These naive T cells migrate to secondary lymphoid organs in the periphery such as the spleen and lymph nodes.

1.3.1 Antigen Presentation and T Cell Activation

A T cell-mediated immune response is initiated by the interaction of a naive T cell with the peptide:MHC complex presented by an antigen-presenting cell (APC). This initial T cell-APC interaction is transient, allowing the T cell to investigate multiple MHC molecules present on the APC surface (Figure 1.2). This transient binding is through CD2, LFA-1 and ICAM-3 on the T cell and ICAM-1, ICAM-2, CD58 and
CD209 on the APC (44). If the T cell recognises a peptide:MHC complex on the
APC, a conformational change in LFA-1 is induced to increase affinity of ICAM-1
and ICAM-2. This stabilises the interaction between the two cells giving time for
subsequent T cell activation.
Figure 1.2. Initial interaction between the T cell and antigen-presenting cell. The interaction is transient, involving CD2, ICAM-3 and LFA-1 on the T cell and CD58, CD209, ICAM-1 and ICAM-2 on the APC.
Upon recognition of a peptide:MHC complex, the T cell is activated via three signals (Figure 1.3). Signal 1 is the binding of the T cell receptor (TCR) complex on the T cell with the peptide:MHC complex on the APC. This activates the TCR. The MHC:peptide-TCR interaction is one hundred-fold more potent in the presence of the co-receptor CD4 (44). The intracellular portion of CD4 interacts with the tyrosine protein kinase Lck, resulting in a cascade of signalling pathway mechanisms.

The signalling pathway begins with the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) located on the TCR complex (Figure 1.4). The activation of these ITAMs on the TCR complex is the first immunological signal that antigen has been encountered. This leads to the activation of signalling molecule ZAP70, and phosphorylation of scaffold proteins LAT and SLP-76, which both function to recruit phospholipase C (44).

Phospholipase C initiates the breakdown of the PIP₂ molecule into DAG and IP₃. These secondary messenger molecules are required for the remaining signalling events. DAG activates protein kinase C and Ras G protein, leading to the activation of NFκB and AP-1 transcription factors, respectively. IP₃ induces a significant calcium influx, which in turn activates calcineurin, leading to the activation of the NFAT transcription factor. NFκB, AP-1 and NFAT transcription factors initiate gene transcription leading to cellular proliferation and differentiation (44).
Figure 1.3. Activation of T cells. Activation of a T cell by an APC has three main signals. Signal 1 involves the interactions between the MHC:peptide complex and the TCR complex, Signal 2 involves costimulation with CD28-B7 molecule interactions to ensure survival of the T cell. Signal 3 involves the production of cytokines leading to differentiation of the T cell into one of the T cell subsets.
Figure 1.4. Phosphorylation pathway of Signal 1 in the activation of T cells. CD4-Lck interactions initiate a cell-signalling pathway to activate important transcription factors required for cellular proliferation and differentiation.
Signal 2 involves costimulation to support the survival of T cells and subsequent proliferation. Interactions between CD28 on naive T cells and costimulatory molecules B7.1/B7.2 (CD80/CD86) on APCs enhance the interactions between the cells via Lck stimulation and Ras g protein activation. As described in signal 1, this allows for the production of transcription factors leading to proliferation of the T cell (44). CD28-B7 interactions also increase PIP$_2$ production to enhance cell survival.

These costimulatory signals send the T cell into the G$_1$ phase of the cell cycle and initiates production of IL2 and the $\alpha$ portion of the IL2 receptor (CD25). IL2 binds to the IL2 receptor and drives the cell into the remainder of the cell cycle, therefore promoting survival and differentiation of the T cell.

Interactions between alternative molecules can also provide costimulatory signals. The ligation of CD40 on APCs with CD154 (CD40L) on T cells increases the expression of costimulatory molecules and increases proliferation. Murine models lacking CD154 cannot undergo T cell clonal expansion (44), thus indicating an integral role of this T cell marker. In addition, CD27 on T cells can bind CD70 on the APC to sustain and enhance proliferation. CTLA-4 can also bind to B7 molecules on the APC, but this association forms an inhibitory signal to decrease the proliferative capacity of the T cell. The lack of costimulatory signals in T cell activation will lead to anergy.

Signal 3 promotes the cytokine-mediated differentiation of T cells into different effector T cell subsets. CD8$^+$ T cells differentiate into cytotoxic lymphocytes (CTLs). CD4$^+$ T cells can differentiate into T$_{H1}$, T$_{H2}$, T$_{H17}$ and regulatory T cells. The differentiation of an activated T cell into a specific subset depends on its cytokine exposure. For example, CD4$^+$ T cells differentiate into T$_{H1}$ cells in the presence of IFN$\gamma$ and IL12. Activated T$_{H1}$ cells secrete the T$_{H1}$ cytokines IFN$\gamma$, IL2, GM-CSF, TNF-\(\alpha\) and TNF-\(\beta\). This T cell subset is involved in the immune response to extracellular pathogens and bacterial infection in macrophages. T$_{H2}$ cells are involved in immune responses against parasites and regulate immune responses to allergens. CD4$^+$ T cells differentiate into T$_{H2}$ cells in the presence of IL4 and secrete the T$_{H2}$ cytokines IL3, IL4, IL5, IL9 and IL13. T$_{H17}$ cells are involved in immune responses against extracellular bacterial and fungal infections. T cells differentiate
into \( T_{H17} \) cells in the presence of TGF-\( \beta \), IL1, IL6, IL21 and IL23 and produce the \( T_{H17} \) cytokines IL17A, IL21, IL22 and TNF\( \alpha \).

### 1.3.2 The \( T_{H1}/T_{H2} \) Paradigm

\( T_{H1} \) cells are involved in macrophage activation and delayed-type hypersensitivity reactions, with secretion of IFN\( \gamma \), IL2 and IL12. \( T_{H2} \) cells are involved in enhanced antibody responses and inhibition of macrophage function (68), with secretion of IL3, IL4, IL5, IL9 and IL13.

Research into these subsets identified human T cell responses to the antigens *Mycobacterium tuberculosis* and *Toxocara canis* revealed distinct \( T_{H1} \) and \( T_{H2} \) cytokine responses, respectively (69), thus identifying \( T_{H1}/T_{H2} \) polarisation of the immune response. Similarly, \( T_{H1} \) responses became associated with autoimmune and inflammatory diseases such as type 1 diabetes, rheumatoid arthritis and Crohn’s disease. In contrast, \( T_{H2} \) responses became associated with immune responses to allergens and parasites. Therefore, it was hypothesised that immune responses may involve functionally polarised and distinct responses depending on the cytokine environment and genetic factors. Further research identified the presence of IFN\( \gamma \) and IL4 is required for polarisation of \( T_{H1} \) and \( T_{H2} \) responses, respectively.

Atopic individuals have an exaggerated immune response to allergens (7) with increased production of IgE antibodies and IL4, IL5, IL9 and IL13 \( T_{H2} \) cytokine production from \( T_{H2} \) cells. In contrast, non-allergic individuals mount a typically low-grade \( T_{H1} \)-skewed immune response to allergens consisting of IgG antibodies and IFN\( \gamma \) production from \( T_{H1} \) cells. Therefore, \( T_{H2} \) cells were implicated as a predominant feature of allergic disease and their role in the initiation and maintenance of atopy has been thoroughly investigated.

### 1.3.3 The Role of \( T_{H2} \) Cells and \( T_{H2} \) Cytokines in Allergy

\( T_{H2} \) cells are heavily implicated in the pathogenesis of allergic disease. Activated CD\( 4^+ \) T cells are identified in the peripheral blood, bronchial mucosa and BAL of atopic individuals (70-72). This marked infiltration of T cells at sites of allergic inflammation correlates with disease severity (73) and illustrates high \( T_{H2} \) expression (74). Furthermore, \( T_{H2} \) cytokines are elevated in the asthmatic airway (75) with increased expression of \( T_{H2} \) transcription factors STAT6 and GATA3.
within bronchial biopsies (76). Numerous research papers also demonstrate the presence of T_{H2} cells and T_{H2} cytokines in peripheral blood of allergic individuals following allergen stimulation using a variety of methodologies (77-81).

Considerable evidence for a role of T_{H2} cells in allergic disease also arises from murine studies. Introduction of T_{H2} cells in mice causes airway eosinophilia, hyperreactivity and mucus hypersecretion (82). This is also evident in transgenic mice genetically created to overproduce T_{H2} cytokines (83, 84). In addition, T_{H2}-deficient mice are unable to elicit allergic reactions (85, 86).

<table>
<thead>
<tr>
<th>T_{H2} Cytokine</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>IL4</td>
<td>IgE isotype switching in B cells</td>
</tr>
<tr>
<td></td>
<td>Increased expression of MHC class II molecules on B cells</td>
</tr>
<tr>
<td></td>
<td>Major stimulus for T_{H2} cell development</td>
</tr>
<tr>
<td></td>
<td>Suppression of T_{H1} cell development</td>
</tr>
<tr>
<td>IL5</td>
<td>Activation, proliferation and survival of eosinophils</td>
</tr>
<tr>
<td>IL9</td>
<td>Inhibition of T_{H1} cytokine production</td>
</tr>
<tr>
<td></td>
<td>Proliferation of mast cells</td>
</tr>
<tr>
<td></td>
<td>Mediates mucus secretion by bronchial epithelial cells</td>
</tr>
<tr>
<td>IL13</td>
<td>IgE isotype switching in B cells</td>
</tr>
<tr>
<td></td>
<td>Activation, recruitment and survival of mast cells</td>
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<tr>
<td></td>
<td>Goblet cell hyperplasia and mucus hypersecretion</td>
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**Table 1.3. Role of T_{H2} cytokines in allergic disease**

The family of T_{H2} cytokines, including IL4, IL5, IL9 and IL13, are important in the initiation and maintenance of allergic disease (Table 1.3). IL4 and IL13 are responsible for the induction of IgE isotype switching in B lymphocytes and regulate the clonal expansion of IgE-producing B cells. In addition, IL4 increases the expression of MHC class II molecules on B cells to promote antigen presentation to T cells by minimal concentrations of allergen (44). IL4 is a major stimulus for the development of T_{H2} cells and concurrent suppression of T_{H1} cell development. IL5 mediates the activation, proliferation and survival of eosinophils. The presence of T_{H2} cells secreting IL5 contributes to the induction of airway hyperreactivity in asthmatic individuals in which the levels of IL5 correlate with disease severity (87). IL9 inhibits T_{H1} cytokine production and promotes the production of IgE by B cells. In addition, IL9 mediates the proliferation of mast cells and mucus secretion by bronchial epithelial cells. IL13 mediates the activation, recruitment and survival of...
mast cells. Genetic polymorphisms in the IL13 pathway leads to increased exacerbations of asthma, total IgE concentration and eosinophilia in peripheral blood. IL13 is also involved in goblet cell hyperplasia, mucus hypersecretion and airway hyperresponsiveness (44).

Although the role of TH2 cells and TH2 cytokines in the pathogenesis of allergic disease is evident, this cell population cannot be solely responsible. The hygiene hypothesis describes a lack of TH2 to TH1 deviation in allergy, but this cannot account for the recent increase in TH1-mediated diseases such as multiple sclerosis and type I diabetes. Recent studies are now identifying roles for TH1, TH17 and regulatory T cells in allergic disease.

1.3.4 The Role of TH1 Cells and TH1 Cytokines in Allergy

TH1 cells and TH1 cytokines are reported to play a role in allergy. Atopic infants exhibit reduced IFNγ responses to allergens compared to non-atopic controls (88), and TH1 cells have been shown to suppress TH2 responses in an asthma model (89). Individuals who have outgrown milk allergy demonstrate higher IFNγ production compared to milk-allergic individuals (81). Furthermore, allergen-specific CD4 T cells from non-allergic individuals express significantly higher levels of IFNγ compared to allergic subjects (78, 81, 90-92). These findings support the research hypothesis that TH1-related immune responses correlate with a lower prevalence of allergic disease. However, some literature has identified IFNγ production in atopic infants is associated with decreased lung function in asthma and a high inflammatory airway response (88). In addition, IFNγ is present at notable levels in the BAL of asthmatic individuals (93, 94), with similar frequencies of IFNγ mRNA between atopic and non-atopic individuals (95).

There is evidence to suggest that IFNγ may work in conjunction with TH2 cytokines to contribute to allergic inflammation. Indeed, increases in both TH1 and TH2 responses to allergens have been identified in egg-allergic individuals (96) and the increased production of IFNγ, IL4 and IL5 has been noted in asthmatics, correlating with disease severity and decreased lung function (88). Although it is likely the cytokines produced by both TH1 and TH2 cells counter-regulate each other, IFNγ may also act as an important contributing factor in chronic allergic inflammation.
1.3.5 The Role of TH17 Cells and TH17 Cytokines in Allergy

TH17 cells, first described by Infante-Duarte et al. (97), are characterised by the production of IL17A and IL22 and expression of the cell surface chemokine receptor CCR6 (98). These cells function in the mucosal host defence against extracellular bacterial and fungal infections and are required for the production of the mucosal mediators β-defensins and CXCL8.

TH17 cells have been recently linked to the allergic immune response in both murine and human models. Induction of TH17 cells and airway hyperreactivity are noted in mice following epicutaneous allergen challenge (99, 100) and transgenic mice unable to produce IL17 do not develop airway hyperreactivity following allergen exposure (101). In rodents, IL17 inhalation contributes to airway neutrophilia, rather than eosinophilia, perhaps implicating TH17 cells in the pathogenesis of the “non-TH2 type asthma” recently described (65).

Impaired TH17 responses are a key feature of hyper-IgE syndrome due to mutations in STAT3; this rare primary immunodeficiency disorder is characterised by eczema, as well as susceptibility to staphylococcal infection, recurrent abscesses and pneumonia (102). TH17 cells are reported in the eczematous lesions of atopic dermatitis patients during acute inflammatory reactions (103) with increased IL17 production compared to chronic disease (104). Decreased IL17 expression in the skin of individuals with atopic dermatitis has been correlated to reduced expression antimicrobial peptides (105).

IL17 mRNA is reported at higher levels in the sputum, lungs, serum and BAL of asthmatic individuals (106-108), with the levels of IL17 correlating with severity of airway inflammation and hyperreactivity (109). IL17 is also detectable within the nasal fluids of individuals with allergic rhinitis (110) in which serum levels may successfully distinguish severely birch-allergic individuals from healthy controls (111). Sublingual immunotherapy has been reported to decrease serum IL17 in allergic subjects (112). Although the evidence demonstrates a role for IL17 in allergic disease, the exact involvement of TH17 cells in allergy is still largely unresolved.
1.3.6 *Regulatory T Cells*

Regulatory T cells (T\(_{\text{Regs}}\)) were characterised in the 1990’s by Sakaguchi and colleagues based on the high expression of CD25 (113). These “naturally occurring” T\(_{\text{Regs}}\) constitute approximately 5-10% of all T cells and commit to the regulatory T cell lineage in the thymus. Naturally occurring T\(_{\text{Regs}}\) have poor proliferative capacity. Depletion of these cells has demonstrated augmentation of tumour immunity and development of autoimmune diseases such as type I diabetes and Crohn’s disease (114, 115). Naturally occurring T\(_{\text{Regs}}\) suppress immune responses in a contact-dependent manner via cytotoxic T lymphocyte antigen-4 (CTLA-4) signalling mechanisms (116) and secretion of the suppressive cytokines TGF-β and IL10. Naturally occurring T\(_{\text{Regs}}\) also suppress antigen-driven *in vitro* proliferation.

However, activated CD4 T cells also express CD25 and CTLA-4, and the identification of a specific cell marker constitutively expressed on T\(_{\text{Regs}}\) has been problematic. Sakaguchi and colleagues identified a transcription factor, forkhead box P3 (FOXP3), expressed on all naturally occurring CD4\(^+\)CD25\(^+\) T\(_{\text{Regs}}\) (117) that acts as a silencer of cytokine gene promoters (118). Mutations in this protein in the rare syndrome IPEX (immunedysregulation polyendocrinopathy enteropathy X-linked) are associated with autoimmune and inflammatory diseases. FOXP3 is now used as the gold standard marker to identify naturally occurring CD4\(^+\)CD25\(^+\) T\(_{\text{Regs}}\).

<table>
<thead>
<tr>
<th>Subset</th>
<th>Origin</th>
<th>Cell Markers</th>
<th>Cytokines Secreted</th>
<th>Differentiation Factors</th>
<th>Suppressive Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naturally occurring T(_{\text{Regs}})</td>
<td>Thymus</td>
<td>CD4, CD25, FOXP3</td>
<td>IL10, TGF-β</td>
<td>Thymus-derived</td>
<td>Contact-dependent, cytokine secretion</td>
</tr>
<tr>
<td>T(_R)1 cells</td>
<td>Periphery</td>
<td>CD4</td>
<td>IL10, IFNγ, IL5, TGF-β</td>
<td>IL10, IFNa</td>
<td>Cytokine secretion</td>
</tr>
<tr>
<td>T(_H)3 cells</td>
<td>Periphery</td>
<td>CD4</td>
<td>TGF-β, IL4, IL10</td>
<td>TGF-β, IL4</td>
<td>Cytokine secretion</td>
</tr>
<tr>
<td>IL10-Producing T(_{\text{Regs}})</td>
<td>Periphery</td>
<td>CD4</td>
<td>IL10</td>
<td>IL10, dexamethasone, vitamin D3</td>
<td>Cytokine secretion, cell-cell contact</td>
</tr>
</tbody>
</table>

Table 1.4. Regulatory T cell populations.

Additional subsets of T\(_{\text{Regs}}\) have also been described (Table 1.4). These “adaptive” or “inducible” T\(_{\text{Regs}}\) are produced in the periphery from naive CD4 T cells following antigen presentation. These include the T\(_H\)3, T\(_R\)1 and IL10-producing T\(_{\text{Regs}}\) that exhibit suppressive function through cytokine-dependent mechanisms.
Th3 regulatory cells were first described by Weiner and co-workers (119). These regulatory cells, induced by oral antigen, are predominantly located in the gastrointestinal tract and suppress immune responses within the mucosal barrier via production of IL4, IL10 and high levels of TGF-β. A deficiency in Th3 cells is associated with autoimmune diseases of the gastrointestinal tract and inflammatory bowel disease. Th3 cells promote IgA isotype switching of B cells (120) and have been shown to induce suppressive immune responses in bystander cells (121).

Roncarolo et al. illustrated that activation of CD4 T cells in the presence of IL10 induced a subset of adaptive TRegs, of poor proliferative capacity, able to suppress the proliferation of CD4 T cells, called TR1 cells (122). TR1 cells differentiate in the presence of IL10 and IFNα and exhibit suppressive function through TGF-β, IFNγ and IL10 secretion. These cells express low levels of CD25 and do not express FOXP3.

IL10-producing TRegs are generated in the periphery from naive CD4 T cells following antigen presentation and differentiate in the presence of IL10, IFNα or addition of dexamethasone or vitamin D3 (123, 124). These cells exhibit suppressive function through secretion of IL10 and possibly cell-cell contact (125).

1.3.7 The Role of Regulatory T Cells in Allergy

A large number of murine and human studies implicate TRegs in tolerance to allergens and defective responses to allergic disease. Naturally-occurring TRegs are able to inhibit airway eosinophilia in a murine model (126) and allergen-specific TRegs engineered to produce the suppressive cytokine TGF-β protected mice from developing Th2 immune responses to allergen in vitro (127). In addition, TRegs are reported to suppress Th17-associated airway inflammation by regulation of neutrophil and B cell infiltration in the allergic lung of rodents (128).

Individuals with IPEX syndrome, a mutation in the transcription factor FOXP3, exhibit severe atopic dermatitis, high levels of IgE, food allergies and eosinophilia (116, 129). TRegs inhibit activation of Th2 cells and Th12 cytokine production (77, 130, 131), suppress innate cells, including eosinophil and mast cell degranulation (132) and suppress IgE in favour of IgG4 production (133). In addition, TRegs inhibit the migration of effector T cells into sites of allergic inflammation through cytokine-
dependent mechanisms (134). $T_{\text{Reg}}$ also prevent airway tissue injury by inhibiting the apoptosis of bronchial epithelial cells initiated by $T_{\text{H}1}$ cell activity (135, 136). Grindebacke and colleagues demonstrated $T_{\text{Reg}}$ from allergic individuals are less efficient at suppressing $T_{\text{H}2}$ cytokine responses compared to non-allergic controls, in whom both $T_{\text{H}1}$ and $T_{\text{H}2}$ cytokine responses are efficiently suppressed (129). The frequency of $T_{\text{Reg}}$ in both allergic and non-allergic subjects was comparable.

Multiple studies illustrate the induction of naturally occurring $T_{\text{Reg}}$ and secretion of suppressive cytokines IL10 and TGF-β following specific immunotherapy (125, 130, 137-139). These observations support the hypothesis that a deviation to $T_{\text{Reg}}$ responses during specific immunotherapy is important in the development of tolerance to allergens (140). Multiple studies demonstrate that $T_{\text{Reg}}$ are the predominant allergen-specific T cell in healthy individuals, indicating that a balance between $T_{\text{H}1}$, $T_{\text{H}2}$ and $T_{\text{Reg}}$ cells is important in the development of allergic disease (77, 130, 131, 141, 142). There are multiple theories postulated on the mechanism by which healthy individuals may tolerate allergens, but the most common hypotheses an increased ability of $T_{\text{Reg}}$ to suppress both $T_{\text{H}1}$ and $T_{\text{H}2}$ responses (109, 143).

The roles of suppressive cytokines IL10 and TGF-β in allergic tolerance are currently under investigation (106, 139). T cells produce IL10 in response to birch allergen stimulation in healthy, but not in allergic, individuals (144). In addition, the BAL of asthmatic individuals illustrates significantly reduced IL10 and IL10 mRNA compared to healthy controls (145). In a murine model, airway hyperresponsiveness and inflammation were reduced upon TGF-β exposure (127) and blocking of TGF-β secretion exacerbated symptoms (146).

Research suggests IL10 is important for the modulation and maintenance of tolerance to allergens (147) through inhibition of IgE isotype switching in B cells and reduction in eosinophil survival (148). Healthy individuals exhibit a strong IL10 and IFN$\gamma$ response to house dust mite with no $T_{\text{H}2}$ cytokine secretion, compared to allergic individuals producing predominantly $T_{\text{H}2}$ cytokines with little or no IFN$\gamma$ (148). The importance of IL10 is also evident in individuals who become tolerant to allergens in adulthood. Individuals tolerant to cow’s milk produce higher levels of IL10 and IFN$\gamma$ compared to allergic individuals (141, 149).
In light of advances in T_{Reg} research in allergy, the hygiene hypothesis has been revisited. This hypothesis states both improved hygiene and low microbial burden contribute to an increase prevalence of allergic disease in urban populations. However, the T_{H1}/T_{H2} paradigm alone cannot account for the simultaneous rise in T_{H1} and T_{H2}-mediated disease. It has been postulated that T_{Reg} activity is impaired with increased hygiene and decreased microbial exposure, causing a subsequent increase in both T_{H1} and T_{H2} responses via a lack of T_{Reg} activity (98).

1.3.8 T Cells in Allergy – A Summary

It is well established that an enhanced T_{H2} immune response, in conjunction with T_{H2} cytokine secretion, contributes significantly to the allergic phenotype. It was initially hypothesised that reduced microbial exposure caused a reduction in T_{H1} immune responses, and as a result, T_{H2} cells were the predominant T cell subset. However, evidence now suggests reduced stimulation, or impaired responses, of T_{Regs} may result in an increase in T_{H2} immune responses. It seems a delicate balance between T_{H1}, T_{H2} and T_{Regs} immune responses is important in the initiation and maintenance of allergic disease.

1.4 Allergens

1.4.1 Characteristics of Allergens

Allergens are a group of heterogenous soluble proteins (150) with varying biochemical structure and function. There are very few common features of all allergens and distinguishing allergenic proteins from non-allergenic proteins is difficult due to their heterogenous properties. Generally, allergens are proteins with carbohydrate side chains (44) of low molecular weight, which are highly soluble and mostly stable with the ability to stimulate T cells, containing peptides for antigen processing and presentation.

Allergen sources typically contain multiple allergenic molecules. For example, Bet v 1 is the major allergenic component of birch pollen however; individuals may also be sensitised to Bet v 2. Therefore, native allergen extracts can include both major and minor allergen components as well as some non-allergenic components.
1.4.2 Pollens

Tree and grass pollens are extremely common in the UK and are significant contributors to the typical symptoms of allergic rhinitis and asthma. Grass pollens from species such as seasonal Timothy and Bermuda grass, are a highly abundant top plant allergen of complex structure, containing multiple allergenic components able to induce IgE-mediated immune responses. These allergens are the most common contributor to allergic rhinconjunctivitis (151). Grass pollen is most abundant from mid-May to September, with peak production in June, and may persist for some time after the pollen season in collaboration with indoor dust (152). The daily variation of pollen production depends on many factors including rain, wind and temperature (7) but in general, grass pollens are released early morning, therefore contributing to higher frequency and severity of allergic symptoms at this time. In urban areas, symptoms may be more problematic in the evenings due to the time taken for pollen to migrate from rural areas (7). Major allergenic components within grass pollens include Phl p 1 and Phl p 5 of Timothy grass, Lol p 1 and Lol p 2 of rye grass and Cyn d 1 and Cyn d 7 of Bermuda grass.

Tree pollens are also top plant allergens contributing to allergic rhinitis and asthma. Birch pollen is the major tree allergen in Northern Europe and epidemiological studies indicate 10-20% of individuals in this area are sensitised to birch (153). Birch pollen is most abundant from March to May with peak production in April. The allergenic components of tree pollens are mostly stored within the cytoplasm of the pollen grain however; some may be contained within the pollen wall. Bet v 1, the major birch pollen allergen, has 55% homology to that of the pathogenesis-related 10 (PR-10) protein family, an acidic group of proteins developed ubiquitously by plants as a defence mechanism in response to pathogen infection (154, 155). Allergenic proteins in birch pollen are able to cross-react with homologous proteins in food products. For example, antibodies directed against the major birch pollen allergen Bet v 1 can bind to homologous proteins from members of the Rosaceae fruit family including apples, peaches and cherries.

1.4.3 Animal Allergens

Allergy to cat, and to a less extent dogs, has been of considerable scientific interest. Pet allergens are ubiquitous in the environment, especially in regions of high pet
ownership, and are difficult to remove from the domestic environment. In addition, pet sensitised pet owners exhibit higher asthma severity scores compared to pet sensitised individuals without pets at home (156). Pet allergens may contribute to symptoms of asthma and perennial allergic rhinitis in sensitised individuals.

Allergy to cats is one of the most common animal allergies in the UK, with allergenic components present in the saliva, dander, pelt, serum and urine. It is possible male cats contribute to allergic symptoms more so than females due to increased production of the major cat allergen Fel d 1 (7). This allergenic molecule is responsible for 90% of IgE-mediated immune responses in cat allergy.

Allergy to dogs is frequent in the UK, although it is far less common than cat allergy. Can f 1, the major dog allergen, accounts for 92% of positive SPTs in allergic individuals (157), although different breeds of dog may illustrate varying concentrations of Can f 1 allergen. This allergenic component is mostly found in dog saliva, dander and pelt.

1.4.4 Food Allergens

Food allergy forms a significant contribution to allergic disease in both children and adults; food allergen-induced reactions can involve multiple physiological systems including the skin, gastrointestinal tract and respiratory tract. Common food allergens include milk, wheat, eggs, nuts, shellfish and fruits. Most food allergens are believed to cause sensitisation through the gastrointestinal tract. Some food allergens are heat-labile and susceptible to degradation; therefore cooking the proteins eliminates the allergenicity and subsequent response. Cross-reactivity from aeroallergens with homologous proteins in food illustrates a common cause of food allergy in adults called pollen-food syndrome.

1.4.4.1 Pollen-Food Syndrome

It was noted in the mid to late 1900’s that individuals sensitised to tree pollens experienced adverse reactions following ingestion of nuts, fruits and vegetables (158-161), generally confined to oral symptoms of the lips, mouth and throat, in a phenomenon later coined as the Pollen-Food Syndrome (PFS). This condition is estimated to affect 2% of the UK population (162), with symptoms, such as itching, swelling, pain and oedema, appearing within minutes of ingesting the food.
Symptoms are also more prevalent during the pollen seasons although serious symptoms such as anaphylaxis are uncommon (1-2%) (163). In Northern Europe, PFS most often affects birch pollen allergic individuals who react to homologous panallergens in the group of *Rosaceae* fruits, including apples, pears, apricots, cherries, peaches and plums (155).

PFS is caused by cross-reactivity and sensitisation to homologous panallergens common in both pollen and edible plant products. IgE antibodies produced against one allergen can bind homologues originating from different allergens (164). The major allergens responsible for this condition fall into three main families: the Pathogenesis-Related 10 (PR-10) protein family, Lipid Transfer Proteins (LTPs) and profilins.
Figure 1.5. Three-dimensional structures of the allergenic PR-10 proteins, LTPs and profilins; all models were obtained from the structural database of allergenic proteins http://fermi.utmb.edu/SDAP/.
1.4.4.1.1 PR-10 Protein Family

The exact biological function of the PR-10 protein family, also referred to as the Bet v 1 cluster, is unknown, however the levels of these proteins are reported to increase when the plants are under stress, perhaps suggesting a role in defence mechanisms (165). Multiple food products, including fruits of the Rosaceae family, contain PR-10 proteins, thus exhibiting structural homology with the major birch pollen allergen Bet v 1. These include Mal d 1 (apple), Fra a 1 (strawberry), Pru av 1 (cherry) Pyr c 1 (pear) and Pru ar 1 (apricot) (155). PR-10 proteins are heat-labile and susceptible to digestion; therefore, some individuals are tolerant of these foods once cooked. The structures of the PR-10 protein family members exhibit an alpha/beta fold with high homology within the amino acid sequences responsible for the IgE cross-reactivity (Figure 1.5).

1.4.4.1.2 Lipid Transfer Proteins

The LTPs, also referred to as the PR-14 protein family, have an important function in plant defence against microorganisms with antimicrobial properties that transfer phospholipids from the liposomes to mitochondria (166). LTPs confer structural homology of approximately 80% within the Rosaceae fruits (155), with examples including Pru p 3 (peach) and Pru ar 3 (apricot). LTPs are heat-stable; therefore, patients may react to cooked or processed foods. However, the LTPs are often present in the peel of fruits and some individuals are tolerant of these foods when peeled (155). The structure of LTPs consists of four alpha helices stabilised by disulphide bonds responsible for the IgE cross-reactivity (Figure 1.5).

1.4.4.1.3 Profilin

Profilins function within the cytosol in actin filament polymerisation (164), with cross-reactive examples including Mal d 4 (apple), Pyr c 4 (pear) and Pru av 4 (cherry). Profilin sensitisation usually relates to grass pollinosis and is evident throughout Europe (167, 168). Profilins are highly susceptible to heat degradation and digestion; therefore, some individuals are tolerant of these foods once cooked. Cross-reactivity of IgE in profilins is reported to relate to a structural homology rather than similarities within the amino acid sequence (165).
1.5 Treatment of Allergic Disease

1.5.1 Allergen Avoidance

Removal and avoidance of allergens where possible is a logical treatment modality. Clearly, strict avoidance of food allergens minimises the risk of a harmful allergic reaction. Encasement of bedding and the use of high efficiency particulate air (HEPA) filters reduces the levels of airborne indoor house dust mite allergens and is associated with decreased bronchial hyperreactivity (169). However, allergen avoidance is not always possible. Individuals allergic to pollens and moulds are unable to avoid the allergens that exacerbate rhinitis or asthmatic symptoms. Allergen avoidance may improve symptoms in some situations but cannot treat completely or modify the disease.

1.5.2 Antihistamines

Upon mast cell degranulation, histamine binds to H\textsubscript{1-4} receptors expressed throughout the body, contributing to a significant proportion of allergic symptoms, including itching, rhinorrhoea, rashes, congestion and difficulty breathing. Antihistamines are antagonists of the H\textsubscript{1} receptor, blocking the binding of histamine, therefore relieving allergic symptoms and improving the quality of life. Antihistamines have been categorised into first generation (sedating antihistamines such as chlorphenamine) and second generation (non-sedating antihistamines such as cetirizine and loratidine). Second generation antihistamines have been reported to reduce allergic rhinitis symptoms in a large number of clinical studies (170). Administration of antihistamines can also prevent local reactions during specific immunotherapy and may reduce urticaria and airway hyperreactivity in anaphylaxis alongside adrenaline. Antihistamines treat the symptoms of allergic disease, but they have no effect on the underlying disease mechanisms.

1.5.3 β\textsubscript{2} Agonists

β\textsubscript{2}-adrenergic receptors are highly expressed in the lung, airway smooth muscle and alveolar regions, in which catecholamines and adrenaline can bind, leading to bronchodilation and smooth muscle relaxation. β\textsubscript{2} agonists, such as short-acting salbutamol or long-acting salmeterol, simulate this sympathetic stimulation of the bronchioles, therefore relieving the contraction of airway smooth muscle cells. β\textsubscript{2}-agonists are also able to increase mucociliary clearance, reduce the effects of
histamine in vascular permeability and inhibit mediator release from mast cells (171). However, β2-agonists are purely symptomatic and do not modify the course of the disease.

1.5.4 Corticosteroids

Corticosteroids bind to the intracellular glucocorticoid receptors within the cytoplasm and inhibit inflammatory genes including NF-κB and NF-AT. Corticosteroids are tolerated in the majority of individuals and are effective in multiple allergic diseases including asthma (172), atopic dermatitis (173) and allergic rhinitis (174). Corticosteroids also reduce production of pro-inflammatory cytokines IL1, TNF-α, GM-CSF, IL3, IL4 and IL5 and production of prostaglandins and leukotrienes. Unfortunately, toxicity issues are a major limiting factor. In addition, a small proportion of individuals do not respond to corticosteroid treatment, particularly in severe or complicated asthma.

1.5.5 Leukotriene Receptor Antagonists

Leukotrienes are potent pro-inflammatory lipid mediators synthesised upon allergen cross-linkage on mast cell-bound IgE through the metabolism of arachidonic acid. Leukotriene receptor antagonists (montelukast, zafirlukast) block the actions of leukotrienes through binding the cysteinyl leukotriene 1 receptor (CysLT1) within the lung and bronchioles. This leads to reductions in airway hyperresponsiveness (175), eosinophilia (176, 177) and symptom severity (178). This treatment exhibits short-acting bronchodilator activity, utilised alongside corticosteroid treatment, for prevention of allergen and exercise-induced bronchoconstriction. Although not as successful as inhaled corticosteroids, leukotriene receptor antagonists may provide an alternative treatment to those individuals unable to use corticosteroids. However, adverse reactions may occur, including gastrointestinal disturbances, seizures, sleep disorders and joint pain.

1.5.6 Cytokine-Directed and Biological Approaches

Numerous cytokines are involved in the pathogenesis of allergic disease, particularly the Th2 cytokine family. Therefore, research into biological approaches has attracted considerable interest.
A soluble human IL4 receptor (altrakincept) improved lung function in moderately severe asthma (179), although this finding was not confirmed in further clinical trials. Anti-IL4 and anti-IL13 antibodies are currently under development. An IL13 receptor, IL13Rα2, may act as a decoy receptor to bind active IL13, and is currently being investigated (180).

Treatment with infliximab, an anti-TNFα antibody, and etanercept, a soluble TNFα receptor, has demonstrated reduced airway hyperresponsiveness and improved symptoms in severe asthmatics compared to steroid therapy (181). Infliximab is also a common treatment used in rheumatoid arthritis and other inflammatory diseases. Although anti-TNFα strategies show promise, they have been associated with some rare, but severe side effects including tuberculosis reactivation. Inhibitors of the TNFα-converting enzyme (TACE), required for TNFα release, are being examined.

The monoclonal anti-IgE antibody, omalizumab, has formed the central focus of biological approaches to allergy therapy, forming complexes with unbound IgE to block its interaction with mast cells and basophils as well as reducing circulatory IgE levels. It does not bind to cell-bound IgE, therefore avoiding IgE-mediated reactions and inhibiting the production of IgE by B cells. Treatment with omalizumab is usually administered by subcutaneous injection every 2-4 weeks. Improved symptoms have been noted during omalizumab treatment in a number of allergic diseases. In seasonal allergic rhinitis, omalizumab therapy results in decreased ocular and nasal symptoms (182) in a dose-dependent manner. Omalizumab treatment also allows increased exposure to peanut antigen in peanut-allergic individuals (183). Multiple large-scale studies have illustrated a reduction in asthmatic exacerbations (184), improved nasal symptoms (185), higher quality of life scores (186) and an inhibitory effect on early and late phase asthmatic reactions (187). In addition, acute reactions during ragweed immunotherapy are reduced when individuals are administered omalizumab prior to immunotherapy (188). In the UK, the main indication is severe asthma with frequent hospital admissions.

1.5.7 **Specific Immunotherapy**

Specific immunotherapy (SIT) involves the subcutaneous or sublingual administration of a particular allergen in escalating concentrations to produce clinical tolerance. This is the only disease-modifying and allergen-specific approach to the
treatment of allergy. The target of this therapy is to induce a state of allergen-specific anergy. Prautnitz and Köstner instigated research into SIT in the 19th century, revealing injection of allergen-specific IgE into a non-allergic participant caused sensitisation to that particular allergen. Soon after, Noon and Freeman performed the first series of immunomodulatory injections of grass pollen extract resulting in improved rhinitis symptoms and a reduction in medication usage for more than one year after injections were terminated (189). Recent studies revealed grass pollen immunotherapy improves lower airway symptoms, reduces medication use and protects against the development of asthma in individuals with seasonal allergic rhinitis (190-192).

The major drawback of SIT regards safety concerns; the allergen preparations utilised for SIT retain B cell epitopes therefore conferring a risk of IgE-mediated reactions. Benefits of immunotherapy can disappear after one year of treatment, with three years treatment providing optimal results; therefore, it is a long and expensive therapy. There is also concern with the use of SIT in some patient groups, such as asthmatic individuals and those with autoimmune disorders, immunodeficiencies and cardiac disease. Therefore, patient selection is crucial.
Figure 1.6. Mechanisms of specific immunotherapy. SIT shifts the immune balance between T\textsubscript{H}2 and T\textsubscript{H}1 cells, favouring T\textsubscript{H}1 responses and downregulating T\textsubscript{H}2 cells and T\textsubscript{H}2 cytokines. Expression of IL10 induces B cells to produce IgG antibodies to inhibit IgE-mediated allergen presentation and mast cell activation.
1.5.7.1 Mechanisms of SIT

The immunological mechanisms of SIT are not completely established, however, it is hypothesised to induce changes in the T and B cell response, antibody production and mast cell and eosinophil function (Figure 1.6). In addition, SIT prevents the development of new allergic sensitisations and the progression of more severe allergic disease.

A large number of publications identify a reduction in T cell responses to allergens in both peripheral blood and tissues (193-196), exemplified by a reduction in $T_{H2}$ cytokines, reduced proliferation and in some cases, an increase in IFNγ production. As previously discussed, the development of allergic disease is associated with $T_{H2}$-skewed immune responses compared to the “protective” $T_{H1}$ responses in tolerant individuals. Therefore, a reduction of $T_{H2}$ responses following SIT addresses this important cytokine balance in allergic tolerance (197-199). However, a reduction in $T_{H2}$ responses has not been consistently replicated (137, 200). This may relate to differences in methodology and the inability to standardise allergens, however, it seems more likely the deviation of $T_{H2}$-skewed immune responses is not the fundamental immunological event occurring following successful SIT. Not surprisingly, several studies report a role of regulatory T cells in the tolerance mechanisms of SIT.

Increased numbers of CD25$^+$FOXP3$^+$ naturally occurring $T_{Regs}$ are detected in the nasal mucosa following grass pollen immunotherapy (201), in which the frequency of these cells correlates with clinical efficacy and suppression of seasonal allergic inflammation (138). FOXP3 expression is also induced in the peripheral blood for up to 12 months following peanut oral immunotherapy (202). These FOXP3-expressing $T_{Regs}$ may be impaired in suppressing $T_{H2}$ responses in allergic individuals compared to healthy controls (203). Alongside the suppression of $T_{H2}$ responses (204, 205), $T_{Regs}$ are reported to inhibit allergen-specific IgE production, mast cell and eosinophil activity and induce a state of anergy following immunotherapy (195, 206, 207). Decreased proliferative responses of T cells to allergens are reported following immunotherapy (208), perhaps reflecting this anergic state.

The frequency of $T_{R1}$ cells is reported to increase significantly during the first 3-6 months of immunotherapy, with a subsequent decrease at 12 months, indicating a
prominent role of these regulatory cells during the initial phases of specific immunotherapy (195). The expression of IL10 following immunotherapy has been consistently confirmed in the literature for a number of allergens (130, 137, 195, 197). IL10 mRNA is detectable in the nasal biopsies of individuals undergoing grass pollen immunotherapy (138, 139). In addition, IL10-producing T cells are identified in the skin biopsies of individuals undergoing hymenoptera venom immunotherapy (209). IL10 is known to suppress TH2 cytokines, such as IL5, and directly inhibit IgE-mediated mast cell activation by inducing IgG4 production plasma B cells (210, 211).

Changes in mast cell and eosinophil function are also described following SIT. Research has demonstrated a reduction in the recruitment of mast cells and basophils during the pollen seasons to the nasal mucosa following grass pollen immunotherapy (212, 213). In addition, several studies have indicated a reduction in eosinophil recruitment in skin biopsies and nasal lavage fluid following grass and ragweed immunotherapy, respectively (214, 215). Following successful SIT, both the early and late phase responses to allergens are reduced in the skin, nose and bronchial mucosa; SIT increases the threshold of allergen required to elicit these responses within the tissues (195).

Allergen-specific IgE levels are initially increased at the beginning of SIT but decrease to pre-treatment levels once the maintenance dose has been achieved. Increased concentrations of IgG1, IgG4 and IgA isotypes have been identified following SIT (130, 201); IgG4 demonstrates the most consistent and prominent increase and competes with IgE to prevent mast cell receptor cross-linkage and activation. IgG4 is also associated with the blocking of IgE-mediated allergen presentation to T cells. Increased concentrations of IgG4 are also associated with natural allergen exposure seen in non-allergic beekeepers (195).

In summary, SIT induces significant alterations in the T cell response to allergens, with increased production of IL10 and abrogation of TH2 responses, in conjunction with reduced mast cell activation, IgE-mediated allergen presentation to T cells and amplified IgG/IgG4 production. This leads to tolerance of the particular allergen and a virtually abolished late phase response.
1.6 Detection of Allergen-Specific Lymphocytes in Peripheral Blood

The investigation of allergen-specific CD4 T cells at the single cell level is important for a better understanding of tolerance, sensitisation and desensitisation to environmental allergens. However, the low frequency of allergen-specific CD4 T cells in peripheral blood makes their detection problematic. Previous techniques described include the expansion of T cell clones, cytokine capture, ELISPOT, MHC tetramers, dye dilution for proliferation analysis and upregulation of surface activation markers detected by flow cytometry.

1.6.1 T Cell Clones

T cell clones have been frequently used to characterise allergen-specific T cells (141, 216-223), confirming high T\textsubscript{H}2 cytokine expression in allergic individuals. However, the use of T cell clones is time-consuming, involving multiple antigenic stimulations and addition of exogenous cytokines, leading to altered antigen responsiveness and phenotype (224).

1.6.2 Cytokine Capture Assays

Cytokine capture assays involve the secretion and subsequent capture of cytokines from activated T cells and detection by addition of fluorochrome-labelled secondary antibodies specific to the cytokine of interest. Akdis and colleagues used this method to identify subsets of T\textsubscript{H}1-, T\textsubscript{H}2- and T\textsubscript{R}1-like cells represented by IFN\textgamma{}, IL4 and IL10-secreting T cells in allergic and non-allergic individuals (77). This study demonstrated an increased frequency of allergen-specific T\textsubscript{R}1 cells in non-allergic individuals and T\textsubscript{H}2 cells in allergic individuals, implying a balance between these T cell subsets exhibits a key role in the pathogenesis of allergy. Despite the fundamental findings of this study, the method is unable to identify antigen-specific cells that do not produce cytokines after activation and cannot directly provide information on the phenotype of the cell without prior cell sorting. The sensitivity of cytokine capture assays is questionable and they involve arduous and time-consuming techniques.

1.6.3 Dye Dilution

Today, much of the literature regarding allergen-induced proliferation utilises cell-tracking dyes, overcoming the limitations of DNA synthesis techniques, and
allowing detailed analysis of responding cell frequency and cytokine expression. Examples of fluorescent cell division dyes include the Carboxyfluorescein succinimidyl ester (CFSE) and Paul Karl Horan (PKH) dyes. These dyes are equally distributed between daughter cells during division, with low fluorescence indicating divided or proliferating cells, compared to high fluorescence representing undivided cells. Cells can retain the dye for several weeks, giving the opportunity for long-term culture, without adverse effects on viability, cellular growth and proliferation. They are simple and quick to use and cells are easily isolated for further experiments. This approach has permitted investigations into an array of allergic sensitisations including peanut, egg, grass pollen, cow’s milk and drug allergy (80, 81, 92, 225, 226). However, detection of intracellular cytokines often requires mitogen restimulation and dye dilution techniques take longer to complete compared to other methods such as MHC class II tetramers or cytokine capture.

Detection of daughter cells depends on the remaining fluorescence of these cells, and as a result, after many divisions there may be little dye left. If increasingly high concentrations of dye are used, breakdown of important proteins in antigen recognition and intracellular pathways may occur (227). The staining intensity of PKH dyes is more stable compared to CFSE because the hydrophobic interactions between the dye and phospholipids of the cell membrane remain stable for longer periods. The reproducibility of PKH dyes is high, and unlike CFSE, there is no waiting period for the staining intensity to stabilise (227). In addition, it is easier to accomplish brighter homogenous staining without causing damaging effects to protein and cell function. The loss of dye out of the cell, leading to culture artefact, is minimal.

1.6.4 MHC Tetramers

The introduction of stable MHC class II tetramers has allowed accurate detection and characterisation of T cells directed against a number of allergens in both allergic and healthy individuals. MHC tetramers are a complex of four MHC molecules related to a specific peptide bound to fluorescently labelled streptavidin (228) that are capable of binding the T cell receptor and therefore identifying peptide-specific T cells in conjunction with flow cytometry.
The first report of MHC tetramers in allergen-specific T cell research identified rye grass-specific T cells in allergic, but not in non-allergic, individuals, but no differences in tetramer+ cell frequencies were noted in and out of pollen season and tetramer binding was not identified when cells were examined ex vivo (229, 230). More encouraging results were identified with the major cat allergen Fel d 1, where tetramer binding was achieved without in vitro allergenic amplification, illustrating a higher frequency of Fel d 1-specific CD4 T cells in allergic individuals compared to healthy controls (90). These experiments were also developed to successfully analyse the phenotype of responding T cells to identify a central memory tetramer+ cell population. These results have been independently confirmed in subsequent Fel d 1 tetramer research as CD4 tetramer binding methods improved (231). Similarly, tetramer+ CD4 T cell populations have been identified for wasp (Ves p 5), house dust mite (Der p 1/Der p 2), mugwort (Art v 1), cow allergen (Bos d 2) and birch pollen (Bet v 1) (78, 79, 91, 232-238).

However, MHC tetramers can only detect T cells for selected epitopes in individuals with particular haplotypes and the manufacturing of tetramers is time-consuming, complicated and expensive. The method may not be ideal for the detection of low frequency events, with many groups using cell expansion and addition of exogenous cytokines. This in vitro amplification may introduce phenotypic modifications and culture artefact. In addition, T cell responses can involve more than one type of epitope and immune responses distinct from this immunodominant epitope may not be identified, perhaps limiting detection of the total allergen-specific T cell population. In addition, this method cannot identify intracellular cytokines without mitogen stimulation.

1.6.5 Flow Cytometry

Flow cytometry is the measurement of cells in a flow system designed to pass cells through a laser emitting a beam of monochromatic light in a single stream. As the cells pass through the laser, light is scattered and fluorescently-conjugated monoclonal antibodies attached to the cell are excited and emit fluorescence. Highly sensitive photomultiplier tubes (PMTs) are able to detect the scattered light to analyse the size and granularity of the cells, and the fluorescence emissions, to give information on the bound monoclonal antibodies and subsequent cell surface and
intracellular protein expression. Flow cytometry requires a single particle suspension for analysis therefore information on tissue architecture and relationships between cells cannot be analysed (239). In addition, information regarding cell shape and the distribution of organelles within the cell cannot be acquired. However, this technique allows the analysis of multiple subpopulations of cells at the single cell level with numerous applications in immunophenotyping, DNA analysis, cell proliferation and apoptosis.

There are two types of flow cytometer: one type can analyse the cells whereas the other can analyse and sort the cells simultaneously. The basic flow cytometer consists of a laser, a flow cell, optical components to focus the light and electronic components to process the signals (239). The flow cell delivers cells in a single file through the laser by injection of the cell sample into a sheath fluid, which hydrodynamically focuses the sample stream (239). A lens system is used to focus the laser beam onto the sample stream and the scattered light and fluorescence emissions are focused onto the detectors (PMTs) through optical filters that select the correct wavelengths of excitation (239). These dichroic filters select out light of different wavelengths. For example, the first dichroic filter in Figure 1.7 may select light with a wavelength less than 500nm (blue) that passes through a barrier filter selecting blue light to fall on the first PMT. This is known as the side scatter. The second dichroic filter may select light with a wavelength less than 540nm (green) that passes through a barrier filter selecting green light to fall on the second PMT.
Figure 1.7. Layout of a simple flow cytometer.
The emission spectrum of fluorochromes is large and there is spectral overlap between these emissions when multiple fluorochromes are utilised (Figure 1.8). For example, the emission spectrum of fluorescein isothiocyanate (FITC) overlaps with that of phycoerythrin (PE) therefore some light will be transmitted by the filter detecting PE.

![Wavelength spectrum of common fluorochromes](image)

**Figure 1.8. Spectral overlap of commonly used fluorochromes. Adapted from Ormerod (1999) Flow Cytometric microscopy handbooks (239, 240)**

Spectral overlap is corrected by subtraction of a part of the FITC signal from the PE signal and vice versa (239). Spectral overlap is calculated for each combination of fluorochromes used in a flow cytometry experiment by the use of compensation controls.
Figure 1.9. Light scatter dot plot. FSC and SSC can be used to distinguish between lymphocytes, monocytes and granulocytes by analysing cell size and granularity.

Analysis of the light scatter can provide information on the size and granularity of the cells. The forward light scatter (FSC) is influenced by cell size whereas the side scatter (SSC) is influenced by cell granularity. These two parameters can be utilised simultaneously to distinguish between lymphocytes, monocytes and granulocytes using a dot plot (Figure 1.9).
Figure 1.10. Analysis of two cell populations. Negative, positive and double positive populations can be analysed using flow cytometric dot plots that can allow gating on a particular subpopulation for further analysis.

The correlation between two parameters, such as the CD4 and CD8 co-receptors on T cells, can be shown using a flow cytometric dot plot in which positive and negative populations can be identified (Figure 1.10). Specific populations can be “gated” based on these dot plots for further analysis of subpopulations. Gates on populations can be combined for analysis to look at cells producing multiple parameters (e.g. multiple cytokine expression). This is called Boolean gating. The definition of positive and negative populations is facilitated by the use of unstained or unstimulated cells in which the negative population is easily identified to improve gating strategies.

1.6.6 Activation Markers

Flow cytometry is well suited to the detection of T cells expressing activation markers. Expression of cytokines, such as IFNγ, have been utilised to detect antigen-specific lymphocytes, but this resulted in detection of only classically effector memory populations, and not identifying cell populations that not producing this cytokine, including TH2, TH17 and TReg cells.
CD154 is a 33kDa integral membrane protein of the tumour necrosis factor (TNF) gene family and activation marker of T helper cells. It is transiently expressed upon ligation of the T cell receptor, thereby providing direct access to an antigen-specific T cell population (241, 242). This assay has been successfully used to track changes during experimental ragweed immunotherapy (243) and was recently evaluated alongside a dye dilution system (244). This detection method may provide advantages over previous techniques including speed and simplicity of the protocol, reduction in culture artefact and compatibility with further intracellular and intranuclear cytokine staining, allowing for detailed interrogation of functional T cell subsets. CD154 expression is more specific compared to detection of other T cell activation markers such as CD69, CD25, CD134, CD137 and HLA-DR, which can be induced independent of TCR ligation (241), with minimal bystander activation of cells in the first few hours of antigenic stimulation (245).

Bonvalet et al. examined the combination of activation markers and MHC tetramers to identify and characterise Bet v 1-, Der p 1- and Phl p 1-specific CD4 T cell responses. The correlation between tetramer+ and CD154+ cells was poor, however this group used an expanded cell population; prolonged cell culture is known to abrogate the specificity of CD154 (244). Nevertheless, in combination these techniques may allow discrimination of functional, anergic or non-specifically activated T cells (246). The use of CD154 is both cheaper and easier and is able to analyse a larger range of epitope responses without haplotype restrictions associated with tetramers. In addition, in vitro cellular expansion and/or mitogen stimulation is not required for cytokine analysis.

1.7 Superantigens and Allergy

1.7.1 Superantigens

Superantigens are a family of microbial toxins able to potently activate T cells upon simultaneous extracellular binding of the MHC class II molecule on APCs and the TCR on T cells. This occurs outside the peptide-binding site, therefore bypassing normal antigen processing and presentation in an immune response (247, 248). The binding site of superantigens on the T cell is the variable region of the β chain, coined the Vβ region. Superantigens have a particular Vβ specificity in which they will trigger the activation and expansion of Vβ-specific T cells (248). For example,
the superantigen Staphylococcal Enterotoxin B (SEB) from *Staphylococcus aureus* generates activation and proliferation of T cells bearing Vβ1.1, 6.4 and 15.1 and predominantly Vβ3.2 (247).

Superantigens are active at very low concentrations and are able to stimulate 20-30% of T cells (249), resulting in widespread secretion of pro-inflammatory cytokines (TNFα, IFNγ, IL6 and IL12), recruitment of B cells, T cells and neutrophils, and Vβ-specific Th1-skewed proliferation (248). Consequently, this is followed by anergy of the T cells and in some cases, Vβ-specific apoptosis and generalised immunosuppression.

### 1.7.2 Superantigens and Allergy

The role of superantigens in allergic diseases has attracted considerable interest. Colonisation of superantigen-producing staphylococci is common in allergic diseases such as atopic dermatitis (250), allergic rhinitis (251, 252), nasal polyposis (253) and asthma (254). Evidence increasingly favours an important role for superantigens as a co-factor in the pathogenesis of allergic diseases. Superantigens may provoke Th2-mediated inflammation and allergen-specific Th2 responses (255) and induce B cell class switching to IgE (256). In atopic dermatitis, staphylococcal colonisation is virtually ubiquitous and correlates with disease severity. The cutaneous application of superantigen induces skin inflammation in both atopic and healthy individuals (257) and in animal models, skin exposure to staphylococcal superantigen enhances Th2 responses to subsequent peanut sensitisation (255). Superantigens are able to induce allergic inflammation without the presence of an allergen (258) and treatment of *S. aureus* infection improves the symptoms of atopic dermatitis (259). Studies have demonstrated superantigens can induce airway hyperresponsiveness (260) and increased sensitivity to aeroallergens (261, 262) in allergic airway disease, but the mechanism by which superantigens facilitate allergic immune dysfunction remain to be fully elucidated.

The susceptibility factors favouring staphylococcal colonisation in atopic diseases remain unknown, but have been postulated to relate to Th2 polarisation in the tissue microenvironment of allergic inflammation, perhaps reflecting mucosal barrier dysfunction or defective innate defences (250). However, very little is known of circulating T cell responses to superantigens in atopic individuals, with existing
studies demonstrating conflicting results. Much of the literature focuses on atopic dermatitis; however, the exact atopic status of individuals has not been accurately defined in these studies (263-266). Several groups have identified increased expression of IL4 and a concomitant decrease in IFNγ expression, suggesting TH2-biased immune responses to superantigens in atopy (263, 265, 266). However, prolonged culture techniques in these studies introduce artefact and reduce the specificity of cytokine analysis.

1.8 Aims and Original Contribution

The central aim of this project was to investigate the cellular mechanisms of allergic immune responses. The first study addressed the requirement of a sensitive and suitable assay to detect and phenotype allergen-specific T cells without the need for in vitro amplification and/or mitogen stimulation. This work sought to utilise the T cell activation marker CD154 to identify and characterise allergen-specific T helper cells ex vivo following allergenic stimulation. Although this method has been used to track changes during experimental ragweed immunotherapy (243) and alongside a dye dilution technique (244), this is the first example of detailed phenotyping using CD154 to date. In addition, the analysis of sensitised and atopic non-allergic individuals provides an additional novel aspect.

Following the development of a suitable CD154 assay, this project also aimed to analyse modulation of T cell responses in two situations: [1] following allergoid immunotherapy and [2] during peak pollen season compared to out of season. In addition, this project sought to develop a successful allergen proliferation assay using PKH cell-tracking dyes to further interrogate CD154+ allergen-specific T cells in allergic and non-allergic individuals.

Whilst studying allergen-specific T cell responses to allergens, a chance observation illustrated differences in the T cell cytokine response to the staphylococcal superantigen SEB between allergic and non-allergic individuals. Little is known regarding T cell responses to superantigens in atopy and health. Therefore, this project sought to further define the T cell response to the staphylococcal superantigen SEB in a group of highly atopic polysensitised individuals using multiparametric flow cytometry.
The final aim of this project was to investigate the sensitisation profiles of adults with pollen-food syndrome using ISAC microarray technology. Data relating to the sensitisation profiles of adults with PFS is lacking in the UK and this study aimed to define patterns of sensitisation using component-resolved diagnosis. This study is complementary to larger ongoing studies investigating adult sensitisation profiles (162, 267) within UK and European populations.

1.9 Hypotheses

- CD154 is a suitable marker for the identification and characterisation of allergen-specific T cells

- Allergic and non-allergic individuals differ in cytokine profile and phenotype of CD154⁺ allergen-specific T cells

- T cell responses to allergens differ in and out of the peak pollen season and following allergoid immunotherapy

- T cell responses to the staphylococcal superantigen SEB differ between highly atopic and non-atopic individuals

- Data on PFS and component resolved diagnosis will reveal similarities to the Northern European pattern of birch pollen associated PFS with cross-reactivity between the major birch pollen allergen Bet v 1 and homologues in food proteins
Chapter 2: Materials and Methods

2.1 Statement of Contribution

I designed the study protocols with considerable assistance from Dr Tarzi, Professor Kern, Dr Taylor and Dr Powell. Dr Tarzi, Prof. Frew and Dr Gray performed all clinical procedures such as skin-prick testing, clinical history, sensitisation profiles and participant recruitment. Dr Tarzi, Dr Rashid and I, performed the microarray experiments for component resolved diagnosis. Mr Saleh and Miss Lavender from the Royal Sussex County Hospital Diagnostic Immunology Laboratory performed serum birch-specific IgE and IgG4 analysis. Dr Taylor performed a significant proportion of the superantigen-induced regulatory T cell experiments and analysis. Dr Gray performed a proportion of the in-season birch and specific immunotherapy experiments. I performed all remaining experimental work involving cell culture, antibody staining, flow cytometry and cytokine analysis. Full contributions are stated within the project acknowledgements on page iii.

2.2 Ethical Approval

The National Research Ethics Service (South East Coast, Brighton and Hove) and the University of Sussex Ethics Research Committee approved all studies and all volunteers provided informed written consent.

2.3 Participant Recruitment

Healthy non-allergic participants were recruited as volunteers from Brighton and Sussex Medical school students and staff, characterised based on no history of clinical symptoms of type I allergy and negative skin prick tests and/or negative specific IgE to a variety of common aeroallergens (early tree pollinating mix, late tree pollinating mix, birch pollen, grass pollen, cat dander, house dust mite). Allergic participants were recruited from the adult Allergy Clinic at Royal Sussex County Hospital with sensitisations and clinical phenotypes confirmed by history and skin prick testing. All participants were between 18 and 65 of age. Exclusion criteria for participants included pregnancy, breastfeeding and those taking parenteral steroids up to eight weeks prior to donation.
2.4 Materials

2.4.1 Cell Culture Media

RPMI 1640: Roswell Park Memorial Institute 1640 (Gibco, Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS, Invitrogen), 2mM L-glutamine (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen) stored at 4°C and discarded after one month.

PKH RPMI 1640: Roswell Park Memorial Institute 1640 (Gibco, Invitrogen) supplemented with 5% autologous serum, 2mM L-glutamine (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen) stored at 4°C and discarded after each experiment.

2.4.2 Antigens

BPE: Freeze-dried lyophilised native Birch Pollen Extract (Allergopharma) filtrated through 0.22µM Millipore Millex GV filters and reconstituted in sterile phosphate buffer saline (PBS) at 2x10^5 Protein Nitrogen Units (PNU)/ml and stored at -20°C in aliquots until use.

CDE: Freeze-dried lyophilised native Cat Dander Extract (Allergopharma) filtrated through 0.22µM Millipore Millex GV filters and reconstituted in sterile PBS at 2x10^5 Protein Nitrogen Units (PNU)/ml and stored at -20°C in aliquots until use.

GPE: Freeze-dried lyophilised native timothy Grass Pollen Extract (Allergopharma) filtrated through 0.22µM Millipore Millex GV filters and reconstituted in sterile PBS at 2x10^5 Protein Nitrogen Units (PNU)/ml and stored at -20°C in aliquots until use.

LPS: Lipopolysaccharide (Sigma Aldrich) from *Escherichia coli* reconstituted in sterile PBS to 1mg/ml and stored at -20°C until use.

Recombinant Bet v 1: rBet v 1.0101 Isoform A (Biomay), produced by heterologous expression in *E.coli*, purified and lyophilised recombinant major allergen of birch, *Betula verrucosa*, reconstituted in sterile water to 1mg/ml and stored at -20°C until use.

PPD: Purified Protein Derivative (Sigma Aldrich) from *Mycobacterium tuberculosis* reconstituted in sterile PBS to 1mg/ml and stored at -20°C until use.
PHA: Phytohaemagglutinin (Sigma Aldrich) from *Phaseolus vulgaris* reconstituted in sterile PBS to 1mg/ml and stored at -20°C until use.

SEB: Staphylococcus Enterotoxin B (Sigma Aldrich) from *Staphylococcus aureus* reconstituted in sterile PBS to 2mg/ml and stored at -20°C until use.

SPE-K/L: Recombinant Streptococcal Pyrogenic Exotoxin K/L, produced by heterologous expression in *E.coli*, purified and lyophilised, and reconstituted in sterile PBS to 1mg/ml and stored at -20°C until use, provided by Thomas Proft, University of Auckland, New Zealand.

2.4.3 **Cell Culture Reagents**

PBS: Phosphate Buffer Saline, 140mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, 1.8mM KH$_2$PO$_4$, pH 7.4

Ficoll Paque Plus: Ficoll PM400 5.7g, Diatrizoate Sodium 9.0g plus Edetate Calcium Disodium in Purified Water (GE Healthcare Science) stored at room temperature.

BFA: Brefeldin A (Sigma Aldrich) from *Penicillium brefeldianum* dissolved in 1ml dimethyl sulphoxide (DMSO) to 5mg/ml and stored at -20°C until use. 2µl of BFA is used per 1ml cell sample to give a final concentration of 10µg/ml.

Human Recombinant IL2: Recombinant expressed in *Escherichia coli* (Sigma Aldrich) reconstituted in sterile PBS to 1x10$^5$U/ml and stored at -20°C until use.

Diluent C: iso-osmotic solution (Sigma Aldrich), pH 5-7.5, containing no salts, buffers or detergents used as a labelling vehicle for PKH-staining of PBMCs and stored at room temperature.

PKH67 Dye: PKH67 cell linker in ethanol Midi Kit (Sigma Aldrich) stored at room temperature in the dark until use with resuspension at 4µg/ml in Diluent C.

2.4.4 **Buffers and Solutions**

PBS: Phosphate Buffer Saline, 140mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, 1.8mM KH$_2$PO$_4$, pH 7.4.
**EDTA**: Ethylenediaminetetraacetic acid, stored as a stock solution at 500mM at room temperature until use; diluted to 20mM in FACS buffer and stored at 4°C.

**ELISA Wash Buffer**: Sterile Water, PBS, 0.05% Tween-20 (Fisher Scientific) pH 7.4 stored at room temperature and discarded after one month.

**ELISA Stop Solution**: 1M H$_2$SO$_4$ stored at room temperature and discarded after one month.

**FACS Buffer**: Sterile water, PBS, 0.5% bovine serum albumin (BSA, Acros Organics) and 0.1% sodium azide (Sigma Aldrich) pH 7.4, stored at 4°C and discarded after one month.

**Lysing Solution**: X10 concentration (BD Biosciences) ammonium chloride-based solution (pH 7.1-7.4) and stored at 4°C until use; diluted 1:10 in distilled water and stored at room temperature.

**Permeabilisation II Solution**: X10 concentration (BD Biosciences) and stored at 4°C until use; diluted 1:10 in distilled water and stored at room temperature.

**Paraformaldehyde**: 0.5% PFA solution in FACS buffer, stored at 4°C until use, discarded after 3 months.

**Human FOXP3 Buffer A**: X10 concentration (BD Biosciences) and stored at 4°C until use; diluted 1:10 in sterile water and stored at room temperature.

**Human FOXP3 Buffer B**: X50 concentration (BD Biosciences) and stored at 4°C until use; diluted 1:50 in human FOXP3 buffer A and stored at room temperature.

### 2.4.5 Antibodies

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<td>Anti-human activated T cell marker</td>
<td>Mouse IgG1</td>
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</tr>
<tr>
<td>CD154-APC-Cy7</td>
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<td>Mouse IgG1</td>
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<td>Biolegend</td>
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<tr>
<td>CD154-APC</td>
<td>Anti-human activated T cell marker</td>
<td>Mouse IgG1</td>
<td>10</td>
<td>BD Biosciences</td>
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<tr>
<td>IL4-FITC</td>
<td>Anti-human IL4 cytokine marker</td>
<td>Rat IgG1</td>
<td>10</td>
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<tr>
<td>IL4-PE</td>
<td>Anti-human IL4 cytokine marker</td>
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<tr>
<td>IL10-APC</td>
<td>Anti-human IL10 cytokine marker</td>
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<td>Anti-human T cell maturity marker</td>
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<td>Live/Dead Fixable Aqua Dead Cell Stain Kit (Aqvid)</td>
<td>Distinguishes dead cells</td>
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<td>Invitrogen</td>
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<tr>
<td>FOXP3-AlexaFluor488</td>
<td>Intranuclear anti-human regulatory T cell marker</td>
<td>Mouse IgG1</td>
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<td>BD Biosciences</td>
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</tbody>
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Table 2.1. List of antibodies.

<table>
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<tr>
<th>Antibody</th>
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<th>Isotype</th>
<th>µl/Sample</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3-AlexaFluor647</td>
<td>Intranuclear anti-human regulatory T cell marker</td>
<td>Mouse IgG1</td>
<td>15</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>TCRVβ1-PE</td>
<td>Variable chain of the β receptor of T cells</td>
<td>Rat IgG1</td>
<td>10</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>TCRVβ3-FITC</td>
<td>Variable chain of the β receptor of T cells</td>
<td>Mouse IgM</td>
<td>2.5</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>Granzyme A-PerCP-Cy5.5</td>
<td>Anti-human protease marker</td>
<td>Mouse IgG1</td>
<td>5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Granzyme B-AlexaFluor647</td>
<td>Anti-human protease marker</td>
<td>Mouse IgG1</td>
<td>5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Perforin-Pacific Blue</td>
<td>Anti-human cytolytic protein</td>
<td>Mouse IgG2</td>
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<td>Biolegend</td>
</tr>
<tr>
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<td>Anti-human T cell activation marker</td>
<td>Mouse IgG1</td>
<td>20</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CTLA-4-PE</td>
<td>Anti-human T cell activation marker</td>
<td>Mouse IgG2</td>
<td>5</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

2.4.6  ImmunoCAP ISAC Allergen Microarray Technology Kit

All reagents were provided in the ISAC microarray kit (Immunocap ISAC).

**ISAC chips**: stored at 4°C until use; slides were prepared by washing with vigorous stirring in component A for 60 minutes followed by a 5 minute washing step in sterile water and left to dry completely.

**Component A Solution**: diluted 1:20 in sterile water before use and discarded after each washing step.

**IgE Detection Antibody**: stored at 4°C and protected from light until use.

**Control Serum**: stored at 4°C and protected from light until use.

2.4.7  TNFα ELISA Kit

All reagents were provided in the TNFα ELISA MAX™ Deluxe kit (Biolegend).
Human TNFα ELISA Capture Antibody: diluted 1:200 in 1X coating buffer and stored at 4°C until use.

Human TNFα ELISA Detection Antibody: diluted 1:200 in 1X assay diluent and stored at 4°C until use.

Human TNFα Standard: reconstituted in 1X assay diluent to 500pg/ml stored at 4°C until use and discarded after one month.

Avidin-HRP: Phosphate buffered solution, pH7.2, containing 0.1% Proclin 150, diluted 1:1000 in 1X assay diluent and stored at 4°C.

TMB Substrate Solution: Tetramethylbenzidine, equal mix of substrate solution A and substrate solution B, stored at 4°C and discarded after use.

Coating Buffer A: 5X concentrated carbonate buffer, pH9.5, diluted to 1X with deionised water and stored at 4°C until use.

Assay Diluent: 5X concentrated phosphate buffered solution containing bovine serum, diluted to 1X with PBS, stored at 4°C until use and discarded after one month.

2.5 Cell Culture Protocols

2.5.1 Serum Sample Preparation

Collect whole blood in red/gold topped vacutainers (Vacuette 5ml Serum Sep Clot Activator Grenier bio-one 456073).

Invert the vacutainer 4 times and leave upright for 30 minutes at room temperature.

Centrifuge at 1800xg for 10 minutes at room temperature to separate the serum (the gel layer will move up the vacuette to allow simple separation).

Carefully pipette out the serum layer into a labelled eppendorf tube and store at -80°C until use.
2.5.2 **PBMC Isolation**

2.5.2.1 **Overnight Allergen Stimulation**

Pour blood from vacutainer into 50ml Falcon tubes, rinse the vacutainer with sterile PBS and add to the Falcon tube to provide diluted sterile blood at 1:1 dilution in PBS.

Add 15ml Ficoll Paque Plus into separate 50ml Falcon tubes; holding the Ficoll-containing tubes at a slant position, pipette the blood slowly onto the Ficoll layer using a sterile Pasteur pipette.

Separate the PBMC by centrifugation at 1000xg, no brakes, for 20 minutes at room temperature.

Carefully aspirate the PBMC and transfer these cells into a fresh 50ml Falcon tube; add sterile PBS up to 50ml and centrifuge for 10 minutes at 300xg at room temperature to wash the cells.

Decant the supernatant, resuspend the cell pellet gently using a pipette.

Add sterile PBS to 50ml and centrifuge at 200xg for 10 minutes at room temperature to wash the cells.

Decant the supernatant and resuspend PBMC in 1ml RPMI 1640 culture media supplemented with 10% FCS, 2mM L-glutamine and 1% Penicillin-Streptomycin.

Count the cells using the haemocytometer at a 1:10 dilution in trypan blue (to exclude dead cells) and resuspend the cells at 5x10^6/ml in RPMI 1640 culture media.

Incubate the cells at 37°C and 5% humidified CO₂ at a slant position for 24 hours before allergen stimulation.

2.5.2.2 **Seven-Day PKH T Cell Proliferation Assays**

Add 5ml Ficoll Paque Plus to 15ml Falcon tubes; holding the Ficoll-containing tubes at a slant position, pipette the blood onto the Ficoll layer straight from the vacutainer; do not dilute blood in sterile PBS.

Separate the PBMC by centrifugation at 1000xg, no brakes, for 20 minutes at room temperature.
Carefully aspirate the PBMC and transfer these cells into a fresh 50ml Falcon tube; add sterile PBS up to 50ml and centrifuge for 10 minutes at 300xg at room temperature to wash the cells.

Decant the supernatant, resuspend the cell pellet gently using a pipette, add sterile PBS to 50ml and centrifuge at 200xg at room temperature for 10 minutes to wash the cells.

Decant the supernatant and resuspend the cells in 1ml sterile PBS (do not resuspend PBMC in RPMI 1640 culture media).

Count the cells using the haemocytometer at a 1:10 dilution in trypan blue (to exclude dead cells) and take note of the cell number before PKH-staining the PBMC.

2.5.3 PKH Staining of PBMCs

Isolate PBMC as described in section 2.5.2.2 and determine cell number.

Centrifuge the cells at 400xg for 5 minutes into a loose pellet; decant the supernatant leaving no more than 25µl of suspension.

Resuspend the cell pellet in 1ml Diluent C and pipette gently to ensure complete dispersion.

Do not leave the cells in Diluent C for extended periods and do not vortex the samples during the remaining staining protocol.

Immediately before staining, prepare a 2x working dye solution (4x10⁻⁶ M) in diluent C by adding 4µl of PKH67 dye to 1ml of Diluent C in a 15ml Falcon tube and mix well to disperse.

Rapidly add the PBMC suspension (no more than 2x10⁷ PBMC) to the dye solution and immediately mix the sample by pipetting.

Incubate the PBMC/dye suspension for 2 minutes with periodic mixing using the pipette.

Stop the staining by adding an equal volume (2ml) of FCS; mix by pipetting and incubate for 1 minute.
Centrifuge the cells for 10 minutes at 400xg at room temperature; decant the supernatant, resuspend the cell pellet in 10ml RPMI 1640 culture media supplemented with 5% autologous serum, 2mM L-glutamine and 1% Penicillin-Streptomycin and transfer into a fresh 15ml Falcon tube.

Centrifuge for 10 minutes at 400xg at room temperature; decant the supernatant, resuspend the cell pellet and add 10ml RPMI 1640 culture media; repeat this washing step twice.

Resuspend the cell pellet in 1ml RPMI 1640 culture media for cell counting; resuspend cells in RPMI 1640 culture media to 1x10^6 PBMCs/ml and rest at 37°C and 5% humidified CO₂ until stimulation.

2.5.4 **PBMC Stimulation**

2.5.4.1 **Overnight Antigenic Stimulation**

PBMC are rested for 24 hours at 37°C and 5% humidified CO₂ before overnight antigenic stimulation.

Stimulation of PBMC are set up in 100µl volumes in triplicates in 5ml polypropylene FACS tubes; vortex the antigens and add to RPMI 1640 culture media to this volume as described in **Table 2.2**.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Volume of Antigen (µl)</th>
<th>Volume of Medium (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>SEB</td>
<td>0.5</td>
<td>99.5</td>
</tr>
<tr>
<td>PPD</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>PHA</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>BPE</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>CDE</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>GPE</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>Bet v 1</td>
<td>15</td>
<td>85</td>
</tr>
</tbody>
</table>

**Table 2.2. Volumes of antigen for overnight stimulation of PBMC**

Add 400µl of cell suspension (5x10^6 PBMC/ml) to each stimulation tube (approximately 2x10^6 cells/tube); vortex the FACS tubes and incubate for 2 hours at 37°C and 5% humidified CO₂ at a slant position.

Dilute BFA in RPMI 1640 culture media (2µl BFA + 498µl RPMI 1640 culture media per FACS tube); add 500µl BFA suspension to each sample to result in 10µg/ml BFA in each tube.
Vortex the samples and incubate at a slant position for 14 hours at 37°C and 5% humidified CO₂.

2.5.4.2 Seven-Day PKH T Cell Proliferation Assays

Add 200µl PKH cell suspension (500,000 cells) to the required number of wells of a 96-well round-bottomed sterile culture plate (3-10 replicates per antigen).

RPMI 1640 culture media used is that described for PKH assays in section 2.4.1.

Vortex the antigens and add directly to the well as described in Table 2.3.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Volume of Antigen (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0</td>
</tr>
<tr>
<td>SEB</td>
<td>0.1</td>
</tr>
<tr>
<td>PPD</td>
<td>2</td>
</tr>
<tr>
<td>PHA</td>
<td>0.2</td>
</tr>
<tr>
<td>BPE</td>
<td>5</td>
</tr>
<tr>
<td>CDE</td>
<td>5</td>
</tr>
<tr>
<td>GPE</td>
<td>5</td>
</tr>
<tr>
<td>Bet v 1</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.3. Volumes of antigen for 7-day stimulation of PBMC

Mix each well by gentle pipetting

Incubate at 37°C and 5% humidified CO₂ for 7 days: do not refresh cells in fresh RPMI 1640 culture media or vortex the samples

To harvest the cells, add 100µl EDTA in FACS buffer to each well and incubate for 10 minutes at room temperature

Gently pipette out each well into a corresponding FACS tube and wash the wells with 200µl FACS buffer and re-harvest

2.6 Experimental Protocols

2.6.1 Surface and Intracellular Staining of PBMCs

Add 3ml FACS buffer to FACS tubes and centrifuge at 400xg for 8 minutes at 4°C; decant the supernatant completely by blotting on paper and vortex the samples.

Prepare the surface staining antibody mix by adding antibodies to a final volume of 100µl FACS buffer for each tube.
Add 100µl of surface staining antibody mix to each tube, vortex, cover with foil, and incubate for 30 minutes at 4°C.

Add 1ml of 1X BD FACS lysing solution to each tube, vortex and incubate at room temperature in the dark for 10 minutes.

Add 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C; decant the supernatant and vortex the samples.

Add 1ml of 1X BD FACS permeabilisation solution to each tube, vortex and incubate at room temperature in the dark for 10 minutes.

Add 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C; decant the supernatant completely by blotting on paper and vortex the samples.

Prepare the intracellular staining antibody mix by adding antibodies to a final volume of 100µl FACS buffer for each tube.

Add 100µl of intracellular staining antibody mix to each tube, vortex, cover with foil and incubate for 30 minutes at 4°C.

Add 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C; decant the supernatant, vortex the samples and store covered in foil at 4°C until acquisition.

If acquisition is not immediate, add 1ml 0.5% PFA to each FACS tube and incubate at room temperature in the dark for 5 minutes; add 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C, decant the supernatant, vortex the samples and store at 4°C protected from light until acquisition.

Samples should be acquired within 7 days after fixing in PFA.

2.6.2 FOXP3 Intranuclear Staining of PBMCs

Wash cells in 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C; decant supernatant completely.

Prepare the surface staining antibody mix by adding antibodies to a final volume of 50µl FACS buffer for each tube.

Add 50µl of surface antibody mix to each tube, vortex and incubate for 30 minutes at 4°C protected from light.
Bring the human FOXP3 buffer set to room temperature and prepare working solutions: dilute FOXP3 buffer A (10X concentrate) 1:10 in room temperature mQ sterile water.

To make a working solution of FOXP3 buffer C, dilute FOXP3 buffer B into 1X FOXP3 buffer A at a ratio of 1:50 (Buffer B:Buffer A).

Add 3ml FACS buffer to tubes, centrifuge at 400xg for 8 minutes and decant supernatant.

Add 2ml 1X FOXP3 buffer A to each tube, vortex, and incubate for 10 minutes at room temperature protected from light.

Centrifuge at 500xg for 5 minutes at 4°C and carefully decant the supernatant.

Add 0.5ml of FOXP3 buffer C to each FACS tube, vortex and incubate for 30 minutes at room temperature protected from light.

Prepare the intracellular staining antibody mix by adding antibodies to a final volume of 50µl FACS buffer for each tube

Add 50µl of intracellular (and intranuclear FOXP3) antibody mix to tubes, vortex and incubate for 30 minutes at room temperature protected from light.

Add 3ml FACS buffer to tubes, centrifuge at 500xg for 5 minutes at 4°C and decant supernatant.

Prepare the intracellular staining antibody mix by adding antibodies to a final volume of 50µl FACS buffer for each tube

Add 50µl of intracellular (and intranuclear FOXP3) antibody mix to tubes, vortex and incubate for 30 minutes at room temperature protected from light.

Add 3ml FACS buffer to tubes, centrifuge at 500xg for 5 minutes at 4°C and decant supernatant.

Acquire immediately.

If acquisition is not immediate, add 1ml 0.5% PFA to each FACS tube and incubate at room temperature in the dark for 5 minutes; add 3ml FACS buffer and centrifuge
at 400xg for 8 minutes at 4°C, decant the supernatant, vortex the samples and store at 4°C protected from light until acquisition.

Samples should be acquired within 7 days after fixing in PFA.

2.6.3 Compensation Controls

Label FACS tubes with the compensation controls to be made – one tube for each fluorochrome.

Add 1 drop of positive and 1 drop of negative mouse IgG1 compensation control beads (BD Biosciences) into each tube and vortex.

Add the appropriate antibody to each tube and make up to 100µl using FACS buffer (e.g. 3µl CD4-ECD with 97µl FACS buffer) and vortex.

Incubate at 4°C for 20 minutes covered in foil.

Add 3ml FACS buffer to each tube, centrifuge at 400xg at 4°C for 8 minutes; decant the supernatant and vortex; store at 4°C covered in foil until use.

2.6.3.1 PKH Compensation

For the PKH compensation control, wash unstained PBMC and PKH-stained PBMC in 3ml FACS buffer, centrifuge at 400xg at 4°C for 8 minutes and decant the supernatant

Pool the unstained PBMC and PKH-stained PBMC into a fresh FACS tube

Add 3ml FACS buffer, centrifuge at 400xg at 4°C for 8 minutes and decant the supernatant; vortex and store at 4°C covered in foil until use.

2.6.3.2 Aqvid Compensation

For the Aqvid compensation control, wash PBMC in 3ml FACS buffer and centrifuge at 400xg at 4°C for 8 minutes.

Decant the supernatant and permeabilise half the PBMC by adding 1ml of 1X permeabilisation II solution for 10 minutes in the dark; add 3ml FACS buffer, centrifuge at 400xg for 8 minutes at 4°C and decant the supernatant.
Pool the permeabilised PBMC and the live PBMC into a fresh FACS tube, add 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C; decant the supernatant completely and stain with 0.75µl Aqvid in 99.25µl FACS buffer at 4°C for 30 minutes protected from light.

Add 3ml FACS buffer and centrifuge at 400xg for 8 minutes at 4°C, decant the supernatant, vortex and leave at 4°C covered in foil until use.

2.6.4 ImmunoCAP ISAC Allergen Microarray Technology

Prepare the humidity chamber by placing a wet paper towel into the chamber for 1 hour with a closed lid.

Add 35ml of component A into 665ml of sterile mQ water.

Number the slides and draw a plan of the participant layout for each slide: use only pencil to label the slides and do not touch the reaction sites.

Immerse the slides in 220ml of solution A and incubate for 60 minutes at room temperature with vigorous stirring.

Place slides into 220ml of sterile water for 5 minutes with vigorous stirring.

Allow slides to completely dry: throw away all used washing solutions.

Place slides in the humidity chamber with the reaction sites facing upwards; add 20µl of participant serum sample to each reaction site on the slide and close the chamber; incubate at room temperature for 2 hours.

Do not touch the reaction sites with the pipette tip, use one sample of control serum per experiment and close the chamber carefully to avoid mixing of samples.

Tap the slides at a 90° angle to remove excess sample.

Immerse the slides in 220ml solution A and wash for 10 minutes with vigorous stirring.

Remove the slides, place into 220ml sterile water, and wash for 5 minutes with vigorous stirring.

Place the slides onto paper towel and allow to dry completely.
Place slides into the humidity chamber with the reaction sites facing upwards and add 20µl of IgE detection antibody to each chip; close the chamber and incubate for 60 minutes protected from light.

Tap the slides at a 90° angle to remove excess sample.

Immerse the slides in 220ml solution A and wash for 10 minutes with vigorous stirring.

Remove the slides, place into 220ml sterile water, and wash for 5 minutes with vigorous stirring.

Allow the slides to completely dry and store in the dark at 4°C until acquisition.

Analysis of the slides is on the Genepix 4000B microarray reader with optimised settings (532nm, 100% power, PMT gain 590).

Export the microarray images to the MIA software to generate sensitisation reports; measurements >0.3 ISAC Standardised Units (ISU) are deemed as positive IgE antibody responses.

2.6.5 **TNFα ELISA**

Isolate PBMC as per protocol and incubate for 24 hours at 37°C and 5% humidified CO₂.

Stimulate PBMC as per protocol with PBS, BPE, CDE, GPE and LPS (log¹⁰ dose response 10-0.001ng/ml) without the addition of BFA: incubate for 18 hours at 37°C and 5% humidified CO₂.

Prepare the coating buffer and pre-titrated capture antibody (section 2.4.7): add 100µl capture antibody solution to the wells of a 96-well flat-bottomed ELISA plate, seal, and incubate for 18 hours at 4°C.

Bring all reagents to room temperature and prepare solutions as described (section 2.4.7).

Centrifuge the stimulated PBMC at 400xg for 8 minutes and pipette the supernatant into labelled eppendorf tubes: discard the cell pellet.
Centrifuge the sample supernatant at 13,000xg for 5 minutes and pipette the supernatant into labelled eppendorf tubes: discard any remaining cell pellet.

Wash the coated ELISA plate four times in ELISA wash buffer.

Add 200μl assay diluent to each well of the ELISA plate, seal, and incubate for 1 hour with shaking at 200rpm on a plate shaker.

Prepare 6 two-fold serial dilutions of the 500pg/ml top TNFα standard with assay diluent with resultant concentrations at 500, 250, 125, 62.5, 31.3, 15.6 and 7.8pg/ml using assay diluent alone as a zero standard.

Wash the ELISA plate four times in ELISA wash buffer.

Add 100μl of the TNFα standards and supernatant samples to the wells, seal the plate and incubate at room temperature for 2 hours with shaking at 200rpm.

Wash the ELISA plate four times in ELISA wash buffer.

Add 100μl of detection antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking at 200rpm.

Wash the ELISA plate four times in ELISA wash buffer.

Add 100μl avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking at 200rpm.

Wash the ELISA plate five times in ELISA wash buffer: immerse for 30 seconds-1 minute per washing step.

Add 100μl of TMB substrate solution to each well and incubate in the dark for 15 minutes.

Stop the reaction by adding 100μl ELISA stop solution to each well.

Read absorbance at 450nm within 30 minutes on the Bio-tek Synergy HT plate reader.
2.6.6 **LAL Test**

The endotoxin content of the native birch (BPE) allergen extract was analysed by Dr Helga Kahlert of Allergopharma in a Limulus Amebocyte Lysate (LAL) test using the test kit pyrochrome C180 (Pyroquant, Mörfelden-Walldorf, Germany).

2.7 **Analysis of Data**

In all experimental work, samples were analysed using the BD Biosciences LSR II flow cytometer. FACS data was analysed using FACSdiva software v6.1.3 and FlowJO software v9.9.3. Fluorescence-minus-one (FMO) controls were performed to ensure compatibility of fluorochromes and to eliminate antibody effects in cytokine expression.

2.8 **Statistical Analysis**

All data was analysed using Graphpad Prism v5.03 and Microsoft Office Excel 2007/2010. Evaluation of data distribution was assessed using the D’Agostino and Pearson omnibus normality test. Duplicate values of tests during optimisation and proliferation values across wells were expressed as means. All other values are expressed as medians where appropriate for non-parametric data. Statistical comparisons between participant groups were compared with the Mann Whitney U test and student t test for non-parametric and parametric data, respectively. Statistical comparisons within participant groups were compared with the Wilcoxon matched-pairs signed rank test for non-parametric data. Correlation analyses were performed using the non-parametric spearman rank test. A p value of less than 0.05 was considered significant. P values between 0.05 and 0.09 were considered a statistical trend. All cell frequency values were background-corrected by subtraction of the unstimulated cell frequency from the stimulated cell frequency.

To calculate the T\(_{H2}:T\(_{H1}\) ratio, the frequency of CD154\(^+\)IL4\(^+\) T\(_{H2}\) cells was divided by the frequency of CD154\(^+\)IFN\(_{\gamma}\)\(^+\) T\(_{H1}\) cells. Where participants had no detectable CD154\(^+\)IFN\(_{\gamma}\)\(^+\) expression, a ratio was defined by allocating a predicted IFN\(_{\gamma}\) frequency value based on the regression equation of all responding participants of that particular participant group. To avoid bias, a similar method was applied where no CD154\(^+\)IL4\(^+\) cell population was detected. Statistical analysis of data was reviewed by a statistician.
Chapter 3: Assay Optimisation

3.1 Detection and Analysis of Allergen-Induced CD4\(^+\) CD154\(^+\) T Cells

3.1.1 Introduction

The low frequency of allergen-specific CD4 T cells in peripheral blood makes their detection problematic. To date, much of the literature refers to cultured cell populations, which introduce bystander activation and culture artefact.

CD154 is a T cell activation marker transiently expressed upon ligation of the T cell receptor, therefore providing direct access to an antigen-specific population following \textit{ex vivo} stimulation (241, 242). This detection method may provide advantages over previous techniques including speed and simplicity of the protocol, reduction in culture artefact and compatibility with further intracellular and intranuclear cytokine staining. CD154\(^+\) T cells can be sorted (FACS or MACS), allowing for further interrogation of functional T cell subsets. CD154 expression is more specific compared to other T cell activation markers such as CD69, CD25, CD134, CD137 and HLA-DR, which can be induced independent of TCR ligation (241), with minimal bystander activation of cells in the first few hours of antigenic stimulation (245).

The expression of CD154 can be detected on the cell surface or intracellularly. Surface detection of CD154 is fast and easy to use, but the frequency of antigen-specific T cells detected is significantly lower than that of intracellular CD154 detection (245). Similarly, surface detection also invokes a higher coefficient of variation, and thus larger dispersion of individual results, therefore an intracellular CD154 system is far more suitable for high standard immune monitoring of CD4 T cell responses. However, detection of intracellular CD154 diminishes cell viability for functional studies following permeabilisation of the cells.

Previously, detection of cytokines was problematic using intracellular cytokine staining as the cytokines were released into the supernatant of cell cultures. The introduction of Brefeldin A (BFA), a protein transport inhibitor, has overcome this issue. BFA, a fungal metabolite, targets the Arf1 protein. This protein is responsible for the recruitment of coating proteins required for transport vehicle formation, therefore inhibiting transport of proteins from the endoplasmic reticulum to the golgi
apparatus. This causes subsequent accumulation of proteins within the endoplasmic reticulum. This allows for the intracellular detection of CD154 and cytokines using flow cytometry following appropriate permeabilisation.

Various optimal conditions for the *ex vivo* detection of antigen-specific CD154⁺ T cells have been determined including cell preparation, antigen sources and stimulation time periods (241, 242, 245, 268, 269). This assay can be performed using either whole blood or PBMC. Whole blood is well suited for fast analysis of CD4 immune responses in which samples can be directly stimulated. In contrast, PBMC are more appropriate for the standardisation of cell concentration and higher frequencies of antigen-specific CD4 T cells are identified using this method (245). PBMC density is equally important in detection of antigen-specific CD4 T cells, in which 5x10⁶/ml PBMC induce the highest frequency of detection without enhancing bystander activation (245). It is important to note the physiological activation of T cells takes place within secondary lymphoid tissue, in which cell density is significantly higher than that of peripheral blood. Therefore, a cell density of less than 1x10⁶/ml does not provide useful results because the sample cell frequency is too low.

Investigations into stimulation time periods for optimal detection of antigen-specific CD154⁺ T cells indicate both six- and sixteen-hour antigenic stimulations are comparable (245). Although a shorter stimulation period decreases the frequency of bystander activation, sixteen-hour stimulation is more convenient for this assay in order to both stimulate and antibody stain within practicality and time constraints.

This chapter focuses on further optimisation experiments performed to identify optimal antigens, allergen concentration, costimulatory requirements and antibody staining panels.

### 3.1.2 Dose Responses

Allergens utilised for this study were native birch pollen (BPE), grass pollen (GPE) and cat dander (CDE) extracts and recombinant Bet v 1, the major birch pollen allergen. Briefly, PBMC from three non-allergic participants were stimulated with 0.5µg/ml Bet v 1 or 30-1000PNU/ml BPE, CDE and GPE for 16 hours. Brefeldin A (BFA) was added for the last 14 hours. Responding cells were identified by
multiparametric flow cytometry following antibody staining for surface (anti-human CD3-AlexaFluor700 and CD4-ECD) and intracellular (anti-human CD154-APC and IFNγ-PeCy7) markers. All results were gated on CD3⁺CD4⁺ single lymphocytes following a minimum collection of 100,000 CD4 events. All data was background corrected.

Figure 3.1. CD154 expression (% of CD4) in Bet v 1, BPE, CDE and GPE stimulated CD4 T cells at various concentrations of allergen

The highest frequencies of CD154⁺ T cells were reported after 16-hour stimulation with 15μg/ml recombinant Bet v1 and 500PNU/ml BPE, CDE and GPE (Figure 3.1). The dose response for recombinant Bet v 1 demonstrated the typical dose response curve seen with allergen titrations, but the CD154⁺ T cell responses were small compared to the native extracts. It is probable that recombinant Bet v 1 stimulates a smaller range of CD4 T cells than the native allergen extracts that contain a larger number of allergenic components. An optimal concentration of
500PNU/ml for BPE, CDE and GPE allergen stimulation was used in all experimental protocols investigating allergen-induced CD154^+ T cells.

3.1.3 **LPS Content of Allergens**

Lipopolysaccharide (LPS), also known as endotoxin, is the foremost cell wall constituent of gram-negative bacteria and is biologically active at very low concentrations. LPS is a potent stimulator for TNFα production by macrophages (270). Literature has indicated that LPS is able to indirectly stimulate both CD4^+ and CD8^+ T cells (271, 272) through cytokine-mediated interactions. In addition, injection of LPS into mice resulted in increased expression of T cell activation markers (271). If endotoxin is present within the native allergen extracts, this may cause stimulation of T cells unrelated to the allergen. To determine the concentration of LPS within the native allergen extracts, a TNFα ELISA bioassay was performed. PBMC were isolated from three non-allergic participants and stimulated with 500PNU/ml BPE, CDE, GPE or 0.001-10ng/ml LPS without addition of BFA. The supernatant was harvested from all samples via centrifugation and the cell pellet was discarded. A standard 96-well ELISA plate was coated with pre-titrated capture antibody for 16 hours before incubation of the supernatant samples and TNFα standard controls in triplicate wells. TNFα detection antibody was added to each well before addition of TMB substrate and measurement of absorbance at 450nm on the Biotek Synergy HT plate reader.

In addition, the endotoxin content of the native birch allergen extract was analysed in a Limulus Amebocyte Lysate (LAL) test using the test kit pyrochrome C180 (Pyroquant, Mörfelden-Walldorf, Germany). The LAL test derives from the clotting mechanism of the amebocyte blood extract from horseshoe crabs (*Limulus polyphemus*) upon encountering endotoxin. This lysate is extremely sensitive and can detect very low levels of endotoxin, providing a useful technique for allergen extract testing. The amebocyte lysate is conjugated with a chromophore in which colour develops as an endotoxin-induced gel clot is formed. Therefore, the rate of colour development reports the level of endotoxin within the sample. Endotoxin levels are denoted as Endotoxin Units (EU). 9EU represents approximately 1ng/ml of endotoxin.
Figure 3.2. TNFα concentration of (a) LPS and (b) BPE, CDE and GPE allergens
Using the TNFα ELISA, the LPS concentration in the final BPE, CDE and GPE stimulation system was estimated to be 4.9ng/ml, 6.5ng/ml and 7.1ng/ml, respectively (Figure 3.2, Figure 3.3).

**Calculation Box 1: LAL Test of BPE**

Endotoxin level measured: 41.439EU in 100μg BPE

500PNU/ml BPE = 33.4μg/ml

100μg/33.4μg/ml = 2.99

41.439EU/2.99 = 13.86EU

13.86/9 = 1.54ng endotoxin in 500PNU/ml

Using the LAL test, the LPS content in the final BPE stimulation system was estimated to be 1.54ng/ml (Calculation Box 1). The LAL test analysis for CDE and GPE is not yet available.

The TNFα ELISA identified a higher level of endotoxin within the allergen extracts compared to the LAL test. However, utilising TNFα production in the ELISA is an indirect approach to the measurement of LPS content and is therefore less robust than the direct endotoxin analysis of the LAL test. The levels of endotoxin measured were considered acceptable for the studies.
3.1.4 Resting Cells and Costimulation

Resting PBMC for 24 hours prior to allergen stimulation may produce an increased CD154 response due to increased MHC class II expression. Addition of CD28 and CD49D during allergen stimulation may also increase CD154 expression because both molecules are known to increase transcription of cytokine mRNA upon ligation of B7 molecules (273). These costimulatory molecules also strengthen the interactions between CD4 T cells and APCs, perhaps allowing for higher avidity ligation of the TCR and a subsequent increase in expression of CD154. Previous literature has used both CD28 and CD49D as costimulatory molecules when stimulating PBMC with antigen to analyse CD154 expression (241, 242, 268).

To investigate the effects of resting cells overnight and costimulation on CD154 expression, PBMC were isolated from 1 allergic and 2 non-allergic participants and designated to one of four groups:

Group 1 – PBMC immediately stimulated with BPE

Group 2 – PBMC rested for 24 hours at 37°C and 5% humidified CO₂ prior to stimulation with BPE

Group 3 - PBMC immediately stimulated with BPE with addition of 1μg/ml CD28 and 1μg/ml CD49D during stimulation (16 hours)

Group 4 – PBMC rested for 24 hours at 37°C and 5% humidified CO₂ prior to stimulation with BPE-A with addition of 1μg/ml CD28 and 1μg/ml CD49D during stimulation (16 hours)

BFA was added for the last 14 hours of allergen stimulation in all groups. Responding cells were identified by multiparametric flow cytometry following antibody staining for surface (anti-human CD3-AlexaFluor700 and CD4-ECD) and intracellular (anti-human CD154-APC and IFNγ-PeCy7) markers. All results were gated on CD3⁺CD4⁺ single lymphocytes following a minimum collection of 100,000 CD4 events. All data was background corrected.

In a separate experiment, PBMC from five non-allergic participants were immediately antibody stained or rested for 24 hours at 37°C and 5% humidified CO₂
prior to antibody staining with anti-human HLA-DR-PerCP-Cy5.5 and anti-human CD14-V450 to assess expression of MHC class II molecules on monocytes.

**Figure 3.4.** CD154 and HLA-DR expression in rested/unrested and costimulated/non-costimulated cell conditions

Resting the cells overnight increased CD154 expression in BPE-stimulated T cells compared to those immediately stimulated (Figure 3.4) and reduced CD154 background staining, therefore improving the measurable response. An increase in MHC class II expression noted in CD14+ monocytes may explain the mechanism of augmentation, allowing increased peptide presentation to CD4 T cells. Costimulation did not improve the CD154 response in CD4 T cells and increased background CD154 staining in unstimulated PBMC. It is possible the addition of CD28 and CD49D caused non-specific activation of CD4 T cells leading to a higher background of CD154 expression in unstimulated cells that masked true CD154 expression in allergen-stimulated T cells. In conclusion, PBMC were rested for 24 hours.
hours at 37°C and 5% humidified CO₂ before allergenic stimulation without the addition of CD28 or CD49D in all future experiments.

3.1.5 Preliminary Experiments

Optimisation of the assay revealed the appropriate conditions for the allergenic stimulation of PBMC and ex vivo detection of CD154⁺ T cells. Preliminary experiments were performed based on these optimisation studies to determine a suitable antibody-staining panel for ex vivo detection and phenotyping of allergen-specific T cells based on CD154 expression.

PBMC were isolated from six allergic and two non-allergic participants, rested for 24 hours, and stimulated with 500PNU/ml BPE, CDE or GPE in the presence of BFA for the last 14 hours. Responding cells were identified by flow cytometry following antibody staining for surface and intracellular markers:

**Surface:**

- CD3-AlexaFluor700
- CD4-PerCP
- CD27-PE
- CD45RA-ECD

**Intracellular:**

- CD154-APC
- IFNγ-PeCy7
- IL4-FITC

All results were gated on CD3⁺CD4⁺ single lymphocytes following a minimum collection of 200,000 CD4 events. Data points for BPE, CDE and GPE allergens were pooled together for preliminary analysis. All data was background corrected.
Participants in preliminary experiments were defined as non-allergic or allergic using the following criteria:

**Non-allergic (n = 2)** – no clinical history of atopic disease

**Allergic (n = 6)** – positive SPTs, specific IgE and clinical symptoms to the test allergen

A CD154$^+$ T cell population was resolved in all allergic and non-allergic subjects after *ex vivo* allergen stimulation (**Figure 3.5**). The CD154$^+$ response represented a median of 0.29% and 0.12% of the total CD4 T cell population in non-allergic and allergic participants, respectively. The majority of CD154$^+$ responding T cells were of a central memory phenotype in both allergic and non-allergic participants (**Figure 3.7**). CD154$^+$ T cells were also analysed for intracellular cytokine expression of IFN$\gamma$ and IL4. A higher frequency of CD154$^+$IFN$\gamma^+$ T cells was reported in non-allergic participants, whereas allergic participants exhibited a higher frequency of CD154$^+$IL4$^+$ T cells (**Figure 3.6**), revealing possible T$_{H1}$ and T$_{H2}$ polarised immune responses, respectively.

These preliminary experiments identified several issues to address regarding cell frequencies and appropriate antibody staining panels. Collection of a significantly larger number of CD4 events on the flow cytometer is required when investigating rare events and small responding cell frequencies. In future experiments, allergen stimulation was set up in triplicates and pooled before antibody staining to provide a significantly increased frequency of CD4 T cells for analysis. In addition, the antibody-staining panel was extended to investigate allergen-induced IL10 responses. CD154$^+$IL4$^+$ cells were difficult to distinguish in preliminary experiments due to the dim staining of the anti-human IL4-FITC antibody. In addition, the inclusion of a live/dead cell stain allowed investigation of cell viability after allergen stimulation. The antibody-staining panel was altered to rectify these issues and improve characterisation of the cellular responses to allergen.
Figure 3.5. Flow cytometric data in one representative non-allergic participant illustrating CD154 expression in (a) unstimulated and (b) BPE-stimulated T cells and (c) IL4/IFNγ expression.
Figure 3.6. Birch-stimulated CD154, CD154^IFNγ^ and CD154^IL4^ T cell frequencies
Figure 3.7. Phenotypic analysis of CD154^+ T cells based on CD27 and CD45RA expression
3.1.6 Antibody Staining Panel

The new antibody-staining panel investigated was as follows:

Surface:

CD3-AlexaFluor700
CD4-PerCP
CD25-APC-Cy7
CD27-FITC
CD45RA-ECD

Intracellular:

CD154-PacificBlue
IFNγ-PeCy7
IL4-PE
IL10-APC

Fluorescence-minus-one (FMO) controls were performed to ensure the compatibility of fluorochromes and to eliminate antibody effects in cytokine expression (Appendix). In all experiments, cells were gated on CD3⁺CD4⁺ single lymphocytes and at least 400,000 CD4 events were recorded per sample.
Figure 3.8. Flow cytometric comparison of (a) CD154-APC and CD154-PB, (b) IL4-FITC and IL4-PE and (c) Aqvid live/dead cell stain in CD154⁺ T cells
The alteration in CD154-APC to CD154-PB antibody allowed for the inclusion of IL10-APC into the phenotyping panel to assess the presence of allergen-induced IL10 expression (Figure 3.8a). CD25-APC-Cy7 was also added to the staining panel, in conjunction with IL10-APC, for analysis of T_{Reg} cell populations. Anti-human IL4-PE stained at a much brighter intensity than anti-human IL4-FITC allowing improved identification of CD154^{+}IL4^{+} T_{H2} cells (Figure 3.8b). This antibody was also significantly cheaper compared to its FITC counterpart. The Aqvid live/dead cell stain demonstrated high cellular viability and was no longer included in the antibody-staining panel (Figure 3.8c). The FMO controls did not illustrate problems in fluorochrome compatibility, indicating the antibodies within the staining panel were suitable for the ex vivo allergen stimulation study (Appendix).

3.1.1 Gating Strategies

For ex vivo analysis of allergen-specific T cell responses, CD4 T cells were gated from single lymphocytes (SSC vs. FSC, FSC-A vs. FSC-H). Activated cells were gated individually for each T cell cytokine (CD154, IFNγ, IL4 and IL10) and then Boolean gating combinations were computed (Figure 3.9). Cells were also gated for maturity using CD27 and CD45RA.
Figure 3.9. Flow cytometric gating strategy for *ex vivo* analysis of CD154⁺ T cells
Detection and Analysis of Superantigen-Induced T Cells

3.2.1 Preliminary Experiments

During preliminary experiments investigating CD154+ allergen-specific T cells, a chance observation demonstrated a difference in cytokine responses to the positive control SEB. This initial finding was further explored to investigate T cell responses to SEB in highly atopic individuals and to develop a suitable antibody staining panel for subsequent analysis.

PBMC were isolated from twelve allergic and ten non-allergic participants and stimulated with 1μg/ml SEB in the presence of BFA for the last 14 hours. PHA was used as a positive alternative control. Responding cells were identified by multiparametric flow cytometry following antibody staining for surface and intracellular markers:

Surface:

CD3-AlexaFluor700
CD4-PerCP

Intracellular:

CD154-APC
IFNγ-PeCy7
IL4-PE
IL10-APC

All results were gated on CD3+CD4+ single lymphocytes following a minimum collection of 200,000 CD4 events. All data was background corrected. Statistical comparisons were performed using the Mann Whitney U test for non-parametric data. P values < 0.05 were deemed significant.

Participants in preliminary experiments were defined as non-allergic or allergic using the following criteria:

Non-atopic (n = 10) – no clinical history of atopic disease

Atopic (n = 12) – positive SPTs, specific IgE and clinical symptoms to a number of common aeroallergens
Figure 3.10. SEB-stimulated CD154, IFNγ, IL4 and IL10 T cell responses

There was no difference in the frequency of CD154+ (p=0.12) or IL10+ (p=0.58) T cells between non-atopic and atopic participants, however, atopic individuals exhibited a significantly lower frequency of IFNγ+ (p=0.02) and IL4+ (p=0.01) T cells compared to their non-atopic counterparts (Figure 3.10). Atopic individuals may illustrate reduced T cell cytokine responses to the superantigen SEB compared to non-atopic individuals. However, the antibody staining panel was limited and alterations would allow for more detailed interrogation of SEB-stimulated responses in atopy.

3.2.2 Antibody Staining Panel

Alterations to the antibody staining panel were made to include analysis of CD8 T cells and production of the cytokine IL17. In addition, a second antibody staining panel was developed to investigate TRegs and TCRVβ expression in SEB-induced T cell responses. T cell responses to superantigen are Vβ-specific, therefore we
investigated T cell expression of TCRVβ3 (the major specificity of relevance to SEB) and TCRVβ1 (not specific for SEB). The new antibody staining panels investigated were as follows:

Panel 1

CD3-AlexaFluor700
CD4-ECD
CD8-PerCP
CD154-PB
IFNγ-PeCy7
IL4-PE
IL10-APC
IL17-FITC

Panel 2

CD3-AlexaFluor700
CD4-ECD
CD8-PerCP
TCRVβ1-PE
TCRVβ3-FITC
FOXP3-AlexaFluor647
CD25-APC-H7

Fluorescence-minus-one (FMO) controls were performed to ensure the compatibility of fluorochromes and to eliminate antibody effects in cytokine expression. In all experiments, cells were gated on CD3⁺ single lymphocytes and at least 400,000 CD3 events were recorded per sample.
Figure 3.11. Flow cytometric gating strategy for *ex vivo* analysis of SEB-stimulated T cells
Figure 3.12. Flow cytometric gating strategy for \textit{ex vivo} analysis of SEB-stimulated TCR\(\beta\) specificities and CD25\(^+\)FOXP3\(^+\) regulatory T cells
3.2.3 **Gating Strategies**

For *ex vivo* analysis of allergen-specific T cell responses, CD4 T cells were gated from single lymphocytes (SSC vs. FSC, FSC-A vs. FSC-H). Activated cells were gated individually for each T cell cytokine (CD154, IFNγ, IL4, IL10 and IL17) and then Boolean gating combinations were computed (Figure 3.11). Cells were also gated for T\(_{\text{Regs}}\) using CD25 and FOXP3 and for TCRVβ specificities using TCRVβ1 and TCRVβ3 (Figure 3.12).

### 3.3 Summary of Assay Optimisation

#### 3.3.1 *Ex vivo* Allergen Stimulation

Optimisation of an assay to explore the *ex vivo* detection and phenotyping of CD154\(^+\) allergen-specific T cells identified a number of favourable conditions. PBMC were rested for 24 hours prior to allergenic stimulation; this manoeuvre was demonstrated to improve allergen-induced CD154 expression, possibly relating to the upregulation of MHC class II molecules on CD14\(^+\) monocytes. Additional costimulation was not required to identify CD154\(^+\) T cell responses. Collection of a significantly large number of CD4 events on the flow cytometer is required when investigating rare events and small responding cell frequencies. In future experiments, allergen stimulation was set up in triplicates and pooled before antibody staining to provide a significantly increased frequency of CD4 T cells. The antibody staining panel was optimised to improve the characterisation of the cellular responses to allergen.

#### 3.3.2 SEB Study

A chance observation demonstrated a reduced T cell cytokine response to the superantigen SEB in atopic individuals. The antibody panel in this assay was further developed to analyse T\(_{\text{Regs}}\) and TCRVβ specificities following SEB stimulation in allergy and health.
Chapter 4:  *Ex vivo* analysis of CD154+ T helper cells in atopic birch pollen-allergic and non-allergic individuals

4.1 Introduction

Activated T\(_{H2}\) cells play a key role in the initiation and maintenance of allergic diseases (74). Over the last decade, the T\(_{H2}\) paradigm has been refined to stress the importance of balance between T\(_{H1}\), T\(_{H2}\) and regulatory responses to allergens in determining the outcome (77). However, the nature of the relationship between these three T cell subsets in health and disease remains unclear.

The investigation of allergen-specific CD4 T cells at the single cell level is important for investigating the mechanism of tolerance and sensitisation to environmental allergens. Flow cytometry is an ideal method for the simultaneous enumeration and characterisation of allergen-specific T cells and its application to the *ex vivo* detection of allergen-specific responses became possible only recently, driven by MHC class II tetramer technology (230). However, tetramer-based methods are restricted to immunodominant epitopes in individuals with the appropriate HLA-DR haplotype; furthermore, co-staining for intracellular cytokine expression still requires *in vitro* expansion and/or mitogen stimulation. *Ex vivo* stimulation systems followed by the detection of activated T cells therefore remain attractive and may provide complementary information.

CD154 is a T cell activation marker transiently expressed upon ligation of the T cell receptor, therefore providing direct access to an antigen-specific population following *ex vivo* stimulation (241, 242). This method has been used to track changes during experimental ragweed immunotherapy (243) and has been recently evaluated alongside a dye dilution technique (244), however, detailed phenotyping of CD154+ T cells after *ex vivo* allergen stimulation has not been reported to date. This method reduces culture artefact, is compatible with intracellular and intranuclear cytokine staining, and may act as a complementary tool to MHC tetramer-based techniques.

Allergy to birch pollen results in an IgE-mediated inflammatory response of the nasal mucosa leading to sneezing, itching, nasal discharge and obstruction during the
birch pollen season (March to May). Birch pollen is the major tree allergen in Northern Europe and epidemiological studies indicate 10-20% of individuals in this area are sensitised to birch (153). T cell responses to birch allergens in health and disease have been well reported in the literature, making birch pollinosis an ideal model for investigation.

4.2 Aims

Using peripheral blood samples from birch-allergic and non-allergic individuals, this study sought to identify and phenotype CD154^+ T helper cells ex vivo following birch allergen stimulation.

4.3 Study Protocol

4.3.1 Laboratory Protocol

Techniques utilised for PBMC isolation, stimulation and antibody staining are fully detailed in Chapter 2 (Materials and Methods). PBMC were isolated from 23 birch-allergic individuals and 19 non-allergic healthy controls from citrated whole blood by density gradient centrifugation, washed twice in sterile PBS and resuspended in RPMI 1640 culture media (5x10^6/ml). PBMC were rested for 24 hours at 37°C and 5% humidified CO_2 before 16-hour stimulation with 500PNU/ml birch pollen extract (BPE) in the presence of BFA for the last 14 hours. Unstimulated PBMC were used as a negative control. PHA-stimulated PBMC were used as a positive control to ensure successful intracellular cytokine staining. Responding cells were identified using multiparametric flow cytometry following antibody staining for a variety of surface and intracellular markers to investigate numerous cell functions:

- **T helper cell type**: CD3-AlexaFluor700 and CD4-PerCP
- **Cytokine expression**: CD154-PB, IFNγ-PeCy7, IL4-PE and IL10-APC
- **Phenotypic analysis**: CD27-FITC and CD45RA-ECD
- **Regulatory T cells**: CD25-APC-Cy7, CD127-PB, GITR-PeCy5, CTLA-4-PE, TGF-β-PeCy7, IL10-PE, FOXP3-AlexaFluor488, GranzymeA-PerCP, GranzymeB-AlexaFluor647 and Perforin-PB

In addition, participant serum samples were frozen at -80°C before batch analysis for birch IgE and IgG4 concentration, using a Phadia 100 instrument, according to the manufacturer’s instructions (Thermofisher IDD, Uppsala, Sweden) in 16 non-allergic and 16 birch-allergic individuals.
4.3.2 *Flow Cytometric Analysis*

All results were gated on CD3$^+$CD4$^+$ single lymphocytes following a minimum collection of 400,000 CD4 events. Boolean gating combinations were computed for cytokine and cell marker analysis.

4.3.3 *Statistical Analysis*

The data distribution was non-parametric according to the D’Agostino and Pearson omnibus normality test. Median values were used for comparison throughout. All cell frequency values were background-corrected by subtraction of the unstimulated cell frequency from the stimulated cell frequency. Statistical significance was calculated using the two-tailed Mann-Whitney U test with a significance level of 0.05. Spearman rank correlation analysis was used to investigate statistical dependence between variables.

To calculate the $T_H2:T_H1$ ratio, the frequency of CD154$^+$IL4$^+$ $T_H2$ cells was divided by the frequency of CD154$^+$IFN$\gamma^+$ $T_H1$ cells. Where participants had no detectable CD154$^+$IFN$\gamma^+$ expression, a ratio was defined by allocating a predicted IFN$\gamma$ frequency value based on the regression equation from all responding participants of that subject group. To avoid bias, a similar method was applied where no CD154$^+$IL4$^+$ population was detected.

4.4 *Participant Characteristics*

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*Table 4.1.Key characteristics of participant groups*
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Table 4.2. Participant demographics

The key characteristics of the participant groups are summarised in Table 4.1. Birch-allergic participants (n=23) were recruited from the allergy clinic of the Royal Sussex County Hospital: all participants had a history of spring rhinitis and were SPT-positive to birch pollen extract. Eighteen of the birch-allergic participants (78%) also described typical symptoms of the pollen-food syndrome (Table 4.2).

An approximately age-matched non-allergic population (n=19) was recruited from Brighton and Sussex University staff and student volunteers: these participants had no history of atopic disease and demonstrated negative SPTs to common aeroallergens (cat dander, birch pollen, early-pollinating tree mix, mid-pollinating tree mix, grass pollen).

Atopic non-allergic (ANA) individuals (n=5) were recruited from the allergy clinic of the Royal Sussex County Hospital: these participants had a history of atopy and multiple positive SPTs to common aeroallergens but were SPT-negative to birch pollen extract with no history of spring rhinitis.

Sensitised individuals (n=5) were recruited from the allergy clinic of the Royal Sussex County Hospital: these participants were SPT-positive for birch pollen extract but did not have a history of spring rhinitis or clinical symptoms upon exposure to birch allergen.
Blood samples were taken a minimum of eight weeks outside the birch pollen season and study participants were confirmed to be free of rhinitis symptoms at the time of venupuncture.

4.1 Results

4.1.1 Detection of CD154, TH1, TH2 and TR1-Like Populations

A birch allergen-induced CD4⁺CD154⁺ T cell population was resolved in all study participants (Figure 4.1a, b). TH1 cells were defined as CD154⁺IFNγ⁺ and TH2 cells as CD154⁺IL4⁺ (Figure 4.1c). In addition, we detected a CD154⁺IL10⁺ population that did not co-express IFNγ or IL4 (Figure 4.1d, e) in excess of background signal; for brevity, these cells are referred to as TR1-like. The CD154⁺ T cell response represented 0.34% and 0.48% of the total CD4 T cell population in non-allergic and birch-allergic participants, respectively (p=0.07, Figure 4.1f).
Figure 4.1. Example of original data in one representative non-allergic individual. Dot plots illustrate CD4⁺ CD154 expression in (a) unstimulated (b) birch allergen-stimulated PBMC. (c) Identification of CD154⁺ IFNγ⁺ T_H1 population and CD154⁺ IL4⁺ T_H2 population. CD154⁺ IL10⁺ T cells did not co-express (d) IFNγ or (e) IL4. Plots illustrate log fluorescence intensity for all markers. Percentages represent background-corrected cytokine expression. (f) CD154⁺ T cells (% of CD4) in non-allergic and birch-allergic participants.
4.1.1 *T*<sub>R1</sub>-Like Responses

In non-allergic participants, the predominant T cell response to birch allergen comprised CD154<sup>+</sup>IL10<sup>+</sup> T<sub>R1</sub>-like cells, although IL10 was not detected universally. Non-allergic individuals exhibited a significantly higher frequency of T<sub>R1</sub>-like cells compared to birch-allergic participants (*p*=0.005, **Figure 4.2**). The presence of T<sub>R1</sub>-like cells in birch-allergic individuals was bimodally distributed such that fifteen of twenty-three participants (65%) did not produce detectable IL10, with the remaining eight participants illustrating T<sub>R1</sub>-like cells at a similar frequency compared to the non-allergic group.

T<sub>R1</sub>-like cells were not detected in any sensitised individuals and only one atopic non-allergic subject, probably reflecting small participant numbers in these groups.

The phenotype of the responding IL10<sup>+</sup> T cell population was further interrogated in three representative non-allergic participants for a variety of T<sub>Reg</sub> markers (**Figure 4.3**). Only a small proportion (23.5%) of IL10<sup>+</sup> T cells expressed FOXP3 and very few cells (3.5%) were consistent with a naturally occurring T<sub>Reg</sub> phenotype (FOXP3<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>). The IL10<sup>+</sup> cell population also expressed low levels of CTLA-4 (7.4%), GITR (18.7%), TGF-β (9.4%) and granzyme A (3.6%), with slightly higher expression of granzyme B (21.1%) and perforin (30.4%).

![Figure 4.2](image-url)  
**Figure 4.2.** Frequency of background-corrected CD154<sup>+</sup>IL10<sup>+</sup> per 10<sup>6</sup> CD4 T cells on a log scale.
Figure 4.3. Expression of regulatory T cell markers in birch allergen-induced IL10⁺ T cells in three representative non-allergic participants as a proportion of the total CD4⁺IL10⁺ cell population.
4.1.1 Th1 and Th2 Responses

The CD154^+IL4^+ Th2 response was greater in birch-allergic compared to non-allergic individuals (p<0.001, Figure 4.4a); however, IL4 expression was bimodally distributed in non-allergic participants: 53% did not express detectable levels of IL4 but 47% of participants expressed IL4 comparable to the birch allergic counterparts. In contrast, CD154^+IFNγ^+ Th1 cells were detected in most participants but there was no difference in expression between the non-allergic and birch-allergic groups (p=0.87, Figure 4.4b).

The Th2:Th1 ratio was calculated for each participant by dividing the frequency of CD154^+IL4^+ Th2 cells by the frequency of CD154^+IFNγ^+ Th1 cells. The Th2:Th1 ratio was significantly higher in birch-allergic participants compared to non-allergic controls in whom the ratio was zero or close to zero (p<0.001, Figure 4.4c). Receiver operating characteristics (ROC) analysis demonstrated that this Th2:Th1 ratio discriminates birch-allergic and non-atopic, birch-tolerant participants with 88% accuracy (p<0.001, confidence intervals 77% to 98%, sensitivity=100%, specificity=84%, Figure 4.4d). Slightly better discrimination between the study groups was achieved by use of the Th2:Th1+T1 ratio to 91% accuracy (p<0.001, confidence intervals 82% to 100%, sensitivity=100%, specificity=94%, Figure 4.4e, f).

The Th2:Th1 ratio in atopic non-allergic and sensitised individuals did not significantly differ compared to birch-allergic or non-allergic individuals (Figure 4.4g). ROC analysis demonstrated the Th2:Th1 ratio could potentially still discriminate birch-allergic individuals from non-atopic, birch-tolerant participants when both sensitised and atopic non-allergic groups are included in the birch-tolerant group, albeit with reduced sensitivity (Figure 4.4h). However, participant numbers in both atopic non-allergic and sensitised participant groups are very small and firm conclusions on this ratio as a diagnostic tool cannot be made.

PHA-stimulated PBMCs were used as an alternative and positive control to ensure successful intracellular cytokine staining in each experiment. There were no significant differences in PHA-induced Th1, Th2 or Tr1-like responses between non-allergic and birch-allergic participants (Figure 4.5).
Figure 4.4. (a) CD154^IL4^ T\textsubscript{H2} cells and (b) CD154^IFNγ^ T\textsubscript{H1} cells illustrated as the frequency of positive cells per 10^6 CD4 T cells on a log scale. (c) T\textsubscript{H2}:T\textsubscript{H1} ratio, (d) T\textsubscript{H2}:T\textsubscript{H1} ratio ROC curve, (e) T\textsubscript{H2}: T\textsubscript{H1}+T\textsubscript{R1} ratio and (f) T\textsubscript{H2}:T\textsubscript{H1}+T\textsubscript{R1} ratio ROC curve, (g) T\textsubscript{H2}:T\textsubscript{H1} ratio in atopic non-allergic (ANA) and sensitised individuals and (h) T\textsubscript{H2}:T\textsubscript{H1} ratio ROC curve incorporating atopic non-allergic and sensitised participants into the non-allergic group.
Figure 4.5. Background-corrected PHA-induced cytokine expression in non-allergic and birch-allergic individuals. (a) CD154 expression illustrated as percentage of total CD4 T cells on a log scale. (b) CD154$^{+}$IL4$^{+}$, (c) CD154$^{+}$IFNγ$^{+}$ and (d) CD154$^{+}$IL10$^{+}$ cells illustrated as the frequency of positive cells per $10^6$ CD4 T cells on a log scale.
4.1.1 Humoral Responses

Serum IgE and IgG4 concentration was analysed in sixteen birch-allergic and sixteen non-allergic individuals. In birch-allergic participants, a positive correlation was identified between the absolute concentration of birch-specific IgE and the frequency of CD154$^{+}$IL4$^{+}$ T\textsubscript{H}2 cells ($p=0.03$, $r=0.54$, Figure 4.6). However, a relationship was not found between IgE and the T\textsubscript{H}2:T\textsubscript{H}1 ratio.

IgG4 concentration was significantly higher in birch-allergic participants compared to non-allergic controls ($p=0.01$, Figure 4.7). The concentration of IgG4 displayed a positive correlation with the T\textsubscript{H}2:T\textsubscript{H}1 ratio in birch-allergic subjects, although this did not reach statistical significance ($p=0.087$) and did not correlate with any other parameter in this subject group. In non-allergic subjects, the concentration of IgG4 was very low and no correlations were identified with any cytokine expression.

Figure 4.6. Correlation of CD154$^{+}$IL4$^{+}$ T\textsubscript{H}2 cell frequency with IgE concentration in birch-allergic participants
Figure 4.7. IgG4 analysis. (a) IgG4 levels in non-allergic and birch-allergic individuals and (b) correlation between IgG4 concentration and $T_{H2}:T_{H1}$ ratio in birch-allergic participants.
4.1.1 A Subset of Non-Allergic Participants Expressing IL4

To further explore the bimodal distribution of T\(_H\)2 cell frequency in the non-allergic group, “IL4-responder” and “IL4-non-responder” subgroups were defined for comparison. The frequency of T\(_H\)1 and T\(_R\)1-like cells was significantly greater in the IL4-responder group compared to the IL4-non-responders; both groups maintained a similarly low T\(_H\)2:T\(_H\)1 ratio (Figure 4.8a-e). There was no difference in circulating birch IgG4 concentrations between IL4 responders and non-responders (Figure 4.8f).

4.1.2 A Subset of Birch-Allergic Participants Expressing IL10

Within the birch-allergic participants, expression of IL10 was bimodally distributed into two subsets: “IL10 responders” and “IL10 non-responders”. Further interrogation of cell and cytokine frequencies revealed no differences between the two subgroups (Figure 4.9a-e). However, IgG4 concentration was significantly increased in the IL10 responders compared to the IL10 non-responders group (p=0.03, Figure 4.9f).
Figure 4.8. Background-corrected birch allergen-induced cytokine expression in non-allergic IL4 responders and IL4 non-responders
Figure 4.9. Background-corrected birch allergen-induced cytokine expression in birch-allergic IL10 responders and IL10 non-responders
4.1.1 \( T_{H1}, T_{H2} \) and \( T_{R1} \)-Like Responses Correlate in Health

Within the non-allergic group, positive correlations were noted between the frequency of \( T_{H2} \) cells and the CD154 (\( p=0.02, r=0.54 \)), \( T_{H1} \) (\( p=0.02, r=0.54 \)) and \( T_{R1} \)-like response (\( p=0.004, r=0.63 \)), such that the expression of these cytokines increased together (Figure 4.10). Amongst allergic participants, the relationship between \( T_{H2} \) and \( T_{H1} \) cell frequency was weaker and non-significant (\( p=0.08, r=0.37 \)) and there was no dependence between the frequency of \( T_{H2} \) and \( T_{R1} \)-like lymphocytes.
Figure 4.10. Correlations of CD154, $T_{H1}$, $T_{H2}$ and $T_{H1}$-like responses in (a) non-allergic and (b) birch-allergic individuals.
4.1.1 **CD154, Th1 and Th2 Phenotypes**

Using surface staining for CD27 and CD45RA, CD154⁺ T cells were defined as naive (CD27⁺CD45RA⁺), central memory (CD27⁺CD45RA⁻), effector memory (CD27⁻CD45RA⁻) and revertant (CD27⁻CD45RA⁺). In non-allergic participants, the majority of CD154⁺, Th1 and Th2 responses were distributed between the naive and central memory compartments (**Figure 4.11**). By contrast, effector memory Th1 and Th2 cells were mainly confined to the birch-allergic group.

Surprisingly, a proportion of CD154⁺ cytokine⁺ T cells expressed both CD27 and CD45RA, consistent with a naive phenotype. Further staining in three non-allergic participants confirmed the co-expression of CD62L and CCR7 in these cells (**Figure 4.12**).

There were insufficient numbers of atopic non-allergic and sensitised individuals to investigate the phenotype of responding CD154⁺, Th1 and Th2 cell populations.
Figure 4.11. Phenotypes of (a) CD154, (b) T\textsubscript{H1} and (c) T\textsubscript{H2} T cell responses in non-allergic and birch-allergic individuals based on CD45RA and CD27 cell expression. Data are expressed as the percentage of positive cells of the total responding CD4 T cell population.
Investigations into allergen-specific lymphocytes in peripheral blood are problematic due to low frequency and the absence of a sensitive detection technique without the introduction of culture artefact or phenotypic modifications.

Flow cytometry is an ideal method for the simultaneous detection and characterisation of allergen-specific CD4 T cells, and its application to this field became possible only recently, with the introduction of MHC class II tetramer technology (230). Technical improvements in tetramer-based research, such as the use of Ii-key fused peptides and population enrichment, has led to enhanced sensitivity of this technique and major advancements in detection of allergen-specific T cell responses. However, this method requires identification and characterisation of immunodominant epitopes, HLA blood typing of participants and allergen-driven culture to support co-staining for intracellular cytokine expression. Consequently,
detection of activation markers may act as a complementary tool in the detection of allergen-specific T cells.

CD154 is a T cell activation marker transiently expressed upon ligation of the T cell receptor, thereby providing direct access to an antigen-specific cell population (241, 242). The technique has been used to track changes during experimental ragweed immunotherapy (243) and was recently evaluated alongside a dye dilution technique (244). Bonvalet *et al.* have compared the use of CD154 and tetramer co-staining using an Ii-key peptide culture system, but failed to demonstrate a significant correlation, although they were able to discriminate between functional, anergic or non-specifically activated T cells using the combination of techniques (246). Staining of the CD154+ cell populations with anti-TCR revealed profuse TCR on the cells but in all probability of other specificities (230), confirming that using CD154 alone may not be antigen-specific. However, in this paper, CD154 expression was detected following prolonged *in vitro* expansion and subsequent restimulation; by contrast, CD154 expression has only been demonstrated to detect an antigen-specific population after *ex vivo* stimulation, and prolonged culture was recently reported to abrogate the specificity of this marker (244). Nevertheless, the use of a CD154-based detection system is fast, easy to use, relatively inexpensive and is compatible with further intracellular and intranuclear cytokine staining.

The aim of this study was to identify and characterise birch allergen-specific CD4 T cells *ex vivo* based on CD154 expression. To date, this is the first detailed investigation into the characterisation and phenotype of T cell responses in allergy and health using CD154 detection. This study identified activated T\(_H\)1, T\(_H\)2 and T\(_R\)1-like lymphocytes in varying proportions in birch-allergic and non-allergic participants. Birch tolerance in non-allergic subjects was characterised by a close correlation between the frequencies of these T cell subsets, maintaining a low T\(_H\)2:T\(_H\)1 ratio and an IL10 response appropriate to the T\(_H\)1 and T\(_H\)2 response. This relationship was observed to be dysregulated in allergy, with the T\(_H\)2:T\(_H\)1 ratio maintained at higher levels and the IL10 response abrogated.

Using the CD154-detection based method; this study was able to resolve a CD154+ T cell population in all non-allergic and birch-allergic participants. Birch-allergic
participants demonstrated an increase in CD154$^+$ T cell frequency compared to their non-allergic counterparts, but this did not reach statistical significance.

The presence of CD154$^+$ T cells following ex vivo birch stimulation in non-allergic participants indicates tolerant individuals also elicit allergen-specific T helper cell responses to birch allergen, as confirmed in several tetramer-based studies for a number of allergens (78, 90, 91, 232, 235, 274). In studies failing to demonstrate allergen-specific T cell populations in non-allergic individuals (229, 231), this may relate to tetramer-based techniques detecting only a proportion of the T cell response. In support of this, it has been suggested MHC tetramers are unable to detect cell frequencies of less than 0.2% of the T cell population (224, 275). Much of the literature also confirms a higher frequency of allergen-specific T cells in allergic individuals compared to healthy controls (90, 229, 231, 232, 234, 235, 274), as identified in this study, albeit of weak significance.

Based on the expression of CD154 alone, the cell frequencies of 0.34% and 0.48% in non-allergic and birch-allergic individuals, respectively, is higher than the estimates of 1:100000 to 1:1000000 derived by back calculation for birch pollen stated in a Bet v 1 tetramer system outside of the pollen season (91). The most popular and successful method for analysing the frequency of antigen-specific T cells using MHC tetramers is antigen-driven expansion with subsequent back-calculations of the precursor frequency. However, this technique can cause underestimations of the frequency of antigen-specific precursor cells without accounting for apoptosis or those failing to proliferate in response to antigenic stimulation (276).

MHC class II tetramers utilise recombinant allergen molecules compared to the native allergen extract used in this study, which perhaps stimulates a wider variety of CD4 T cells because they contain a larger number of allergenic epitopes. Furthermore, the affinity of the TCR for the MHC:peptide complex can affect the tetramer avidity. Therefore, peptides with low avidity, such as allergens, can be difficult to detect using MHC tetramers (277). For example, De Long et al. hypothesised Ara h 1-specific T cells in non-allergic individuals were of lower avidity and therefore more difficult to detect compared to those of allergic individuals (274). Therefore, further research into the detection of low avidity binding would improve investigation of the full allergen-specific T cell repertoire.
T cell responses can involve more than one type of epitope and so MHC tetramers may fail to identify a proportion of the T cell response. For example, De Long et al. postulated that MHC tetramers cannot analyse the full allergen-specific repertoire because individual antigenic epitopes are used from a single protein restricted by a single HLA (274). This limited analysis may identify fewer allergen-specific lymphocytes compared to the CD154 detection method using native allergen extracts with multiple allergenic components each containing multiple epitopes without the restriction of HLA haplotypes. This has also been described as a major drawback of MHC tetramers by several review papers (228, 277).

Allergen-specificity has not been formally demonstrated in this instance and it is likely the CD154\(^+\) T cell population are enriched for allergen-specific T cells but not entirely allergen-specific. The major birch pollen allergen Bet v 1 belongs to the PR-10 panallergen family. The T cell response to native birch allergen is therefore likely to include cells responding to plant-derived homologs that represent perennial rather than seasonal allergens, and could account for the high CD154\(^+\) cell frequency. The single-epitope detection of tetramer-based assays clearly differs markedly in this respect. Based on the co-expression of CD154 and cytokines, these results relate to humoral responses to birch allergen and are consistent with previous reports. The expression of CD154 together with any of IL4, IFN\(\gamma\) or IL10 was observed at a frequency of 1:2000 (birch-allergic) and 1:5000 (non-allergic) CD4 T cells, and perhaps represents a more realistic estimate of birch pollen-specific T cell frequency outside of the pollen season.

Activated T\(_{H2}\) cells are known to play an important role in the initiation and maintenance of allergic disease (74) and substantial evidence now suggests that tolerance to airborne allergens is an active process dependent upon a balanced T cell response that may include T\(_{H1}\), T\(_{H2}\) and T\(_{Reg}\) responses (77). This study revealed strong T\(_{H2}\)-polarised immune responses to birch allergen in birch-allergic individuals, consistent with earlier studies of cytokine profiles in birch-allergic individuals (77, 144, 153). However, there was no difference in the frequency of T\(_{H1}\) cells between birch-allergic and non-allergic individuals, indicating a mixed cytokine profile as previously described for a number of allergens (78, 90, 225). The predominant response in tolerant individuals consisted of a CD154\(^+\)IL10\(^+\) T\(_{R1}\)-like cell population.
A close relationship between Th1, Th2 and Tr1-like cells was first reported by Akdis et al. (77) using cytokine capture technology. All three T cell subsets were represented in both the allergic and non-allergic populations: during other in vitro experiments, allergen-specific Th2 responses were further enhanced by inhibiting the function of Tr1 lymphocytes or increasing Th2 cell frequency. The present study supports the paradigm that the relationship between these T cell subsets rather than absolute numbers may determine B cell IgE isotype switching. The ratio between Th2 and Th1 cell frequency after ex vivo birch stimulation was a key parameter that differentiated allergic and non-allergic participants. Regardless of the frequency of Th2 cells, the Th2:Th1 ratio was maintained at low levels in non-allergic individuals compared to the birch-allergic group. By ROC analysis, the Th2:Th1 ratio in this system was 88% accurate in discriminating the two participant groups, with sensitivity of 100% and specificity of 84%. By contrast, Th2 responses illustrated considerable overlap between the allergic and non-allergic groups. A high Th2:Th1 ratio is well known to be associated with birch pollinosis (153), and has been shown to decrease after birch-specific immunotherapy (278), but this is perhaps the first description of accurate discrimination between allergic and non-allergic individuals using a T cell based assay. The findings suggest that such methods may find diagnostic utility in an allergy context, for example food or drug sensitivity. However, discrimination was weaker between individuals truly allergic and those with asymptomatic sensitisation to birch pollen allergen and those with an atopic phenotype who were birch-IgE negative. It is probable with larger participant numbers, this ratio will no longer discriminate these groups.

Interestingly, the Th2 response to birch allergen in non-allergic participants displayed marked bimodal distribution. A significantly higher frequency of Th1 lymphocytes was identified in the IL4-responder subgroup, maintaining a low Th2:Th1 ratio, in addition to an appropriately greater Tr1-like response, all of which presumably favours tolerance to birch allergen in the face of a Th2 response. Birch-specific IgG4 levels were similarly low in both groups, although it would be of interest to repeat this measurement during the birch pollen season. Platts-Mills et al. described a “modified Th2 response” characterised by IL4, IL10 and the production of cat dander-specific IgG4, in a group of cat-tolerant, heavily exposed individuals (279, 280). Although this study is reminiscent of this “modified Th2 response”, non-
allergic individuals were not exposed to birch pollen at the time of venupuncture and sampling of the non-allergic IL4-responder population occurred throughout the year, without clustering after the pollen seasons. However, as previously stated, the major birch pollen allergen Bet v 1 belongs to the PR-10 panallergen family and the T cell response to native birch allergen is therefore likely to include cells responding to plant-derived homologs, representing perennial rather than seasonal allergens.

Multiple *in vitro* and *in vivo* studies have demonstrated a mixed response to allergens that is not consistent with the T\(_{H1}\) or T\(_{H2}\) polarisation definitions, in allergy and health, respectively. Investigations into the role of regulatory T cell biology in the 1990’s challenged the concept of the T\(_{H1}:T_{H2}\) paradigm and there is now increasingly more evidence for a role of T\(_{Reg}\) and IL10 in tolerance to allergens (120, 143). T\(_{Reg}\) that secrete IL10 have a confirmed role in tolerance induction during natural high-dose allergen exposure (133) and allergen immunotherapy, both conventional (130, 137) and experimental (281). Multiple studies have demonstrated a significantly higher frequency of IL10\(^{+}\) allergen-specific T cells in healthy individuals compared to allergic individuals (77, 125, 141, 145).

This study illustrated the predominant T cell response to birch allergen in the non-allergic group comprised CD154\(^{-}\)IL10\(^{+}\) lymphocytes that were negative for T\(_{H1}\) and T\(_{H2}\) cytokines. This is consistent with results generated in an *ex vivo* stimulated, cytokine capture system (77) in addition to more contemporary reports (78, 79, 91). IL10 is known to modulate the function of numerous cells that are involved in allergic inflammation (125), but the exact mechanisms by which IL10-secreting T cells exert their regulatory function is not entirely clear. To further investigate the birch-induced IL10 T cell response, expression of various T\(_{Reg}\) markers was explored. Only a small proportion of IL10\(^{+}\) T cells were consistent with a naturally occurring T\(_{Reg}\) phenotype (FOXP3\(^{+}\)CD25\(^{+}\)CD127\(^{Low}\)). Phenotyping of allergen-induced IL10\(^{+}\) cell populations has been performed by Van Overtvelt and colleagues, in which they identified comparable FOXP3 and GITR expression to this study (and higher CTLA-4 expression) within the CD4 T cell population in non-allergic individuals, although this was not within the IL10\(^{-}\) cell subset (91). The allergen-specific IL10-secreting cells isolated by Akdis *et al.* (77) appeared to operate by a mechanism that was dependent upon PD-1 and CTLA-4 in addition to IL10. Circulating IL10-secreting T cells responding to islet cell antigens have been
identified in healthy non-diabetic blood subjects (282), and operate by perforin and granzyme-mediated killing of APCs in an antigen-dependent fashion (283). It would be interesting to determine whether environmental allergens are subject to similar regulatory mechanisms given the moderate expression of these molecules by T\textsubscript{R1}-like cells in this study.

It is notable that IL10 production was bimodally distributed in the birch-allergic population. The frequencies of T\textsubscript{H1}, T\textsubscript{H2} and T\textsubscript{R1}-like cells after \textit{ex vivo} birch stimulation were similar between these groups, but the levels of birch-specific IgG4 were greater within the IL10-responders subgroup. The production of allergen-specific IgG4 is known to be IL4 and IL10-dependent (284) and has attracted intense interest over recent years for its role in the mechanisms of immunotherapy (285). However, the clinical significance of this observation is unclear, as the two allergic subpopulations did not appear to differ in disease phenotype.

Detailed analysis of these cytokine responses revealed significant correlations between T\textsubscript{H2}, T\textsubscript{H1} and T\textsubscript{R1}-like responses to birch allergen after \textit{ex vivo} stimulation amongst healthy subjects. By contrast, the relationship between these T cell subsets was dysregulated in the birch allergic group, with abrogation of the IL10 response and a weaker, non-significant correlation between T\textsubscript{H2} and T\textsubscript{H1} cell frequency at an increased T\textsubscript{H2}:T\textsubscript{H1} ratio. This mathematical relationship between the frequency of IL4 expression and IFN\gamma/IL10 responses in health further supports what Akdis \textit{et al.} described as a “fine balance between T\textsubscript{H1}, T\textsubscript{H2} and T\textsubscript{R1}-like responses” (77), and a switch in this cytokine profile may play an important role in the allergic phenotype.

This study also observed the concentration of circulating birch-specific IgE correlated closely with the frequency of T\textsubscript{H2} cells rather than the T\textsubscript{H2}:T\textsubscript{H1} ratio, suggesting that the absolute T\textsubscript{H2} lymphocyte frequency represents an important parameter once sensitisation has occurred. Crack \textit{et al.} recently used a cultured ELISPOT system to demonstrate a correlation between the frequency of responding cells and cat allergen IgE grade (286). It seems likely that the advent of more sensitive T cell assays will further refine our understanding of the relationship between humoral and T cell responses to allergens.

In a small proportion of participants, CD154\textsuperscript{+} T cells lacked expression of IFN\gamma, IL4 or IL10. As discussed, this may describe non-specifically activated T cells, or
perhaps are indicative of an activated T cell of different effector cell function, such as T\textsubscript{H}9 or T\textsubscript{H}17. It would be interesting to extend the cytokine panel to investigate a more detailed T cell repertoire.

This study also investigated the phenotype of responding CD154\textsuperscript{+} and CD154\textsuperscript{+}cytokine\textsuperscript{+} T cell populations following \textit{ex vivo} birch stimulation using the expression of cell surface markers CD27 and CD45RA. Subsets were defined as naive (CD27\textsuperscript{+}CD45RA\textsuperscript{+}), central memory (CD27\textsuperscript{+}CD45RA\textsuperscript{+}), effector memory (CD27\textsuperscript{−}CD45RA\textsuperscript{−}) and revertant (CD27\textsuperscript{−}CD45RA\textsuperscript{−}). Naive T cells are small, resting T cells released from the thymus that have not yet encountered antigen. Central memory T cells have a higher sensitivity to antigens compared to naive T cells and up-regulate significantly more CD154 (287). Central memory T cells are the predominant subset in peripheral blood CD4 T cells and differentiate into effector T cells with subsequent vast secretion of cytokines. Effector memory T cells are characterised by rapid effector function (287) and no longer express CD62L and CCR7, compared to naive and central memory counterparts, two markers important for migration of T cells into secondary lymphoid organs. Little is known about the function of CD27\textsuperscript{−}CD45RA\textsuperscript{+} revertant T cells but it has been hypothesised these are multifunctional but short-lived effector memory-like T cells (288). CD154\textsuperscript{+}IFN\gamma\textsuperscript{+} T\textsubscript{H}1 and CD154\textsuperscript{+}IL4\textsuperscript{+} T\textsubscript{H}2 cells were also separately phenotyped into the naive, central memory, effector memory and revertant T cell subsets. This type of investigation has not been performed in such detail and information regarding the phenotype of T\textsubscript{H}1 and T\textsubscript{H}2 responses to allergens is a novel aspect of this study.

In keeping with tetramer-based studies with seasonal allergens, responding cells were predominantly CD45RA-negative, CD27-positive, consistent with an early-differentiated memory phenotype. However, the frequency of CD27-negative T cells within the T\textsubscript{H}1 and T\textsubscript{H}2 compartments was significantly lower in the birch-allergic participants, supporting the view that T cell responses in birch allergy comprise a higher proportion of late-differentiated memory T cells. Using an \textit{ex vivo} tetramer-based detection system to interrogate alder pollen-specific T cells, Wambre and colleagues recently demonstrated that a CD27-negative, CRT\textsubscript{H}2-positive allergen-specific population was specific to allergy, compared to the early-differentiated phenotype of non-allergic subjects that produced IFN\gamma and IL4; furthermore, specific immunotherapy selectively reduced the frequency of this population (79). Overtvelt
et al. similarly defined tetramer-positive T cells as effector memory for birch-allergic individuals compared to central memory for non-allergic individuals (91), although this study used a prolonged culture step that may lead to unrepresentative T cell analysis. It is notable that T cell responses to perennial allergens have been described as early-differentiated in both allergic and non-allergic individuals (90, 246). As previously discussed, Bet v 1 may have some characteristics of a perennial allergen in this assay system, possibly explaining the partial agreement with studies utilising tetramer technology.

It was interesting to observe that effector cytokines were readily detected in apparently naive T cells, supported by co-staining for CD62L and CCR7. Research into naive antigen-specific CD4 T cell responses is limited and previous studies have only been able to demonstrate the presence of a naive antigen-specific population but it has not provided information on the function of these cells. It is notable that IL4 production is seen in multiple murine models and one human model within the naive CD4 T cell population (289-293) which may be the initial source during priming of T\textsubscript{H2} cells (294). It has been hypothesised that allergens may have an intrinsic ability to induce an IL4 response related to toll-like receptor (TLR) 4 interactions (294). For example, house dust mite allergen ligation to TLR4 on lung epithelial cells leads to dendritic cell activation and differentiation of T\textsubscript{H2} cells (295). Der p 2 is structurally homologous to the MD2 LPS-binding component of the TLR4 receptor, and therefore is able to induce allergic inflammation in an allergic asthma model in a TLR4-dependent manner (296). If allergens do in fact have an intrinsic ability to induce IL4 production in both allergic and non-allergic participants, perhaps T\textsubscript{Reg} mechanisms in non-allergic individuals down-regulate this IL4 response but fail to do so in allergic disease. Further investigation into both TLR4 ligation of allergens and T\textsubscript{Reg} function may elucidate this theory. In addition, previous reports have described the ability of naive T cells to acquire memory characteristics, including up-regulation of activation markers and/or effector cytokine activity (297). On this basis, the apparently naive population may actually represent a relatively undifferentiated memory population, capable of secreting cytokines, but maintaining a naive-like phenotype (298).

The investigation of both atopic non-allergic and sensitised individuals is a novel aspect of this study. Atopic non-allergic individuals have a history of allergic
disease, multiple sensitisations and positive SPTs to a variety of common aeroallergens, but a negative SPT to birch allergen extract. Sensitised individuals exhibit a positive SPT to birch allergen extract but do not display clinical symptoms to the allergen. Little is known about these individuals, and their differences in T cell responses compared to birch-allergic or birch-tolerant groups. Macaubas et al. identified very few rye grass-specific lymphocytes in atopic non-allergic participants with an insufficient number of cells for cytokine analysis (229). The findings of this study indicate both atopic non-allergic and sensitised participants may have a similar T cell profile to birch-allergic individuals with TH2-biased immune responses and low IL10 expression, although participant numbers are small. This may suggest the manifestation of clinical symptoms to birch allergen and severity of atopic disease does not relate to the T cell cytokine response. Numerous other factors could be involved such as B cells, defective regulatory mechanisms or differences in mast cell and eosinophil function, to name a few. The skewed TH2 immune response of atopic non-allergic participants compared to the non-allergic TH1/TH1-like response suggests an intrinsic tendency of allergen-specific TH2 cell involvement in the atopic phenotype.

4.3 Conclusions

In summary, this study is the first description of CD154+ T cell responses to birch allergen using an ex vivo stimulation system. This assay explored the fine balance between allergen-specific TH1, TH2 and TR1-like responses, originally proposed by Akdis and colleagues (77), producing further support for the paradigm that the relationship between these parameters defines the humoral response to allergens. It seems likely that the introduction of more sensitive T cell assays will further refine understanding of the relationship between humoral and T cell responses to allergens.

This study also illustrated the suitability of CD154 as a marker of birch allergen-specific CD4 T cells. This technique may be as successful as MHC class II tetramers with similar findings in responding CD154+cytokine+ cell frequency, cytokine responses and phenotypic analysis. Although allergen-specificity of the CD154+cytokine+ T cell population was not formally demonstrated, the ability of the assay to predict humoral responses to allergens and concordance with results derived from different methodologies is supportive of this view. The use of a CD154-based
detection system is both cheaper and easier, and is able to analyse a larger range of epitope responses without haplotype restrictions associated with tetrarners and \textit{in vitro} expansion is not a requirement for cytokine analysis.
Chapter 5:  *Ex vivo* analysis of CD154⁺ T helper cells in cat-allergic, grass-allergic and non-allergic individuals

5.1 Introduction

Investigation of birch-specific CD154⁺ T cells revealed an important balance between T₁h₁, T₁h₂ and T₁r₁-like responses in health, providing supportive evidence for the fundamental work of Akdis and colleagues (77). This study was the first detailed description of CD154⁺ T cell responses to allergens using an *ex vivo* stimulation system, illustrating CD154 as a suitable marker for the detection and characterisation of allergen-specific T cells.

Although allergen-specificity of the CD154⁺cytokine⁺ T cell population was not formally demonstrated, the ability of the assay to predict humoral responses to allergens and concordance with results derived from different methodologies is supportive of this view. It is highly likely the CD154⁺ T cell population is enriched for allergen-specific T cells, but not entirely allergen-specific. The frequency of CD154⁺ T cells following *ex vivo* birch allergen stimulation was higher than the estimates described in previous tetramer-based literature (91). The major birch pollen allergen Bet v 1 belongs to the PR-10 panallergen family. Therefore, the T cell response to native birch allergen is likely to include cells responding to plant-derived homologs that represent perennial rather than seasonal allergens. This may account for the high CD154⁺ T cell frequency.

For that reason, it would be interesting to investigate the differences in cytokine profiles and phenotype of CD154⁺ T cell responses between true seasonal and perennial allergens in more detail.

This chapter focuses on the *ex vivo* identification and phenotyping of CD154⁺ T helper cells following stimulation with cat-dander (perennial) and grass pollen (seasonal) native allergens in cat-allergic, grass-allergic and non-allergic individuals.

Allergy to grass pollen is an IgE-mediated inflammatory response of the nasal mucosa with typical symptoms including sneezing, itching, nasal discharge and obstruction. Grass pollen allergy is present in the summer (mainly June to August) and grass allergens remain the most common contributor to allergic seasonal
rhinconjunctivitis (151). Major allergenic components within the native grass pollen extracts include Phl p 1 and Phl p 5 of Timothy grass.

Allergy to cats is one of the most common animal allergies in the UK, with allergenic components present in cat dander, pelt, saliva, serum and urine. The major allergenic component, Fel d 1, is responsible for 90% of IgE-mediated immune responses in cat allergy. Sensitisation to domestic pets is an important risk factor in the development of asthma, although pet ownership is not a prerequisite for sensitisation. Therefore, we also investigated non-allergic individuals who are tolerant to cat allergen but highly exposed (pet owners).

5.2 Aims

Using peripheral blood samples from cat-allergic, grass-allergic and non-allergic individuals, this study sought to identify and phenotype CD154⁺ T helper cells ex vivo following cat and grass allergen stimulation.

5.3 Study Protocol

5.3.1 Laboratory Protocol

Techniques utilised for PBMC isolation, stimulation and antibody staining are fully detailed in Chapter 2 (Materials and Methods). Briefly, PBMC were isolated from grass-allergic, cat-allergic and non-allergic individuals from citrated whole blood by density gradient centrifugation, washed twice in sterile PBS and resuspended in RPMI 1640 culture media (5x10⁶/ml). PBMC were rested for 24 hours at 37°C and 5% humidified CO₂ before 16-hour stimulation with 500PNU/ml GPE or CDE in the presence of BFA for the last 14 hours. Unstimulated PBMC were used as a negative control. PHA-stimulated PBMC were used as a positive control to ensure successful intracellular cytokine staining. Responding cells were identified using multiparametric flow cytometry following antibody staining for a variety of surface and intracellular markers to investigate numerous cell functions:

- **T helper cell type**: CD3-AlexaFluor700, CD4-PerCP and CD25-APC-Cy7
- **Cytokine expression**: CD154-PB, IFNγ-PeCy7, IL4-PE and IL10-APC
- **Phenotypic analysis**: CD27-FITC and CD45RA-ECD
5.3.2 Flow Cytometric Analysis

All results were gated on CD3$^+$CD4$^+$ single lymphocytes following a minimum collection of 400,000 CD4 events. All data was background-corrected. Boolean gating combinations were computed for cytokine and cell marker analysis.

5.3.3 Statistical Analysis

The data distribution was non-parametric according to the D’Agostino and Pearson omnibus normality test. Median values were used for comparison throughout. Statistical significance was calculated using the two-tailed Mann-Whitney U test with a significance level of 0.05. Spearman rank correlation analysis was used to investigate statistical dependence between variables. The T$_{H2}$:T$_{H1}$ ratio was calculated as previously described (Chapter 4).

5.4 Participant Characteristics

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<th>Non-Allergic Highly-Exposed (Cat Control)</th>
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<th>Non-Allergic (Grass Control)</th>
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</table>

Table 5.1. Participant demographics

The clinical characteristics and sensitisations of participants are summarised in Table 5.1. Cat-allergic (n=20) and grass-allergic (n=19) participants were recruited from the allergy clinic of the Royal Sussex County Hospital: all grass-allergic participants had a history of spring rhinitis and were SPT-positive to grass pollen extract. All cat-allergic participants had a history of cat-induced rhinitis and were SPT-positive to cat dander extract.
Two approximately age-matched non-allergic populations (n=18) were recruited from Brighton and Sussex University volunteers: these participants had no history of atopic diseases and demonstrated negative SPTs to common aeroallergens (cat dander, birch pollen, early-pollinating tree mix, mid-pollinating tree mix, grass pollen). In addition, six highly exposed non-allergic cat-owner individuals were also recruited from university volunteers with no history of atopic disease and negative SPTs to common aeroallergens.

Atopic non-allergic individuals for cat allergen (n=11) and grass pollen allergen (n=3) were recruited from the allergy clinic of the Royal Sussex County Hospital: these participants had a history of atopy and multiple positive SPTs to common aeroallergens but were SPT-negative to either grass pollen or cat dander extract.

Sensitised individuals for cat allergen (n=4) and grass pollen allergen (n=3) were recruited from the allergy clinic of the Royal Sussex County Hospital: these participants were SPT-positive for cat or grass allergen but did not exhibit clinical symptoms upon exposure to the allergen.

Blood samples were taken a minimum of four weeks outside the grass pollen season and study participants were confirmed to be free of rhinitis symptoms at the time of venupuncture.

5.1 Results

5.1.1 Detection of CD154, T\(_{H1}\), T\(_{H2}\) and T\(_{R1}\)-like Responses

A cat and grass allergen-induced CD\(_4^+\)CD154\(^+\) T cell population was resolved in all study participants (Figure 5.1a, b, Figure 5.2a, b). As with the birch-induced response, T\(_{H1}\) cells were defined as CD154\(^+\)IFN\(\gamma\)^+ and T\(_{H2}\) cells as CD154\(^+\)IL4\(^+\) (Figure 5.1c, Figure 5.2c). In addition, a CD154\(^+\)IL10\(^+\) T\(_{R1}\)-like cell population was detected (Figure 5.1d, e, Figure 5.2d, e).

The cat allergen-induced CD154\(^+\) T cell response represented 0.58%, 0.27% and 0.74% of the total CD4 T cell population in non-allergic, highly exposed non-allergic and cat-allergic individuals, respectively (Figure 5.1f). Cat-allergic individuals exhibited a significantly higher frequency of CD154\(^+\) T cells compared to highly exposed individuals (p=0.02), but no difference was evident compared with their non-allergic counterparts (p=0.29). Non-allergic participants displayed a higher
frequency of CD154+ T cells compared to those highly exposed individuals, however this did not reach statistical significance (p=0.07).

The frequency of T cells responding to grass pollen allergen was lower compared to cat allergen, representing 0.11% and 0.33% of all CD4 T cells in non-allergic and grass-allergic individuals, respectively. Grass-allergic individuals exhibited a significantly higher frequency of CD154+ T cells compared to non-allergic controls (p=0.01, Figure 5.2f).
Figure 5.1. Example of original data in one representative non-allergic individual following *ex vivo* cat allergen stimulation. Dot plots illustrate CD4⁺ CD154 expression in (a) unstimulated (b) cat allergen-stimulated PBMC. (c) Identification of CD154⁺IFNγ⁺ T₉₁ population and CD154⁺IL4⁺ T₉₂ population. CD154⁺IL10⁺ T₉₁-like cells did not co-express (d) IFNγ or (e) IL4. Plots illustrate log fluorescence intensity for all markers. Percentages represent background-corrected cytokine expression. (f) CD154⁺ T cells (% of CD4) in non-allergic and cat-allergic participants.
Figure 5.2. Example of original data in one representative non-allergic individual following \textit{ex vivo} grass allergen stimulation. Dot plots illustrate CD4$^+$ CD154 expression in (a) unstimulated (b) grass allergen-stimulated PBMC. (c) Identification of CD154$^+$ IFN\gamma$^+$ T\textsubscript{H}1 population and CD154$^+$ IL4$^+$ T\textsubscript{H}2 population. CD154$^+$ IL10$^+$ T\textsubscript{R}1-like cells did not co-express (d) IFN\gamma or (e) IL4. Plots illustrate log fluorescence intensity for all markers. Percentages represent background-corrected cytokine expression. (f) CD154$^+$ T cells (% of CD4) in non-allergic and cat-allergic participants.
5.1.1  $T_{h1}$ and $T_{h2}$ Responses

The CD154$^+$IL4$^+$ $T_{h2}$ response was greater in cat-allergic individuals compared to non-allergic controls ($p=0.007$, Figure 5.3a).

The frequency of $T_{h2}$ cells was also higher in grass-allergic individuals compared to non-allergic controls ($p=0.006$, Figure 5.3b). However, IL4 expression was bimodally distributed in non-allergic participants following grass allergen stimulation, such that eight of eighteen participants (44%) did not produce detectable IL4, with the remaining ten participants illustrating $T_{h2}$ cells at a similar frequency to the grass-allergic participant group (Figure 5.3b).

CD154$^+$IFNγ$^+$ $T_{h1}$ cells exhibited no difference in expression between the cat-allergic and non-allergic group ($p=0.23$, Figure 5.3c).

The frequency of $T_{h1}$ cells was also similar between grass-allergic individuals and non-allergic controls ($p=0.38$, Figure 5.3d). In grass-allergic subjects, IFNγ expression was also bimodally distributed such that eight of nineteen participants (42%) did not produce detectable IFNγ, with the remaining eleven participants illustrating a $T_{h1}$ cell frequency similar to that of the non-allergic group (Figure 5.3d).

The $T_{h2}$:$T_{h1}$ ratio was calculated for each participant by dividing the frequency of CD154$^+$IL4$^+$ $T_{h2}$ cells by the frequency of CD154$^+$IFNγ$^+$ $T_{h1}$ cells. The $T_{h2}$:$T_{h1}$ ratio was significantly higher in both cat ($p<0.001$) and grass-allergic ($p<0.001$) participants compared to non-allergic controls in whom the ratio was zero or close to zero (Figure 5.3e, f). In addition, non-allergic individuals heavily exposed to cat allergen exhibited a significantly higher $T_{h2}$:$T_{h1}$ ratio compared to non-allergic individuals without high exposure ($p=0.006$, Figure 5.3e).

Receiver operating characteristics (ROC) analysis demonstrated that this $T_{h2}$:$T_{h1}$ ratio discriminated cat-allergic and non-allergic participants with 93% accuracy ($p<0.001$, confidence intervals 74% to 100%, sensitivity=100%, specificity=88%, Figure 5.3g), and grass-allergic and non-allergic participants with 94% accuracy ($p<0.001$, confidence intervals 84% to 100%, sensitivity=95%, specificity=88%, Figure 5.3h).
Figure 5.3. (a, b) CD154⁺IL4⁺TH2 and (c, d) CD154⁺IFNγ⁺TH1 cells illustrated as the frequency of positive cells per 10⁶ CD4 T cells on a log scale, (e, f) TH2:TH1 ratio and (g, h) TH2:TH1 ratio ROC curve following cat and grass allergen stimulation.
PHA-stimulated PBMCs were used as a positive control to ensure successful intracellular cytokine staining in each experiment. There were no differences in PHA-induced T<sub>h</sub>1, T<sub>h</sub>2 or T<sub>r</sub>1-like responses between non-allergic, grass-allergic and cat-allergic participants (Figure 5.4).

Figure 5.4. Background-corrected PHA-induced cytokine expression in non-allergic, cat-allergic and grass-allergic individuals. (a) CD154 expression illustrated as percentage of total CD4 T cells on a log scale. (b) CD154<sup>+</sup>IFNγ<sup>+</sup> T<sub>h</sub>1, (c) CD154<sup>+</sup>IL4<sup>+</sup> T<sub>h</sub>2 and (d) CD154<sup>+</sup>IL10<sup>+</sup> T<sub>r</sub>1-like cells illustrated as the frequency of positive cells per 10<sup>6</sup> CD4 T cells on a log scale.
5.1.1 $T_{reg}$-Like Responses

A CD154$^+$IL10$^+$ $T_{reg}$-like cell population was identified in the majority of cat-allergic, grass-allergic and non-allergic individuals. However, the frequency of $T_{reg}$-like cells did not differ in cat-allergic ($p=0.81$) or grass-allergic ($p=0.41$) participants compared to non-allergic healthy controls (Figure 5.5). In both cat-allergic and grass-allergic participants, the expression of IL10 was bimodally distributed.

![Graph](image)

**Figure 5.5.** Frequency of background-corrected CD154$^+$IL10$^+$ $T_{reg}$-like cells per $10^6$ CD4 T cells on a log scale in (a) cat-allergic and (b) grass-allergic individuals.
5.1.1 Bimodal Distribution of IL4 in Non-Allergic Participants

To further explore the bimodal distribution of Th2 cell frequency in the non-allergic group following grass allergen stimulation, “IL4-responder” and “IL4-non-responder” subgroups were defined for comparison. The IL4 responders exhibited a significantly higher frequency of CD154+ T cells compared to IL4-non-responders (p=0.04, Figure 5.6), however no other differences in cytokine expression were identified between the two subpopulations. The Th2:Th1 ratio was slightly higher in the IL4 responder participants, although this did not reach statistical significance (p=0.05).
Figure 5.6. Background-corrected grass allergen-induced cytokine expression in non-allergic IL4 responders and IL4 non-responders
5.1.1 Bimodal Distribution of IFNγ in Grass-Allergic Participants

To further explore the bimodal distribution of TH1 cell frequency in the grass-allergic participants, “IFNγ-responder” and “IFNγ-non-responder” subgroups were defined for comparison. The IFNγ-non-responders also exhibited a significantly reduced frequency of TR1-like cells (p=0.004, Figure 5.7). Those individuals producing IFNγ in response to grass allergen stimulation demonstrate a significantly higher TH2:TH1 ratio compared to the non-responders (p=0.02, Figure 5.7).
Figure 5.7. Background-corrected grass allergen-induced cytokine expression in allergic IFNγ responders and IFNγ non-responders
### 5.1.1 Bimodal Distribution of IL10 in Allergic Participants

Within the cat and grass allergic individuals, expression of IL10 was bimodally distributed. To further explore this phenomenon, “IL10 responder” and “IL10 non-responder” subgroups were defined for comparison.

Further interrogation of cell and cytokine frequencies revealed IL10-non-responders also illustrated a significantly reduced frequency of $\text{T}_{\text{H}1}$-like cells following both cat (p=0.03, Figure 5.8) and grass allergen (p=0.03, Figure 5.9) stimulation, but no differences were revealed in the $\text{T}_{\text{H}2}:\text{T}_{\text{H}1}$ ratio between the subgroups.
Figure 5.8. Background-corrected cat allergen-induced cytokine expression in cat-allergic IL10 responders and IL10 non-responders
Figure 5.9. Background-corrected grass allergen-induced cytokine expression in grass-allergic IL10 responders and IL10 non-responders
5.1.1 *The Relationship between T\(_H\)1, T\(_H\)2 and T\(_R\)1-Like Responses*

Following *ex vivo* grass allergen stimulation, non-allergic participants exhibited positive correlations between the frequency of T\(_H\)2 cells and the T\(_H\)1 (p=0.003) and T\(_R\)1-like response (p=0.01), such that the expression of these cytokines increased together (Figure 5.10). In addition, positive correlations were noted between the frequency of T\(_H\)2 cells and the T\(_H\)1 (p=0.002) and T\(_R\)1-like (p=0.003) response in non-allergic participants following *ex vivo* stimulation with cat allergen (Figure 5.11).

Amongst grass-allergic participants, there was no dependence between the frequency of T\(_H\)2, T\(_H\)1 and T\(_R\)1-like cells (Figure 5.10). In cat-allergic participants, positive correlations were noted between the frequency of T\(_H\)2 cells and the T\(_H\)1 (p=0.003) and T\(_R\)1-like (p=0.02) response, however at lower significance compared to their non-allergic counterparts (Figure 5.11). There were insufficient numbers of non-allergic highly cat-exposed individuals to investigate dependence between variables.
Figure 5.10. Correlations of CD154, T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>R</sub>1-like responses in (a) non-allergic and (b) grass-allergic individuals.
Figure 5.11. Correlations of CD154, T\(_H\)1, T\(_H\)2 and T\(_R\)1-like responses in (a) non-allergic and (b) cat-allergic individuals.
5.1.1 **CD154, T\textsubscript{H1} and T\textsubscript{H2} Phenotypes**

Using surface staining for CD27, CD154\(^+\) T cells were defined as early differentiated (CD27\(^+\)) or late differentiated (CD27\(-\)). In non-allergic participants, the majority of CD154\(^+\), T\textsubscript{H1} and T\textsubscript{H2} responses consisted of an early differentiated phenotype (Figure 5.12a-c). There were insufficient participant numbers to investigate T cell phenotypes in cat-exposed non-allergic subjects.

In cat-allergic subjects, the majority of T\textsubscript{H1} and T\textsubscript{H2} responses were also distributed within the early-differentiated compartments (Figure 5.12a-c). By contrast, late differentiated CD154\(^+\) and T\textsubscript{H2} cells were mainly confined to the grass-allergic group. Grass-allergic participants illustrated a significantly higher frequency of CD27- late differentiated T\textsubscript{H2} cells compared to their non-allergic counterparts (p=0.001, Figure 5.12c).
Figure 5.12. Phenotypes of (a) CD154, (b) CD154+IFNγ+ T\textsubscript{H1} and (c) CD154+IL4+ T\textsubscript{H2} cell responses in non-allergic, cat-allergic and grass-allergic individuals based on CD27 cell surface expression. Data are expressed as the percentage of positive cells of the total responding CD4 T cell population.
5.1.1 *Atopic Non Allergic and Sensitised Individuals*

Following *ex vivo* cat allergen stimulation, atopic non-allergic and sensitised subjects exhibited a CD154⁺ T cell population comparable to both cat-allergic and non-allergic participants (Figure 5.13a). The CD154⁺IL4⁺ TH2 response in atopic non-allergic and sensitised participants was similar to the cat-allergic subject group (Figure 5.13c). CD154⁺IFNγ⁺ TH1 cells were detected in atopic non-allergic and sensitised participants; reduced IFNγ expression was noted in atopic non-allergic participants compared to non-allergic controls, although this did not reach statistical significance (p=0.07, Figure 5.13b).

CD154⁺IL10⁺ TR1-like cells were detected in very few atopic non-allergic and sensitised participants, therefore demonstrating significantly lower expression compared to both allergic and non-allergic subjects (Figure 5.13d).

Non-allergic individuals exhibited a significantly lower TH2:TH1 ratio compared to cat-allergic (p<0.001), atopic non-allergic (p=0.002) and sensitised (p=0.02) participants (Figure 5.13e).
Figure 5.13. Background-corrected cat-induced cytokine expression in atopic non-allergic and sensitised individuals. (a) CD154 expression illustrated as percentage of total CD4 T cells on a log scale. (b) CD154$^{+}$IFNγ$^{+}$ T_H1, (c) CD154$^{+}$IL4$^{+}$ T_H2 and (d) CD154$^{+}$IL10$^{+}$ T_R1-like cells illustrated as the frequency of positive cells per 10$^6$ CD4 T cells on a log scale.
Following *ex vivo* grass allergen stimulation, atopic non-allergic and sensitised subjects exhibited a CD154⁺ T cell population comparable to both grass-allergic and non-allergic participants (Figure 5.14a). Non-allergic participants demonstrated a significantly reduced frequency of CD154⁺IL4⁺ Th2 cells compared to grass-allergic (p=0.006), atopic non-allergic (p=0.02) and sensitised (p=0.01) individuals (Figure 5.14c). Sensitised individuals illustrated fewer Th1 cells compared to non-allergic participants, but this did not reach statistical significance (p=0.05, Figure 5.14b).

No differences were noted in the frequency of CD154⁺IL10⁺ Tr1-like cells between all participant groups (Figure 5.14d).

Non-allergic individuals exhibited a significantly lower Th2:Th1 ratio compared to grass-allergic (p<0.001), atopic non-allergic (p=0.04) and sensitised (p=0.008) participants (Figure 5.14e).

There were insufficient numbers of atopic non-allergic and sensitised individuals for both cat and grass allergens to investigate the phenotypes of responding CD154⁺, Th1 and Th2 cell populations.
Figure 5.14. Background-corrected grass-induced cytokine expression in atopic non-allergic and sensitised individuals. (a) CD154 expression illustrated as percentage of total CD4 T cells on a log scale. (b) CD154^{IFNγ^{+} T_{H1}}, (c) CD154^{IL4^{+} T_{H2}} and (d) CD154^{IL10^{+} T_{R1-like}} cells illustrated as the frequency of positive cells per 10^6 CD4 T cells on a log scale.
5.2 Discussion

CD154 is a T cell activation marker transiently expressed upon ligation of the T cell receptor, thereby providing direct access to an antigen-specific cell population (241, 242). This technique was previously applied to investigate T cell responses to birch allergen in an \textit{ex vivo} stimulation system in birch-allergic and non-allergic individuals (Chapter 4). An important balance between T\textsubscript{H}1, T\textsubscript{H}2 and T\textsubscript{R}1-like cell responses was identified in tolerant responses to birch allergen. This relationship was observed to be dysregulated in allergy, with the T\textsubscript{H}2:T\textsubscript{H}1 ratio maintained at higher levels and the IL10 response abrogated. This work was also able to discriminate successfully between birch-allergic and non-allergic individuals to a high level of accuracy using the T\textsubscript{H}2:T\textsubscript{H}1 ratio.

As described, the birch-specific CD154\textsuperscript{+} T cell response may include cells responding to plant-derived homologs, thus representing perennial rather than seasonal allergens. Therefore, it would be interesting to compare CD154\textsuperscript{+} T cell responses to true seasonal and perennial allergens in more detail. In this case, \textit{ex vivo} analysis of CD154\textsuperscript{+} T helper cells was performed following stimulation with cat-dander (perennial) and grass pollen (seasonal) native allergens in cat-allergic, grass-allergic and non-allergic individuals. The T cell response of non-allergic individuals highly exposed to cat allergens (cat-owners) was also investigated.

CD154\textsuperscript{+} T cell populations were identified in all cat-allergic, grass-allergic and non-allergic participants, as confirmed in the birch pollinosis model. Interestingly, grass-allergic individuals exhibited a significantly higher frequency of CD154\textsuperscript{+} T cells compared to non-allergic controls; a similar increase was identified in the birch study, although of weaker significance. Differences in CD154 expression were not evident in participant groups following stimulation with the perennial cat allergen. Cat allergen-induced CD154\textsuperscript{+} T cell responses were also larger in comparison to seasonal allergen-induced T cell responses, with grass-specific CD154\textsuperscript{+} T cells illustrating the smallest response.

Differences between the magnitude of CD154\textsuperscript{+} T cell responses following birch, grass and cat allergen stimulation may relate to cross-reactivity to plant-derived homologs within the native allergen extracts. As stated, the T cell response to native birch allergen is likely to include cells responding to plant-derived homologs that
represent perennial rather than seasonal allergens, and could account for the higher CD154⁺ cell frequency. The native grass allergen extract may be more representative of a seasonal allergen, with fewer cross-reactive components. In addition, the grass-induced CD154⁺ T cell response represents a more realistic estimate of allergen-specific T cell frequency when compared to tetramer-based studies (229, 235, 238). Furthermore, multiple tetramer-based papers have confirmed a higher frequency of allergen-specific T cells in allergic individuals compared to healthy controls (90, 229, 231, 232, 234, 235, 274), as identified in this study. Van Hemelen et al. failed to identify a difference in the frequency of grass-specific T cells between allergic and non-allergic individuals using a similar CD154-detection system; however, this is likely to reflect very low participant numbers (244).

A reduction in allergen-specific T cells following avoidance of food allergens (274) and outside of the pollen seasons (78, 238) is reported in the literature; therefore, discrepancies in CD154⁺ T cell frequency between allergens may reflect allergen exposure. Although cat allergen is ubiquitous in the environment, chronic allergen exposure seems an unlikely explanation for increased CD154⁺ T cell responses compared to seasonal allergens. In fact, highly exposed non-allergic individuals exhibited slightly lower CD154 responses compared to non-allergic and cat-allergic individuals, although this was not statistically significant and participant numbers were small.

A recent study by Wambre and colleagues compared the characteristics of seasonal (birch allergen Bet v 1) and perennial (house dust mite allergen Der p l/Der p 2) allergen-specific T cell responses using MHC tetramer technology (78). This work identified T helper cell responses to the perennial house dust mite allergens were directed at a broader range of epitopes compared to the seasonal birch pollen allergen, confirming the findings of other literature (221, 238, 299). This perhaps explains the higher CD154⁺ T cell response identified in the present study following stimulation with the perennial cat allergen compared to seasonal (birch/grass) T cell responses. Of course, it is difficult to directly compare T cell responses to different allergen extracts due to disparities in potency.

Again, allergen-specificity has not been formally demonstrated in this work, and it is likely the CD154⁺ T cell population is enriched for allergen-specific T cells but not
entirely allergen-specific. As with the birch-induced T cell response, expression of CD154 together with IL4, IFNγ or IL10, observed at a frequency of approximately 1:600 (cat-allergic) and 1:30,000 (grass-allergic) CD4 T cells, perhaps represents a more realistic estimate of cat and grass-specific T cell frequency.

Considerable evidence now suggests allergic tolerance depends upon a balanced Th1, Th2 and regulatory T cell response, from both our birch pollen study and previous literature (77). Investigations into cat and grass-induced T cell responses revealed strong Th2 polarisation in allergic individuals, as expected. However, no difference in the frequency of Th1 cells between allergic and non-allergic individuals was revealed, indicating a mixed cytokine profile previously described for a number of allergens (78, 90, 225). The predominant response in tolerant individuals consisted of a mixed Th1/Th1-like cell population. This Th1:Th2 polarisation in non-allergic and grass-allergic individuals has recently been described using the CD154-detection assay (244), however, participant numbers were considerably smaller and the findings did not reach statistical significance.

The ratio between Th2 and Th1 cell frequency following ex vivo cat or grass stimulation was a key parameter that differentiated allergic and non-allergic participants. Regardless of the frequency of Th2 cells, the Th2:Th1 ratio was maintained at low levels in non-allergic individuals compared to the allergic groups, although this was less evident for grass pollinosis. Using ROC analysis, the Th2:Th1 ratio in this system successfully discriminated non-atopic individuals from cat-allergic (93% accuracy) and grass-allergic (94% accuracy) participant groups. As previously mentioned, this is perhaps the first description of accurate discrimination between allergic and non-allergic individuals using a T cell based assay and the findings suggest this ratio may find diagnostic utility in food or drug sensitivity. However, both atopic non-allergic and sensitised individuals exhibited similar Th2:Th1 ratios to grass and cat-allergic participants. This suggests the Th2:Th1 ratio discriminates atopy from health but may not be wholly allergen-specific.

Interestingly, the Th2 response to grass allergen in non-allergic participants displayed marked bimodal distribution and considerable overlap with the allergic participant group. A significantly higher frequency of Th1 lymphocytes was identified in the IL4-responder subgroup, but no differences were identified in the
frequency of T_{R1}-like cells. The T_{H2}:T_{H1} ratio was also higher in the IL4-responder subgroup; however, this did not reach statistical significance. This higher frequency of T_{H1} lymphocytes perhaps favours tolerance to grass allergen in the face of a T_{H2} response, although not to the significant extent identified within the model of birch pollinosis.

There is now substantial evidence for a role of T_{Regs} and IL10 in tolerance to allergens (120, 143) as previously discussed. This study illustrated a predominant T_{H1}/T_{R1}-like cell response to cat and grass allergens in non-allergic participants. However, the frequency of T_{R1}-like cells did not differ between cat/grass-allergic participants and their non-allergic counterparts.

Although a role for T_{Regs} in allergic tolerance is established, findings have not been consistently replicated in the literature. For example, Parviainen et al. displayed no difference in the frequency or function of T_{Regs} between allergic and non-allergic individuals (300). Similarly, Skrindo and colleagues demonstrated the depletion of CD4^{+}CD25^{+} T_{Regs} caused no difference in the proliferative responses to grass pollen (301). Various factors may affect the suppressive activity of T_{Regs} including the allergen in question, exposure to the allergen and atopic status of the individual (129, 300-303). The suppressive function of CD4^{+}CD25^{+} T_{Regs} is significantly reduced with higher allergen concentrations, with different thresholds of regulatory failure associated with both allergenicity and atopic severity of the individual (302). One type of allergen may induce suppression of immune responses through T_{Reg} function whereas another allergen may relate to T_{H1} deviation or immune ignorance as the predominant mechanisms (300), indicating allergenicity may be important in T_{Reg} function.

It has been suggested that T_{Regs} are short-lived unless there is a continuous exposure to allergen (301). This may explain the lack of findings regarding T_{Reg} frequencies out of the grass pollen season; however, this is not reflected in the birch pollen response. In addition, cat-exposed non-allergic participants displayed similar frequencies of T_{R1}-like cells to both non-allergic and cat-allergic subjects, perhaps indicating allergen exposure is not an important factor in this setting.

It is notable that IL10 production was bimodally distributed in the cat and grass-allergic populations. In those allergic individuals with undetectable IL10 responses,
CD154$^{+}$IFNγ$^{+}$ T\(_H\)1 cell populations were also significantly reduced. This perhaps further supports the hypothesis of an important T\(_H\)1-T\(_H\)2-T\(_R\)1-like relationship, such that in allergic individuals, T\(_H\)1 or T\(_R\)1-like responses are unable to counterbalance the T\(_H\)2 response.

Detailed analysis of the T\(_H\)1, T\(_H\)2 and T\(_R\)1-like cytokine responses revealed significant correlations between T\(_H\)2, T\(_H\)1 and T\(_R\)1-like responses to both cat and grass allergen after \textit{ex vivo} stimulation amongst healthy subjects. This confirms the positive correlations noted in the birch pollinosis model in the previous chapter. By contrast, the relationship between these T cell subsets was dysregulated in the grass allergic group, with abrogation of both T\(_H\)1 and IL10 responses. Positive correlations were noted between T\(_H\)2, T\(_H\)1 and T\(_R\)1-like responses in cat-allergic individuals, but to a lower significance than their non-allergic counterparts. As stated, both cat and grass-allergic individuals also exhibited significantly higher T\(_H\)2:T\(_H\)1 ratios. This mathematical relationship between the frequency of IL4 expression and IFNγ/IL10 responses in health further supports the birch work and what Akdis \textit{et al.} described as a “fine balance between T\(_H\)1, T\(_H\)2 and T\(_R\)1-like responses”\((77)\).

This study also investigated the phenotype of responding CD154$^{+}$ and CD154$^{+}$cytokine$^{+}$ T cell populations following \textit{ex vivo} grass and cat allergen stimulation using the expression of cell surface marker CD27. Subsets were defined as early differentiated (CD27+) or late differentiated (CD27-). CD154$^{+}$IFNγ$^{+}$ T\(_H\)1 and CD154$^{+}$IL4$^{+}$ T\(_H\)2 cells were also separately phenotyped.

In keeping with tetramer-based studies investigating seasonal allergens and our birch pollen study, responding grass-specific CD154$^{+}$ T cells were predominantly CD27-positive, consistent with an early-differentiated memory phenotype in both grass-allergic and non-allergic participants. However, the frequency of effector memory T cells within the T\(_H\)2 compartment was significantly higher in grass-allergic participants; this was also evident in birch-allergic participants. This supports the view that T cell responses to seasonal allergens comprise a higher proportion of late-differentiated T cells \((78)\). In contrast, T\(_H\)2 cells in cat-allergic individuals were consistent with an early-differentiated phenotype, confirming the findings of previous tetramer-based literature \((90, 246)\). Wambre and colleagues also demonstrated early- and late-differentiated T cell phenotypes for perennial (house
dust mite) and seasonal (birch) allergens, respectively (78), hypothesising chronic allergen exposure could explain a persistent central memory profile. Indeed, literature investigating virus-specific lymphocytes hypothesised the nature and timing of antigen stimulation may influence the generation of memory T cells (304), in which chronic viral infection, such as cytomegalovirus, is associated with a central memory T cell phenotype (305). In addition, CD27 expression has been utilised to discriminate active and latent tuberculosis infection, in which the loss of CD27 expression marks active infection, compared to latent, chronic infection exhibiting high CD27 expression (306). It would be interesting to assess T\textsubscript{H}2 cell phenotype in a larger number of cat-exposed individuals and within the grass pollen season to investigate chronic exposure in more detail. In addition, research into a more extensive memory marker panel may provide more detailed and accurate insights into the phenotype of these allergen-induced T cells, or perhaps differentiate T cells from sensitised and allergic individuals. This is demonstrated in the work of Wambre and colleagues, who recently utilised a larger combination of cell surface markers including CD27, CCR7, CD62L, CRT\textsubscript{H}2, CCR4, CXCR3 and CD7 to accurately define the phenotypes of tetramer\textsuperscript{+} allergen-specific T cells (79).

This study failed to identify differences in the frequency of cat-specific T cells between cat-exposed and non-exposed non-allergic individuals following allergen stimulation. However, cat exposed individuals did demonstrate a significantly higher T\textsubscript{H}2:T\textsubscript{H}1 ratio compared to non-exposed non-allergic participants. This perhaps suggests increased exposure to allergens may induce an IL4 response, even in non-allergic individuals. However, it should be noted that participant numbers within the highly exposed participant group were small and may not be wholly representative. In addition, cat dander allergen is particularly ubiquitous in the environment, therefore comparisons between highly exposed and non-exposed individuals are not sufficiently standardised.

The investigation of both atopic non-allergic and sensitised individuals is novel; little is known about the T cell response to allergens in these subjects. Atopic non-allergic and sensitised participants may have a similar T cell profile to allergic individuals with T\textsubscript{H}2-biased immune responses and low IL10 expression, although participant numbers are small. This skewed T\textsubscript{H}2 immune response of atopic non-allergic
participants compared to the non-allergic T\textsubscript{H}1/T\textsubscript{R}1-like response suggests an intrinsic tendency of allergen-specific T\textsubscript{H}2 cell involvement in the atopic phenotype.

5.3 Conclusions

In summary, this study further supports the fine balance between allergen-specific T\textsubscript{H}1, T\textsubscript{H}2 and T\textsubscript{R}1-like responses, originally proposed by Akdis and colleagues (77) and identified within T cell responses to birch allergen (Chapter 4). This provides further support for the paradigm that the relationship between these parameters may define the humoral and clinical response to allergens. As stated, it seems likely that the introduction of more sensitive T cell assays will further refine the understanding of the relationship between humoral and T cell responses to allergens.
Chapter 6:  Modulation of T Cell Responses during the Birch Pollen Season and following Allergoid Immunotherapy

6.1 Introduction

In this chapter, I present limited pilot data studying the modulation of T cell responses in two settings: [1] in and out of the birch pollen season and [2] before and after specific immunotherapy.

Publications regarding modulation of T cell responses in and out of the pollen seasons vary significantly (307-309), perhaps influenced by differences in methodology, allergens investigated, allergenic stimulation and blood sampling time points. In addition, alterations in cell phenotype and maturity marker expression in and out of the pollen seasons have not been reported to date. The CD154 assay overcomes these limitations, allowing for reduction in culture artefact, compatibility with further intracellular and intranuclear cytokine staining and detailed interrogation of functional T cell subsets. The birch pollen season in the UK occurs between March and May, with peak pollen production in April. This small pilot study investigated T cell responses to birch pollen allergen during the peak season (April) compared to out of season (August to November).

The modulation of allergen-specific T cell responses during specific immunotherapy has been well studied, with numerous publications coinciding with its 100-year anniversary in 2011 (310, 311). The literature indicates increased production of IL10 (137) abrogation of \( \text{T_H2} \) responses and changes in \( \text{T_H2}:\text{T_H1} \) polarisation (198), in conjunction with reduced mast cell activation, increased IgG/IgG4 production and reduced IgE production (201) following successful immunotherapy. This leads to clinical tolerance and a reduced late phase response to the allergen. The present project has illustrated a sensitive CD154 assay to detect allergen-specific T cells; it would be interesting to assess the ability of this assay to detect changes in the T cell response during immunotherapy to compare with the findings of recent tetramer-based research.
6.2 Aims
Using peripheral blood samples from allergic and non-allergic individuals, this study sought to assess the use of our *ex vivo* CD154 detection system in tracking T cell responses in two situations: [1] in and out of the birch pollen season, and [2] before and after specific immunotherapy.

6.3 Study Protocol

6.3.1 Laboratory Protocol
Techniques utilised for PBMC isolation, stimulation and antibody staining are fully detailed in Chapter 2 (Materials and Methods). PBMC were isolated from birch-allergic individuals, grass-allergic individuals and non-allergic controls and rested overnight before 16-hour stimulation with 500PNU/ml BPE or GPE in the presence of BFA for the last 14 hours. Unstimulated PBMC were used as a negative control. PHA-stimulated PBMC were used as a positive control to ensure successful intracellular cytokine staining. Responding cells were identified using multiparametric flow cytometry following antibody staining for a variety of surface and intracellular markers to investigate numerous cell functions:

- **T helper cell type**: CD3-AlexaFluor700, CD4-PerCP and CD25-APC-Cy7
- **Cytokine expression**: CD154-PB, IFNγ-PeCy7, IL4-PE and IL10-APC
- **Phenotypic analysis**: CD27-FITC and CD45RA-ECD

6.3.2 Flow Cytometric Analysis
All results were gated on CD3^+^CD4^+^ single lymphocytes following a minimum collection of 400,000 CD4 events. All data was background-corrected. Boolean gating combinations were computed for cytokine and cell marker analysis.

6.3.3 Statistical Analysis
The data distribution was non-parametric according to the D’Agostino and Pearson omnibus normality test. Median values were used for comparison throughout. Statistical significance was calculated using the two-tailed Mann-Whitney U test with a significance level of 0.05. Spearman rank correlation analysis was used to investigate statistical dependence between variables. The T\(_{H2}\):T\(_{H1}\) ratio was calculated as previously described (Chapter 4). Statistical comparisons were not performed in immunotherapy experiments due to small participant numbers.
### 6.4 Participant Characteristics

<table>
<thead>
<tr>
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<th>Non-Allergic (Seasonal Study)</th>
<th>Birch-Allergic (Seasonal Study)</th>
<th>Grass-Allergic (SIT)</th>
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<tr>
<td>No. of Participants</td>
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<td>5</td>
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<tr>
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<td>29±14</td>
<td>38±24</td>
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<td>Physician-Diagnosed Allergic Diseases (%):</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>Cat: 0</td>
<td>80</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 6.1. Participant demographics

For investigations into seasonal changes of T cell responses, birch-allergic individuals (n=5) were recruited from the allergy clinic of the Royal Sussex County Hospital: all participants had a history of spring rhinitis and were SPT-positive to birch pollen extract (Table 6.1). An approximately age-matched non-allergic population (n=5) was recruited from Brighton and Sussex University volunteers: these participants had no history of atopic disease and had negative SPTs to common aeroallergens (cat dander, birch pollen, early-pollinating tree mix, mid-pollinating tree mix, grass pollen). Blood samples were taken both during the spring birch pollen season (April 2012) and a minimum of eight weeks outside the birch pollen season (August to November 2010). Study participants were confirmed to be free of rhinitis symptoms at the time of venupuncture outside the birch pollen season.

For the investigations into T cell responses before and after specific immunotherapy, grass-allergic participants (n=3) were recruited from the allergy clinic of the Royal Sussex County Hospital: all participants had a history of spring rhinitis and were SPT-positive to grass pollen extract (Table 6.1). There was no control group for this work. Blood samples were taken a minimum of four weeks outside of the grass and birch pollen seasons.
6.1 Results

6.1.1 Seasonal Changes

UK birch pollen counts remained low to moderate throughout the 2012 season. In non-allergic participants, we observed a statistical trend for a reduced frequency of CD154⁺IFNγ⁺ TH₁ cells in the birch pollen season (p=0.06, Figure 6.1a). There was no difference in the frequency of TH₁ cells in birch-allergic individuals in and out of pollen season (p=0.13). The frequency of CD154⁺IL4⁺ TH₂ cells did not differ in and out of the pollen season in non-allergic (p=0.44) or birch-allergic (p=0.82) participants (Figure 6.1b). However, birch-allergic individuals demonstrated a significantly higher frequency of TH₂ cells during the peak season compared to non-allergic controls (p=0.008, Figure 6.1b).

In non-allergic participants, the TH₂:TH₁ ratio was maintained at low levels in and out of the pollen season (Figure 6.1c). In contrast, this ratio demonstrates a marked increase in season in three of five birch-allergic subjects, although this is not statistically significant (p=0.31, Figure 6.1c). In addition, the TH₂:TH₁ ratio is significantly higher in birch-allergic individuals compared to non-allergic controls outside of the pollen season (p=0.03, Figure 6.1c), but this difference is not evident in season.
Figure 6.1. (a) TH1 and (b) TH2 responses and the (c) TH2:TH1 ratio in and out of the birch pollen season in non-allergic and birch-allergic individuals
There were no differences in the frequency of CD4^+IL10^+ or CD154^+IL10^+ T cells in and out of the pollen season in birch-allergic or non-allergic individuals (Figure 6.2a, b). However, non-allergic individuals did exhibit a significantly higher frequency of CD4^+IL10^+ cells compared to birch-allergic participants outside of birch pollen season (p=0.02); this differences also applied in season, but of weaker significance. (p=0.06, Figure 6.2b).

![Graphs showing CD4^+IL10^+ and CD154^+IL10^+ T cells in and out of birch pollen season](image)

**Figure 6.2.** (a) CD4^+IL10^+ and (b) CD154^+IL10^+ T_{H1}-like cell responses in and out of the birch pollen season in non-allergic and birch-allergic individuals

CD154^+ and T_{H1} cells were mostly of an early-differentiated phenotype in both non-allergic and birch-allergic individuals in and out of the birch pollen season (Figure 6.3, Figure 6.4). T_{H2} cells were also consistent with an early-differentiated phenotype in and out of the pollen season in birch-allergic individuals (Figure 6.5). There was an insufficient number of responding T_{H2} cells to investigate the phenotype of T_{H2} responses in the non-allergic group.
Figure 6.3. Phenotype of CD154+ T cell responses in and out of the birch pollen season in non-allergic and birch-allergic individuals
Figure 6.4. Phenotype of \( T_{H1} \) cell responses in and out of the birch pollen season in non-allergic and birch-allergic individuals
Figure 6.5. Phenotype of T\(_h2\) T cell responses in and out of the birch pollen season in birch-allergic individuals

6.1.1 Allergoid Immunotherapy

Grass-allergen induced CD154\(^+\) T cell populations were resolved both before and after allergoid immunotherapy in all three participants (Figure 6.6a). The frequency of CD154\(^+\)IFN\(\gamma^+\) T\(_{h1}\) cells decreased after immunotherapy in two of three participants (Figure 6.6b). In addition, the frequency of CD154\(^+\)IL4\(^+\) T\(_{h2}\) cells decreased following immunotherapy in all subjects (Figure 6.6c); consequently the T\(_{h2}\):T\(_{h1}\) ratio was also lower in all participants following allergoid immunotherapy (Figure 6.6d). There was no difference in the frequency of CD4\(^+\)IL10\(^+\) or CD154\(^+\)IL10\(^+\) T cells before or after allergoid immunotherapy (Figure 6.6e, f). The percentage of CD4\(^-\)CD25\(^+\) T cells increased in two of three participants following immunotherapy (Figure 6.6g).
Figure 6.6. (a) CD154+, (b) T\(_H\)1 and (c) T\(_H\)2 responses (d) T\(_H\)2:T\(_H\)1 ratio (e) CD154+IL10+ (f) CD4+IL10+ and (g) CD4+CD25+ cells before and after specific immunotherapy in grass-allergic individuals.
6.2 Discussion

The aim of this small pilot study was to investigate the potential utility of the CD154 assay in investigating modulation of T cell responses in two situations: [1] during the birch pollen season and [2] following allergoid immunotherapy.

6.2.1 Seasonal Changes

This preliminary work suggests the CD154 detection system may have utility in tracking the modulation of T cell responses during seasonal allergen exposure. Although we cannot draw firm conclusions from this initial data, the $T_{H2}:T_{H1}$ ratio appears to increase in birch-allergic individuals during the peak pollen season. In contrast, the $T_{H2}:T_{H1}$ ratio remained low in non-allergic subjects both in and out of season. Preliminary results suggest responding CD154$^+$, $T_{H1}$ and $T_{H2}$ cells are perhaps of an early-differentiated phenotype in both non-allergic and birch-allergic individuals, in and out of the pollen season. However, it should be noted the birch pollen season was atypical in 2012 with very little birch pollen and increased rainfall.

Research into the modulation of T cell responses during the pollen season illustrates conflicting results. Multiple papers have demonstrated an increase in $T_{H2}$ cells and $T_{H2}$ cytokine production during the pollen season in allergic individuals (307, 309, 312, 313); however, Van Overtvelt and colleagues failed to detect changes in IFNγ, IL5 or IL10 expression during the birch pollen season using an MHC class II tetramer assay (91). In addition, Koscher et al. and Jepsen et al. identified decreased production of $T_{H2}$ cytokines during the cypress and grass pollen seasons, respectively (308, 314), postulating a seasonal migration of allergen-specific T cells from blood into the tissues. Multiple studies have also illustrated a reduced ability of allergen-specific T cells to proliferate during the pollen seasons (307, 314, 315).

The present study identified a potential increase in the $T_{H2}:T_{H1}$ ratio during the birch pollen season in birch-allergic individuals. In contrast, this ratio remains low in non-allergic individuals both in and out of the birch pollen season. The maintenance of a low $T_{H2}:T_{H1}$ ratio in non-allergic individuals demonstrates the high reproducibility of the CD154 assay and supports previous work indicating the relationship between $T_{H1}$, $T_{H2}$ and $T_{R1}$-like cells is important in allergic tolerance, even during natural allergen exposure. An increased $T_{H2}:T_{H1}$ ratio has also been described by Wosinska-
Becler and colleagues in birch-allergic individuals during peak pollen season in which T_{H2} responses correlated with the clinical symptoms of seasonal allergic rhinitis (313). This confirms previous hypotheses indicating the inflammatory response is increased in allergic individuals following natural exposure to allergen (312).

The conflicting results between the existing literature and the present pilot work may reflect differences in methodology, sampling time points, participant groups or the allergens investigated. Most pre-existing studies measured cytokine concentration in supernatant cultures, sometimes after mitogen re-stimulation. Gabrielsson et al. illustrated a seasonal increase in IL13 production in response to birch allergen, but not in response to PHA or PPD control antigens, suggesting this is an allergen-specific response (307). Therefore, literature investigating mitogen-stimulated T cell responses during pollen seasons may not be reflective of the true allergen-specific T cell response (309, 315).

Interestingly, Lagier et al. identified seasonal variations of IL4 production fluctuated depending on patient sensitisation (309). Polysensitised individuals did not exhibit differences in cytokine expression in and out of the pollen seasons. In contrast, monosensitised grass-allergic and cypress-allergic individuals exhibited increased IL4 production during the respective pollen seasons. This suggests a potential distinction between those polysensitised individuals continuously exposed to allergen and individuals with seasonal allergy. In this case, it would be interesting to compare T cell responses in and out of pollen seasons in individuals with perennial allergic rhinitis associated with a non-seasonal allergen, such as house dust mite.

The present pilot study identified a decreased frequency of CD154^+ responding T cells during the birch pollen season in both allergic and non-allergic participants, although this did not reach statistical significance. In contrast, Wambre and colleagues demonstrated an increased frequency of alder pollen-specific T cells in allergic individuals during the pollen season (79). Expression of CD38 and Ki67, T cell activation and proliferation markers, respectively, were also increased within the tetramer^+ T cell population. The increased expression of Ki67 indicates these T cells are actively proliferating during the alder pollen season, although the cytokine profile of these responding T cells was not investigated.
The role of IL10 in allergic tolerance has been intensively investigated. The previous chapters and the work of Akdis et al. (77) have demonstrated an important balance between T\textsubscript{H}1, T\textsubscript{H}2 and T\textsubscript{R}1-like responses, such that in health, the frequencies of these T cell subsets increased together. In contrast, IL10 responses appear abrogated in allergic individuals.

The frequency of CD4\textsuperscript{+}IL10\textsuperscript{+} and CD154\textsuperscript{+}IL10\textsuperscript{+} T\textsubscript{R}1-like cells did not differ in and out of pollen season in non-allergic or birch-allergic individuals. Multiple papers have demonstrated defective T\textsubscript{Reg} responses during the pollen season in allergic individuals (129, 303); however, Van Overtvelt and colleagues failed to detect changes in IL10 expression during the birch pollen season (91). In addition, Wosinska-Becler identified a significant increase of IL10 expression during the alder pollen season (313). Again, these variations may reflect the very small data set in the present study or differences between allergens and methodology.

Grindebacke and colleagues demonstrated that CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{Reg} in allergic individuals were defective in suppressing T\textsubscript{H}2 cytokine responses but maintained the ability to suppress IFN\gamma: no defects in suppressive capacity were identified in non-allergic participants (129). Outside of the birch pollen season, CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{Reg} were able to suppress T\textsubscript{H}2 cytokine responses in both allergic and non-allergic individuals. This was later confirmed to be an allergen-specific response, with upregulation of FOXP3 expression, although in a smaller data set (303). However, no difference in the frequency of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{Reg} was identified, perhaps suggesting suppressive function of these cells is more important in season than their absolute frequency. Moreover, further investigation illustrated no difference in IL10 expression within the CD4\textsuperscript{+}CD25\textsuperscript{+} T cell population. In addition, it appears different participants were recruited in and out of pollen season, thus illustrating a potential confounding factor. These investigations also utilised \textsuperscript{3}H-thymidine incorporation to analyse allergen-specific T cell responses; background DNA synthesis can alter the true results, thus the ex vivo CD154 detection method described may provide a more representative reflection of IL10\textsuperscript{+} T cell frequencies. Of course, firm conclusions on IL10 responses cannot be drawn from such a small data set in the current pilot work.
In summary, the CD154 assay appears useful in tracking the modulation of T cell responses in and out of the birch pollen season. This ex vivo stimulation system provides advantages over previously adopted techniques, such as MHC tetramers and cytokine capture, including speed and simplicity of the protocol, compatibility with intracellular cytokine staining and no HLA restrictions. The T\textsubscript{H}2:T\textsubscript{H}1 ratio remains low in non-allergic participants despite seasonal allergen exposure. In contrast, the frequency of T\textsubscript{H}2 cells appears to increase in birch-allergic individuals at peak pollen season. However, firm conclusions cannot be made with such a small data set. Further work will involve the recruitment of a larger number of participants and comparison with the grass pollen season. It would also be interesting to evaluate the expression of FOXP3 in and out of season to confirm and compare the findings of Grindebacke and colleagues. It may also be of value to correlate changes in the T\textsubscript{H}2:T\textsubscript{H}1 ratio during peak pollen season with symptom scores, to perform a formal assessment of the pollen counts at the sampling time points, and to investigate T cell responses within the nasal mucosa.

6.2.2 Allergoid Immunotherapy

Alterations in T cell responses during allergen-specific immunotherapy have been intensively studied and extensively re-visited last year with the 100-year anniversary of immunotherapy following the work of Noon in 1911 (310). Literature has indicated induction of IL10-producing T\textsubscript{RegS} (137), abrogation of T\textsubscript{H}2 responses and changes in T\textsubscript{H}2:T\textsubscript{H}1 polarisation (198), reduced mast cell activation, inhibition of IgE-mediated presentation to T cells and amplified IgG/IgG4 production (201) following successful immunotherapy. In the present pilot study, allergoid immunotherapy resulted in a decreased frequency of CD154\textsuperscript{+}IFN\textgamma\textsuperscript{+} T\textsubscript{H}1 cells and CD154\textsuperscript{+}IL4\textsuperscript{+} T\textsubscript{H}2 cells, with a resultant decrease in the T\textsubscript{H}2:T\textsubscript{H}1 ratio, in all three grass-allergic participants. It was not possible to assess phenotypic changes in maturity of responding T cells or the correlation with clinical outcome due to small participant numbers.

A decrease in allergen-specific T\textsubscript{H}2 responses following successful immunotherapy has been reported in many studies (194, 197-199, 222, 316-318), although a few publications note this reduction is predominantly seen in the target organ rather than in peripheral blood (200, 318). This reduction in the T\textsubscript{H}2 response has also been
accompanied by improved clinical outcome (278, 318), although a correlation between these parameters has not been consistently confirmed (137, 319).

In contrast, Till et al. demonstrated no change in T_{H2} cytokine production following immunotherapy (319), which may reflect differences in methodology and the difficulty in standardising allergens. Alternatively, the deviation of T_{H2}-skewed immune responses may not be the fundamental immunological change occurring following successful immunotherapy. More recent studies have focused on a role of T_{Regs} and IL10 production in the tolerance mechanisms of immunotherapy. Two T_{Reg} populations are predominantly described in the mechanisms of immunotherapy: [1] naturally occurring FOXP3-expressing T_{Regs} and [2] inducible T_{R1} cells.

Increased numbers of CD25^{+}FOXP3^{+} naturally occurring T_{Regs} are detected in the nasal mucosa following grass pollen immunotherapy (201), in which the frequency of these cells correlates with clinical efficacy and suppression of seasonal allergic inflammation (138). Allergen-specific FOXP3 expression is also induced in the peripheral blood for up to 12 months following peanut oral immunotherapy (202). These FOXP3-expressing T_{Regs} may be impaired in suppressing T_{H2} responses in allergic individuals compared to healthy controls (203). Alongside the suppression of T_{H2} responses (204, 205), T_{Regs} are reported to inhibit allergen-specific IgE production, mast cell and eosinophil activity and induce a state of anergy following immunotherapy (195, 206, 207). Decreased proliferative responses of T cells to allergens are reported following immunotherapy (208), perhaps reflecting this anergic state.

The frequency of T_{R1} cells is reported to increase significantly during the first 3-6 months of immunotherapy, with a subsequent decrease at 12 months, indicating a prominent role of these regulatory cells during the initial phases of specific immunotherapy (195). Mobs et al. illustrated a significant decline in the T_{H2}:T_{R1} ratio following birch pollen immunotherapy, with the induction of a T_{R1} cell population and associated suppression of T_{H2} cytokines (278). Non-allergic individuals represent an ideal model of induced allergic tolerance. Non-allergic beekeepers are tolerant to the repetitive stings of bee venom associated with a switch to IL10-producing T_{R1}-like immune responses (133). The expression of IL10 following immunotherapy has been consistently confirmed in the literature for a
number of allergens (130, 137, 195, 197). IL10 mRNA is detectable in the nasal biopsies of individuals undergoing grass pollen immunotherapy (138, 139). In addition, increased numbers of IL10$^+$ T cells were identified in the late phase skin responses of individuals undergoing hymenoptera venom immunotherapy (209). IL10 is known to suppress Th2 cytokines, such as IL5, and directly inhibit IgE-mediated mast cell activation by inducing IgG4 production plasma B cells (210, 211).

It seems highly likely the important balance between Th2, Th1 and Tr1-like responses, described by both Akdis and colleagues and the current project, is also relevant in the immunological changes occurring following successful immunotherapy.

However, in the present study, the frequency of CD154$^+$IL10$^+$ Tr1-like cells did not appear to change during allergoid immunotherapy, although the data set was small. The frequency of CD4$^+$CD25$^+$ T cells increased in two of three participants following immunotherapy. However, FOXP3 expression was not analysed and CD25 expression alone is not a sufficient marker for this cell type. Investigations into expression of additional regulatory markers including FOXP3, CD127, GITR or CTLA-4 would provide further insight.

Campbell and colleagues recently evaluated the allergen-specific T cell response following an experimental form of ragweed immunotherapy, also based on the expression of CD154 and cytokines. In this work, the frequency of CD154$^+$ T cells did not differ before and after immunotherapy. However, no differences were evident in Th2 cytokine responses (IL4, IL5, IL13) within the CD154$^+$ T cell population. This may relate to a number of methodological factors. Firstly, anti-CD28 was added into cell cultures. According to optimisation experiments of the current project, this may increase culture artefact and mask the rare cytokine events. In addition, very few events were recorded (6x10$^5$/sample); a significantly larger frequency of CD4 events must be analysed to identify representative CD154$^+$ T cell populations. The samples were collected relatively close to the ragweed pollen season (as little as 2 weeks), in addition to multiple ragweed challenges before PBMC isolation, which may result in higher or skewed frequencies of responding
The recruitment of a larger data set would allow for detailed phenotypic analysis of responding CD154⁺, T\(_{H1}\) and T\(_{H2}\) populations. The work of Wambre et al. demonstrated alder pollen-specific T cells in allergic individuals undergoing immunotherapy were predominantly CD27⁺ early-differentiated cells with a similar cytokine profile to non-allergic participants (79). In comparison, allergic individuals not receiving immunotherapy exhibited effector memory CD27-negative cell populations. It would be interesting to assess phenotypic changes in more detail using the CD154-based assay.

It would also be of benefit to examine the IgE and IgG4 levels before and after allergoid immunotherapy alongside correlations with T\(_{H2}/T_{H1}/T_{R1}\)-like parameters. Allergen-specific IgE levels are initially increased at the beginning of immunotherapy but decrease to pre-treatment levels once the maintenance dose has been achieved. Increased concentrations of IgG1, IgG4 and IgA isotypes have been identified following immunotherapy (130, 201); IgG4 demonstrates the most consistent and prominent increase, but with little inflammatory activity, is referred to as a blocking antibody competing with IgE to prevent mast cell cross-linkage and activation. IgG4 is also associated with the blocking of IgE-mediated allergen presentation to T cells. Increased concentrations of IgG4 are also evident in natural allergen exposure such as that seen in non-allergic beekeepers (195). Both IgG and IgA antibodies from nasal lavage fluid are able to inhibit basophil histamine release following immunotherapy (320). However, the weak correlation between IgG4 and clinical efficacy introduces questions into the exact function of this antibody (321, 322).

In summary, the CD154 assay seems useful in tracking the modulation of T cell responses during specific immunotherapy. Allergoid immunotherapy appears to result in a decreased T\(_{H2}/T_{H1}\) ratio, although this is a very small data set and conclusions presented thus far are purely speculative. An increased data set and addition of a control group would provide further insight. Investigation of phenotype and humoral responses would also be of benefit.
6.3 Conclusions

In conclusion, this small pilot study reveals a possible increase in $T_{H2}$ polarisation during peak pollen season in allergic subjects and a decrease in $T_{H2}$ polarisation following allergoid immunotherapy, although firm conclusions cannot be made in such a small data set. The CD154 assay appears to be highly reproducible and sensitive, illustrating similar findings to that of tetramer-based work, and adopting a number of biological applications.
Chapter 7:  **T cell cytokine responses to Staphylococcal Enterotoxin B in highly atopic and non-atopic individuals**

7.1 Introduction

Superantigens are a family of microbial exotoxins that are able to potently activate T cells, in a Vβ-specific manner, by simultaneously binding the T cell receptor and MHC class II molecule (247). Superantigens are able to stimulate 20-30% of T cells, resulting in widespread secretion of pro-inflammatory cytokines, recruitment of B cells, T cells and neutrophils, and Vβ-specific Th1-skewed proliferation (248).

Colonisation with superantigen-producing staphylococci is common in allergic diseases such as atopic dermatitis (250), allergic rhinitis (251, 252), nasal polyposis (253) and asthma (254). Evidence increasingly favours an important role for superantigens as a co-factor in the pathogenesis and modulation of allergic diseases. Superantigens may provoke Th2-mediated inflammation and allergen-specific Th2 responses (255) and induce B cell class switching to IgE (256). In atopic dermatitis, staphylococcal colonisation is virtually ubiquitous and correlates with disease severity. The cutaneous application of superantigen induces skin inflammation in both atopic and healthy individuals (257) and in animal models, skin exposure to staphylococcal superantigen enhances Th2 responses to subsequent peanut sensitisation (255). Superantigens are able to induce inflammation with microscopic similarities to allergic inflammation without the presence of an allergen (258) and treatment of *S. aureus* infection improves the symptoms of atopic dermatitis (259). Studies have demonstrated superantigens can induce airway hyperresponsiveness (260) and increased sensitivity to aeroallergens (261, 262) in allergic airway disease, but the mechanism by which superantigens facilitate allergic immune dysfunction remain to be fully elucidated.

The susceptibility factors favouring staphylococcal colonisation in atopic diseases remain unknown, but have been postulated to relate to Th2 polarisation in the tissue microenvironment of allergic inflammation, perhaps reflecting mucosal barrier dysfunction or defective innate defences (250). However, very little is known of circulatory T cell responses to superantigens in atopic individuals. Studies have
focused on atopic dermatitis, but results have been conflicting and the exact atopic status of individuals was poorly defined (263-266).

Whilst studying allergen-specific T cell responses to allergens, the superantigen Staphylococcal Enterotoxin B (SEB) from *S. aureus* was initially used as a positive control. By chance, a significantly reduced T helper cell cytokine response to SEB was observed in highly atopic individuals compared to non-atopic healthy controls. A recent report demonstrated that staphylococcal superantigens induce functionally competent T\textsubscript{Reg} in a V\textbeta-specific, dose-dependent manner, causing suppression of T cell responses and a mechanism for evasion of the immune response by superantigen-producing staphylococci (249). Therefore, we hypothesised downregulated T cell cytokine responses to superantigens in atopy may reflect SEB-induced T\textsubscript{Reg} activity and contribute to increased colonisation in the atopic phenotype. To further explore this phenomenon, T\textsubscript{H1}, T\textsubscript{H2}, T\textsubscript{H17} and T\textsubscript{Reg} cell responses were studied following *ex vivo* SEB stimulation using multiparametric flow cytometry in blood samples taken from healthy volunteers, compared to a group of highly atopic polysensitised individuals.

### 7.2 Aims

Using peripheral blood samples from non-atopic and highly atopic individuals, this study sought to investigate T helper cell cytokine and regulatory responses to the staphylococcal superantigen SEB.

### 7.3 Study Protocol

#### Laboratory Protocol

Techniques utilised for PBMC isolation, stimulation and antibody staining are fully detailed in Chapter 2 (Materials and Methods). PBMC were isolated from highly atopic polysensitised individuals and non-atopic healthy controls and stimulated with 1\textmu g/ml SEB in the presence of BFA for the last 14 hours. Unstimulated PBMC were used as a negative control. PHA-stimulated PBMC were used as a positive control. Responding cells were identified using multiparametric flow cytometry following antibody staining for a variety of surface and intracellular markers to investigate numerous cell functions:
- **T helper cell type**: CD3-AlexaFluor700, CD4-ECD, CD8-PerCP
- **Cytokines**: CD154-PB, IFNγ-PeCy7, IL4-PE and IL17-FITC
- **Regulatory T cells**: CD25-APC-H7, IL10-APC, FOXP3-AlexaFluor488
- **TCRVβ Specificity**: TCRVβ1-PE, TCRVβ3-FITC

In addition, SEB-specific IgE was determined in eight atopic individuals, in whom serum was available, using a Phadia 100 instrument, according to the manufacturer’s instructions (Thermofisher IDD, Uppsala, Sweden).

### 7.3.2 Flow Cytometric Analysis

All results were gated on CD3^+^CD4^+^ single lymphocytes following a minimum collection of 200,000 CD4 events. All data was background-corrected. Fluorescence-minus-one (FMO) controls were performed to ensure the compatibility of fluorochromes and to eliminate antibody effects in cytokine expression (Appendix). Boolean gating combinations were computed for cytokine and cell marker analysis. T helper cell subsets were defined based on cytokine expression as described in Table 7.1.

### Table 7.1

<table>
<thead>
<tr>
<th></th>
<th>Th1</th>
<th>Th2</th>
<th>Th17</th>
<th>Th1-Like</th>
<th>Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>N/A</td>
</tr>
<tr>
<td>IL4</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>IL17</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>IL10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>FOXP3</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
</tr>
</tbody>
</table>

*Table 7.1. Definitions of T helper cell subsets according to cytokine expression*

### 7.3.3 Statistical Analysis

The data distribution was non-parametric according to the D’Agostino and Pearson omnibus normality test. Median values were used for comparison throughout. Statistical significance was calculated using the two-tailed Mann-Whitney U test or Wilcoxon matched-pairs signed rank test, where appropriate. A significance level of 0.01 was deemed as significant based on Bonferroni corrections for five possible T cell subset outcomes.
7.1 Results

7.1.1 Participants

<table>
<thead>
<tr>
<th></th>
<th>Non-Atopic</th>
<th>Atopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Participants</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Mean Age</td>
<td>32±10</td>
<td>42±15</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of Males</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>No. of Females</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>Allergic Diseases (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
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<td>44</td>
</tr>
<tr>
<td>Eczema</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Rhinitis:</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>50</td>
</tr>
<tr>
<td>Cat-induced</td>
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</tr>
<tr>
<td>Pollen Food Syndrome</td>
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<td>69</td>
</tr>
<tr>
<td>SPT-positive (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birch</td>
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<td>100</td>
</tr>
<tr>
<td>Grass</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>Cat</td>
<td>0</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 7.2. Participant Demographics

The demographics and clinical information of study participants are displayed in Table 7.2. Highly atopic polysensitised participants (n=16) with seasonal allergic rhinitis in the spring and/or summer, a positive SPT to a seasonal pollen allergen (birch/grass) plus one or more sensitisations to common perennial allergens (cat dander, house dust mite) were recruited from the allergy clinic of the Royal Sussex County Hospital. IgE directed against SEB was analysed in eight of sixteen (50%) highly atopic participants in whom serum was available. SEB-specific IgE was undetected in all cases.

An approximately age-matched non-atopic population (n=12) was recruited from Brighton and Sussex University volunteers: these participants had no history of atopic disease and demonstrated negative SPTs to a variety of common aeroallergens (cat dander, birch pollen, early-pollinating tree mix, mid-pollinating tree mix, grass pollen).

Blood samples were taken a minimum of four weeks outside the grass and birch pollen seasons and study participants were confirmed to be free of rhinitis symptoms at the time of venupuncture.
7.1.2 T Cell Cytokine Responses

A combination of cytokine-positive T\textsubscript{H1}, T\textsubscript{H2}, T\textsubscript{H17} and/or T\textsubscript{R1}-like cell populations were resolved in all participants following ex vivo SEB stimulation (Figure 7.1). Highly atopic individuals exhibited a significantly reduced frequency of IL17\textsuperscript{+} T\textsubscript{H17} cells compared to non-atopic controls (p<0.001, Figure 7.3a). There was no difference in the frequency of CD154\textsuperscript{+} (p=0.18), T\textsubscript{H1} (p=0.06) or T\textsubscript{H2} (p=0.17) cells between atopic and non-atopic participants following ex vivo SEB stimulation (Figure 7.3b-d). Subgroup analysis by allergic disease did not reveal any obvious clustering in patients with co-existing perennial allergic rhinitis, asthma or atopic dermatitis.

In addition, there were no differences in the frequency of CD154\textsuperscript{+} (p=0.47), IFN\gamma\textsuperscript{+} (p=0.98), IL4\textsuperscript{+} (p=0.12) or IL17\textsuperscript{+} (p=0.88) cells within the CD8 T cell population between atopic and non-atopic participants following SEB stimulation (Figure 7.2, Figure 7.4).

There were no significant differences in cytokine expression following PHA stimulation (control antigen) between atopic and non-atopic participants in either the CD4 or CD8 T cell population (Figure 7.5, Figure 7.6).
Figure 7.1. An example of SEB-induced CD4 T cell cytokine expression in one representative non-allergic individual. Percentages represent the proportion of cytokine-positive T cells in the total CD4 T cell population.
Figure 7.2. An example of SEB-induced CD8 T cell cytokine expression SEB in one representative non-allergic individual. Percentages represent the proportion of cytokine-positive T cells in the total CD8 T cell population.
Figure 7.3. SEB-induced (a) $T_{H17}$, (b) $T_{H2}$, (c) $T_{H1}$ and (d) CD154$^+$ CD4 T cell frequency in atopic and non-atopic participants.
Figure 7.4. SEB-induced (a) IL17⁺, (b) IL4⁺, (c) IFNγ⁺ and (d) CD154⁺ CD8⁺ T cell frequency in atopic and non-atopic participants
Figure 7.5. PHA-induced (a) T<sub>H17</sub>, (b) T<sub>H2</sub>, (c) T<sub>H1</sub> and (d) CD154<sup>+</sup> CD4 T cell frequency in atopic and non-atopic participants.
Figure 7.6. PHA-induced (a) IL17⁺, (b) IL4⁺, (c) IFNγ⁺ and (d) CD154⁺ CD8 T cell frequency in atopic and non-atopic participants
7.1.1 Regulatory T cells

It was hypothesised that downregulated T\textsubscript{H}17 responses to SEB in atopic individuals may reflect SEB-induced regulatory T cell activity. Therefore, both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell populations were analysed for the expression of CD25 and FOXP3 in both rested and SEB-stimulated PBMC (Figure 7.7a, Figure 7.8a).

In CD4 T cells, there was no difference in the frequency of resting CD25\textsuperscript{+}FOXP3\textsuperscript{+} T\textsubscript{Reg} between atopic and non-atopic individuals (p=0.81). Following ex vivo stimulation with SEB, FOXP3 expression was upregulated in both atopic (p<0.001) and non-atopic (p<0.001) participants; however, this upregulation of FOXP3 did not differ between the groups (p=0.88). In addition, there was no difference in the frequency of SEB-stimulated CD25\textsuperscript{+}FOXP3\textsuperscript{+} T cells between groups (p=0.95).

In CD8 T cells, there was no difference in the frequency of resting CD25\textsuperscript{+}FOXP3\textsuperscript{+} T\textsubscript{Reg} between atopic and non-atopic individuals (p=0.38). Similarly, FOXP3 expression was upregulated following ex vivo stimulation with SEB in both atopic (p=0.02) and non-atopic (p=0.007) participants, albeit at lower statistical significance in comparison to the CD4 T cell population. This upregulation of FOXP3 expression is slightly higher in non-atopic individuals compared to their atopic counterparts, but this difference did not reach statistical significance (p=0.08). There was no difference in the frequency of SEB-stimulated CD25\textsuperscript{+}FOXP3\textsuperscript{+} T cells between groups (p=0.30).

Expression of IL10 in both CD4 and CD8 T cells following SEB stimulation was also examined in atopic and non-atopic participants. There was no difference in the frequency of CD4\textsuperscript{+}IL10\textsuperscript{+} T cells between the participant groups (p=0.36, Figure 7.7b). In contrast, atopic individuals exhibited a significantly higher frequency of CD8\textsuperscript{+}IL10\textsuperscript{+} T cells compared to their non-atopic counterparts (p=0.008, Figure 7.8b).
Figure 7.7. Frequency of (a) CD25⁺FOXP3⁺ CD4 T cells before and after SEB stimulation and (b) SEB-induced Tₘ1-like cells in atopic and non-atopic participants.
Figure 7.8. Frequency of (a) CD25+FOXP3+ CD8 T cells before and after SEB stimulation and (b) SEB-induced IL10+ CD8 T cells in atopic and non-atopic participants.
7.1.1 *TCRVβ Specificities*

To explore the possibility that downregulated Th17 responses to SEB in atopy relate to differences in TCRVβ specificities, the expression of TCRVβ3 (major target of SEB) and TCRVβ1 (no relevance to SEB) was examined. There was no difference in the frequency of TCRVβ3+ T cells in rested (p=0.15) or SEB-stimulated (p=0.47) PBMC between atopic and non-atopic participants (Figure 7.9). In addition, there was no difference in the frequency of TCRVβ1+ T cells in rested (p=0.77) or SEB-stimulated (p=0.35) PBMC between atopic and non-atopic participants (Figure 7.10).
Figure 7.9. Frequency of TCRβ3⁺ T cells (a) before and (b) after SEB stimulation in atopic and non-atopic participants.
Figure 7.10. Frequency of TCRVβ1+ T cells (a) before and (b) after SEB stimulation in atopic and non-atopic participants
7.2 Discussion

The colonisation of superantigen-producing staphylococci is common in allergic diseases and may contribute to the initiation and maintenance of allergic sensitisation. However, little is known of circulating T cell responses to superantigens in atopic individuals. This study sought to explore T cell responses to the staphylococcal superantigen SEB in highly atopic polysensitised individuals and non-atopic healthy controls. This work demonstrated a significant reduction in TH17 responses in highly atopic individuals compared to healthy non-atopic controls following ex vivo SEB stimulation. In addition, there were no differences in TReg cell frequency or TCRVβ3 expression between participant groups. No differences were observed following PHA stimulation.

Existing studies investigating T cell responses to superantigens have focused on atopic dermatitis, with significant differences in methodology and participant groups, therefore these are not directly comparable to this study. The findings of such studies have also been inconsistent. Multiple papers have demonstrated reduced T cell proliferation to SEB stimulation in atopic dermatitis patients (263, 323); however, both Yudate et al. and Campbell et al. described an increase in proliferation (264, 324), whilst Tanaka and colleagues failed to detect any difference between participant groups (325). Several groups report TH2-biased immune responses to superantigens in atopic dermatitis (265, 266), but Campbell and colleagues failed to identify differences in cytokine expression (264) and Yoshino et al. reported decreased IL4 production to superantigens in atopic dermatitis patients with more severe disease (263).

The production of IL17 in response to SEB stimulation is not described in any previous literature regarding atopic dermatitis, and the omission of details regarding the sensitisation profile and co-morbidities of the study participants complicates interpretation; allergic diseases such as atopic dermatitis, asthma and food allergy tend to co-exist as part of the general atopic phenotype. Therefore, the description of homogenous atopic dermatitis groups in previous studies may be artificial. The study group in the present work had multiple clinically relevant sensitisations and allergic diseases, but segregation by disease (asthma, atopic dermatitis, perennial allergic
rhinitis) did not reveal any clustering; reduced T\textsubscript{H}17 responses were uniformly low in the entire atopic group.

The susceptibility to staphylococcal colonisation is best described in the setting of atopic dermatitis, but colonisation is also evident in asthma, allergic rhinitis and nasal polyposis. It is possible the presence of these superantigens modulates or contributes to the pathogenesis of the disease. In atopic dermatitis, this susceptibility to colonisation has been attributed to impairment of mucosal innate immune defences, including lipid content, pH changes, enhanced staphylococcal adhesion and reduced production of antimicrobial peptides such as the β-defensins, perhaps reflecting a T\textsubscript{H}2-polarised environment (250). However, the exact susceptibility factors for colonisation remain unclear in other atopic diseases. The reduction in T\textsubscript{H}17 responses following SEB stimulation in atopic individuals may represent an alternative explanation for the increased staphylococcal colonisation noted in allergic disease.

T\textsubscript{H}17 cells are characterised by the production of IL17A and IL22 and the expression of the cell surface chemokine receptor CCR6 (98). These cells function in the mucosal host defence against extracellular bacterial and fungal infections and are required for the production of mucosal mediators including β-defensins and CXCL8. Development of T\textsubscript{H}17 cells requires the expression of transcription factor STAT3. Impaired T\textsubscript{H}17 responses are a key feature of hyper-IgE syndrome (326) caused by a dominant-negative mutation in STAT3. This rare primary immunodeficiency disorder is characterised by an increased susceptibility to staphylococcal infection, eczematous lesions, recurrent abscesses, pneumonia and the development of pneumatoceles (102). In the setting of atopic dermatitis, decreased IL17 expression in the skin has been correlated to reduced expression of antimicrobial peptides (105, 327). In addition, a recent study by Hayashida and colleagues also demonstrated a reduced frequency of T\textsubscript{H}17 cells in the peripheral blood of atopic dermatitis patients following PMA/ionomycin stimulation compared to healthy controls, but the frequency of T\textsubscript{H}2 and regulatory T cells was similar (328). Moreover, fungal infections noted in allergic bronchopulmonary aspergillosis (ABPA) relate to exacerbation of asthma and atopic dermatitis and may reflect impaired T\textsubscript{H}17 immune responses; T\textsubscript{H}17 responses in ABPA have not been reviewed to date. However, the reduced T\textsubscript{H}17 response identified in this work cannot suggest atopy is an
immunodeficient state, and this IL17 reduction purely relates to superantigen-induced T cell responses.

The explanation for this reduction in T_{H17} responses to SEB in the atopic group remains unclear. This reduction was not observed following PHA stimulation, suggesting that sequestration of memory T cells at sites of allergic inflammation was not responsible for the reduced T_{H17} response (263). Peripheral blood samples were also taken outside of the pollen seasons (birch, grass), suggesting the results are not related to seasonal allergen exposure. Research has postulated that superantigens may act as allergens (233); SEB-specific IgE binds to mast cells causing subsequent histamine release and inflammation (329). Indeed, Leung et al. have confirmed serum-specific IgE antibodies directed against a number of staphylococcal superantigens in individuals with atopic dermatitis (329), contributing to exacerbation and persistence of allergic inflammation through IgE-mediated immune responses. However, the present work failed to identify SEB-specific IgE in eight of sixteen atopic participants in whom serum was available. Sensitisation to SEB is rare and usually only seen in individuals with severe atopic dermatitis (330), a condition present in only 25% of the highly atopic study group. Therefore, sensitisation to SEB is an unlikely factor involved in the reduced T_{H17} responses in atopy identified in this study.

A recent report by Taylor et al. demonstrated that staphylococcal superantigens induce functionally competent T_{Regs} in a Vβ-specific, dose-dependent manner, causing suppression of T cell responses and a mechanism for evasion of the immune response by superantigen-producing staphylococci (249, 331). Therefore, the downregulated T_{H17} responses to SEB in atopic individuals may relate to the immunosuppressive activity of SEB-induced functional T_{Regs} (249, 332). Indeed, this study identified a significant upregulation of FOXP3 expression following SEB stimulation in both CD4 and CD8 T cell populations. However, differences in the frequency of CD25^+FOXP3^+ or IL10^+ regulatory T cells between atopic and non-atopic individuals were not identified. In addition, the frequency of these regulatory T cells did not correlate with IL17 production or any other parameter. However, our assay was optimised for detection of CD154 and cytokines, rather than FOXP3 expression, and functional suppression experiments were not performed. Chronic superantigen exposure related to the increased colonisation in atopic individuals may
lead to “exhausted” or anergic T cells, perhaps reducing the suppressive activity of regulatory T cells, and conceivably inhibiting superantigen-induced cytokine expression. Indeed, Ou et al. demonstrated that superantigen stimulation was able to abrogate the suppressive function of regulatory T cells. It would be interesting to assess and compare suppressive function of superantigen-induced regulatory T cells in highly atopic individuals compared to healthy controls. In addition, it would be interesting to assess an extended panel of regulatory T cell markers, including CTLA-4, GITR, granzymes and perforin, in a larger data set of atopic individuals.

T cell responses to superantigens are Vβ-specific, therefore the frequency of TCRVβ3+ T cells was investigated (major restriction element for SEB), postulating that downregulated Th17 responses in atopy may relate to reduced TCRVβ3 expression. However, no difference in TCRVβ3 expression was revealed between atopic and non-atopic individuals, consistent with previous work in the setting of allergic rhinitis (333, 334), asthma (335) and food allergy (336); therefore, it can be presumed that reduced Th17 responses to SEB in atopy do not reflect TCRVβ3 expression. Interestingly, Bunikowski et al. identified increased TCRVβ3+ T cells in the skin of atopic dermatitis patients (337), and differences in TCRVβ expression exist between BAL and peripheral blood (335). Therefore, it is possible that a more comprehensive TCRVβ analysis will reveal differences within tissue compartments or in minor SEB targets such as TCRVβ6 or TCRVβ15.

Finally, activation-induced apoptosis may be increased in the setting of atopy (263, 266) and has been related to continuous antigen exposure. Yoshino et al. hypothesised T cells continually stimulated with superantigen in vivo are ready to undergo apoptosis following in vitro SEB stimulation; in contrast, T cells only moderately activated in vivo exhibit stronger in vitro responses to SEB stimulation (263). This study did not observe differences in cell viability or PHA response; however, formal apoptosis studies were not performed. Examination of Fas-FasL interactions, Bcl-2 or Annexin V activity would provide further insight.

In conclusion, this study identified a significant reduction in SEB-induced IL17 production in highly atopic polysensitised individuals compared to healthy non-atopic controls. Although the findings are preliminary, they may represent a mechanism underlying the increased susceptibility to staphylococcal colonisation in
atopic disease. If confirmed, this could have potentially important implications for the pathogenesis and treatment of atopic diseases.

Of course, characterisation of atopic participants in this work was incomplete. Information regarding staphylococcal colonisation rates was not available and SEB-specific IgE was analysed in only half the participant group. This reflects the availability and convenience of participant recruitment during investigation of allergen-specific T cells (Chapters 4-6). In this instance, it would be interesting to examine IL17 expression in a group of well-characterised atopic dermatitis patients, in whom research into superantigen responses is most abundant. This could include analysis of staphylococcal colonisation, superantigen-specific IgE and a more accurate disease scoring system. It would be of benefit to confirm this T\textsubscript{H17} reduction with alternative methods including T cell proliferation assays and supernatant analysis. Alternatively, relevant functional experiments investigating CXCL8 and β-defensin expression could determine IL17 dependence in staphylococcal colonisation. Additional analysis of T\textsubscript{H17} markers IL22 and CCR6 would also be of interest.

7.3 Conclusions

In summary, this study identified a downregulated T\textsubscript{H17} response to the superantigen SEB in highly atopic individuals that does not relate to T\textsubscript{Reg} cell frequency or TCRV\textsubscript{β3} expression. The findings of this study reflect the difficulty in ascertaining cause and consequence of this T\textsubscript{H17} reduction in atopy. It is possible reduced T\textsubscript{H17} responses are a consequence of the atopic phenotype itself, or possibly, may contribute to increased colonisation of superantigen-producing staphylococci. It would be of benefit to test this hypothesis with relevant functional experiments and in the context of other atopic diseases and superantigens.
Chapter 8: Optimisation of a PKH Proliferation Assay to Detect Allergen-Specific T cells

8.1 Introduction

Traditionally, T cell proliferation in response to allergen stimulation was investigated using the incorporation of $^3$H-thymidine or BrdU detection within newly synthesised DNA. However, these methods suffer from a number of drawbacks (338). Radioactive markers tend to decrease cellular viability and cannot provide information on cell phenotype. The dyes can transfer to unlabelled cells and background DNA synthesis from bystander cell populations can modify the true results. In addition, DNA synthesis analysis does not allow separation of viable daughter cells for further interrogation. Furthermore, normalisation of data depends on an equal number of cells in each sample and the use of radiation infers expensive specialised equipment.

Today, cell-tracking dyes have become more popular, overcoming the limitations of DNA synthesis techniques, and allowing detailed analysis of responding cell frequency and cytokine expression. This approach has permitted investigations into an array of allergic sensitisations including peanut, egg, grass pollen, cow’s milk and drug allergy (80, 81, 92, 225, 226).

Fluorescent cell division dyes fall into two categories: reactive compounds and lipophilic compounds. Reactive compounds, such as CFSE, diffuse into the cytoplasm of the cell to form stable amide bonds with cytoplasmic proteins (227). In contrast, lipophilic dyes, such as PKH (named after the discoverer Paul Karl Horan), bind non-covalently to the cell “lipid bilayer” plasma membrane via its aliphatic tails. The entire cell, and often the intracellular organelles, is usually stained due to lateral dye diffusion within the cell membrane. In both cases, the tracking dyes are equally distributed between daughter cells during division, with low fluorescence indicating divided or proliferating cells, compared to high fluorescence representing undivided cells. Cells can retain the dye for several weeks (339), giving the opportunity for long-term culture, without adverse effects on viability, cellular growth or proliferation. They are simple and quick to use and cells are easily isolated for further analysis.
Detection of daughter cells depends on the remaining fluorescence of these cells, and as a result, after many divisions there may be little dye left. If increasingly high concentrations of dye are used, breakdown of important proteins in antigen recognition and intracellular pathways may occur (227). The staining intensity of PKH dyes is more stable compared to CFSE because the hydrophobic interactions between the dye and cell membrane phospholipids remain stable for longer periods. The reproducibility of PKH dyes is high, and unlike CFSE, there is no waiting period for the staining intensity to stabilise (227). In addition, it is easier to accomplish brighter homogenous staining without causing damaging effects to protein and cell function. The loss of dye out of the cell, leading to culture artefact, is minimal.

There are multiple PKH dyes available including PKH2, PKH26 and PKH67. PKH2 was the first PKH dye to be established in proliferation research, however it was reported to reduce the viability of cells (49), was prone to dye transfer (340), and down-regulated surface expression of activation markers such as CD62L (49). In contrast, PKH26 showed minimal toxicity following staining, with the exception of excessive exposure to excitation lights (341). PKH67 is more stable within the lipid bilayer compared to alternative PKH dyes and can be identified on the FITC channel during flow cytometry, conferring simple compatibility with other fluorochromes and straightforward detection. However, detection of daughter cells is more difficult using PKH dyes compared to CFSE, therefore detailed optimisation steps are required for optimal staining and identification of a proliferating cell population (PKH\textsuperscript{Low}). This chapter focuses on the optimisation techniques utilised to improve PKH67 staining of cells, reduction of background proliferation and detection of allergen-induced proliferation.

### 8.2 Aims

The aim of this study was to optimise the PKH-staining of PBMC for the investigation of allergen-induced proliferation of T helper cells in allergic and non-allergic individuals. This included research into PKH dye concentration, culture media, IL2 supplementation and cell culture techniques.
8.3 Optimisation Steps

8.3.1 PKH Dye Concentration

According to the manufacturer’s instructions, 4μM of PKH67 dye is recommended for the staining of 2x10^7 PBMC. It is well established that high concentrations of PKH dyes can be toxic to the cells and cause breakdown or altered function of essential proteins in antigen recognition and intracellular pathways (227). Therefore, a lower concentration of 2μM PKH67 was explored to reduce the risk of toxicity and cost of the experiment.

PBMC were isolated from a non-allergic participant and immediately stained with either 2μM or 4μM PKH67 dye according to the manufacturer’s protocol (described in section 2.5.3). PBMC were resuspended in RPMI 1640 culture media and stimulated with SEB in triplicates for 7 days in 96-well round-bottomed plates at 1x10^6 PBMC/ml (2x10^5 cells/well). PBMC were refreshed with RPMI 1640 culture media on days 3 and 5, harvested into FACS tubes on day 7 and subsequently analysed using flow cytometry following antibody staining for surface markers (CD3-AlexaFluor700, CD4-ECD).

Figure 8.1. PKH^{Low} T cells following SEB stimulation with 2μM and 4μM PKH67 dye
Following SEB stimulation, the percentage of PKH^{Low} CD4 T cells was slightly higher in PBMC stained with 4\mu M PKH67 dye (Figure 8.1). The staining intensity was reduced when using 2\mu M PKH67, which after many divisions, may result in very little PKH dye remaining in extended culture. In addition, background proliferation was higher in PBMC stained with the lower 2\mu l concentration of PKH dye, perhaps suggesting dye is released more readily upon cell division at lower concentrations. Therefore, 4\mu M PKH67 dye was used in all future experiments.

8.3.2 Culture Media Supplementation

Preliminary PKH assays illustrated significantly high background proliferation in unstimulated PBMC, masking true proliferation of small responses often noted with allergenic stimulation. Alterations in culture media supplementation were investigated to reduce culture artefact. Comparisons were performed between three culture media sera supplementations:

- RPMI 1640 + 10\% FCS + 2mM L-glutamine + 1\% Penicillin-Streptomycin
- RPMI 1640 + 5\% human AB serum + 2mM L-glutamine + 1\% Penicillin-Streptomycin
- RPMI 1640 + 5\% autologous serum + 2mM L-glutamine + 1\% Penicillin-Streptomycin

PBMC were isolated from a non-allergic participant and immediately stained with 4\mu M PKH67 dye according to the manufacturer’s instructions. PBMC were resuspended in RPMI 1640 culture media supplemented with either 10\% FCS, 5\% human AB serum or 5\% autologous serum (plus 2mM L-glutamine and % penicillin-streptomycin) and stimulated with SEB in triplicates for 7 days in 96-well round-bottomed plates at 1x10^6 PBMC/ml (2x10^5 cells/well). PBMC were refreshed with the corresponding RPMI 1640 culture media on days 3 and 5, harvested into FACS tubes on day 7 and subsequently analysed using flow cytometry following antibody staining for surface markers (CD3-AlexaFluor700, CD4-ECD).

The supplementation of RPMI 1640 culture media with 5\% autologous serum reduces background PKH proliferation and is easily separated from participant peripheral blood samples (Figure 8.2). It is also a cheaper alternative to human AB serum.
Figure 8.2. PKH\textsuperscript{low} T cells following SEB stimulation in RPMI culture media supplemented with either FCS, human AB or autologous serum.
8.3.3 T Cell Proliferation Time Course

Antigen-induced proliferation is usually analysed after 7 days in culture (80, 81). However, a recent paper by Rimaniol and colleagues reported optimal proliferation detection following 11 days of grass pollen stimulation using a PKH26 assay (226). Therefore, a time course was performed to investigate the most favourable length of allergenic stimulation for the detection of a PKH\textsuperscript{Low} allergen-specific T cell population.

PBMC were isolated from a non-allergic participant, stained with 4µM PKH67 dye and stimulated with 500PNU/ml BPE in triplicates for up to 10 days in 96-well round-bottomed plates at 1x10^6 PBMC/ml (2x10^5 cells/well). PBMC were refreshed with RPMI 1640 culture media on days 3, 5 and 7, harvested into FACS tubes on days 3, 5, 7 and 10 and subsequently analysed using flow cytometry following antibody staining for surface markers (CD3-AlexaFluor700, CD4-ECD).

The highest frequency of PKH\textsuperscript{Low} CD4 T cells was identified on days 7 and 10 (Figure 8.3). The proliferation on days 3 and 5 were considerably smaller, although background proliferation was lower in unstimulated PBMC. The frequency of dead cells (Aqvid-positive) was slightly higher on day 10 compared to day 7, suggesting this time point is too long to retain cellular viability at reproducible levels (data not shown). Therefore, allergenic stimulation for 7 days is the optimal length for detection of PKH\textsuperscript{Low} T cell populations.
Figure 8.3. Frequency of PKH\textsuperscript{low} T cells following BPE stimulation for 3, 5, 7 and 10 days.
IL2 Supplementation and Culture Media Refreshment

IL2 is necessary for the growth and survival of T cells. Previous literature has utilised IL2 addition in prolonged cell culture in combination with refreshing cell culture media to improve proliferation, differentiation and survival of T cells (80). Therefore, this experiment sought to investigate the requirement of IL2 supplementation in PKH allergen-stimulated experiments.

PBMC were isolated from a non-allergic participant, stained with 4µl PKH67 dye and stimulated with 500PNU/ml BPE in triplicates for 7 days in 96-well round-bottomed plates at 1x10^6 PBMC/ml (2x10^5 cells/well), with or without addition of 20U/ml IL2 on days 3 and 5. PBMC were refreshed with RPMI 1640 culture media on days 3 and 5, harvested into FACS tubes on day 7 and subsequently analysed using flow cytometry following antibody staining for surface markers (CD3-AlexaFluor700, CD4-ECD).

Addition of IL2 during cell culture increased PKH proliferation within unstimulated T cells but did not increase the frequency of PKH^Low^ T cells following in vitro birch allergen stimulation (Figure 8.4). IL2 supplementation did seem to increase the frequency of live, viable PKH^Low^ T cells (Figure 8.4), although this did not seem to compensate for the increase in background proliferation. Therefore, IL2 was not added to cell culture in further PKH proliferation assays. In addition, cells were no longer refreshed with RPMI 1640 media on days 3 and 5 to reduce the removal of important growth factors in culture. The Aqvid live/dead antibody was used in all PKH experiments to distinguish and remove dead cells from the analysis.
Figure 8.4. Frequency of live and dead PKH<sup>low</sup> T cells following BPE stimulation with or without IL2 supplementation
8.3.5 **Culture Set Up**

Various allergen and cell concentrations were investigated to determine the optimal conditions for PKH proliferation analysis.

PBMC were isolated from three non-allergic participants, stained with 4μM PKH67 dye and stimulated with 500PNU/ml BPE in triplicates for 7 days at 0.125x10^6 – 2.5x10^6 PBMC/ml in 96-well round-bottomed plates. To investigate various allergen concentrations, PBMC were stimulated with 30-1000PNU/ml BPE in triplicates for 7 days at 1x10^6 PBMC/ml (2x10^5 cells/well). PBMC were harvested into FACS tubes on day 7 and subsequently analysed using flow cytometry following antibody staining for surface markers (CD3-AlexaFluor700, CD4-ECD).

<table>
<thead>
<tr>
<th>Cell Concentration (PBMC/ml)</th>
<th>Frequency of PKH^{Low} T Cells (% of CD4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125x10^6</td>
<td>0</td>
</tr>
<tr>
<td>0.25x10^6</td>
<td>0</td>
</tr>
<tr>
<td>0.5x10^6</td>
<td>0.8</td>
</tr>
<tr>
<td>1x10^6</td>
<td>2.34</td>
</tr>
<tr>
<td>2x10^6</td>
<td>1.99</td>
</tr>
<tr>
<td>2.5x10^6</td>
<td>1.84</td>
</tr>
</tbody>
</table>

*Table 8.1. Frequency of PKH^{Low} T cells at various cell concentrations in background-corrected BPE-stimulated CD4 T cells*

The optimal cell and allergen concentration for the detection of PKH^{Low} allergen-stimulated T cells was 1x10^6 PBMC/ml (2x10^5 cells/well) and 500PNU/ml, respectively (Table 8.1, Figure 8.5).
Figure 8.5. Frequency of PKH\textsuperscript{low} T cells at various BPE concentrations

<table>
<thead>
<tr>
<th>BPE Concentration</th>
<th>PKH\textsuperscript{low} T Cell Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>23.9%</td>
</tr>
<tr>
<td>30PNU/ml</td>
<td>23.2%</td>
</tr>
<tr>
<td>100PNU/ml</td>
<td>19.4%</td>
</tr>
<tr>
<td>500PNU/ml</td>
<td>11.5%</td>
</tr>
<tr>
<td>1000PNU/ml</td>
<td>23.2%</td>
</tr>
</tbody>
</table>

209
8.3.6 Restimulation Experiments

The purpose of this work was to optimise a PKH assay to use in combination with intracellular cytokine staining for the analysis of allergen-stimulated proliferating CD154\(^+\) T cells in allergic and non-allergic individuals. In preliminary experiments, restimulation experiments were performed to investigate CD154 expression in PKH\(^{\text{Low}}\) T cells.

Briefly, PBMC were isolated from a non-allergic participant, stained with 4µM PKH67 dye and stimulated with 500PNU/ml BPE, 10µg/ml PPD or 1µg/ml SEB in triplicates for 7 days at 1x10\(^6\)/ml (2x10\(^5\) cells/well) in 96-well round-bottomed plates. On day 7, PBMC were restimulated with antigen for 6 hours in the presence of Brefeldin A for the last 4 hours. PBMC were harvested into FACS tubes and subsequently analysed using flow cytometry following antibody staining for surface (CD3-AlexaFluor700, CD4-ECD) and intracellular (CD154-PB) markers.

CD154 was upregulated following restimulation with PPD or SEB in the corresponding antigen-stimulated PKH\(^{\text{Low}}\) T cells (Figure 8.6). In contrast, restimulation with BPE did not induce CD154 expression (Figure 8.6). This may necessitate the need for autologous APCs during restimulation to improve presentation of allergen to T cells after long-term culture.
Figure 8.6. CD154 expression in PKH^{Low} T cells following antigen restimulation

8.4 Optimised Study Protocol

Following analysis of the PKH proliferation assays, an optimised study protocol was devised. Briefly, PBMC were isolated and immediately stained with 4μl PKH67 dye according to the manufacturer's instructions. PBMC were resuspended in RPMI 1640 culture media (5% autologous serum, 2mM L-glutamine, 1% Penicillin-
Streptomycin) at 1x10⁶/ml (2x10⁵ cells/well) and stimulated with 500PNU/ml native allergen extract in triplicates in 96-well round-bottomed plates for 7 days without addition of IL2 or refreshment of cell culture media. Unstimulated PBMC were used as a negative control to assess background proliferation levels. PBMC were analysed by flow cytometry following antibody staining for surface markers (CD3-AlexaFluor700, CD4-ECD). All results were gated on single CD4⁺ T cells following a minimum collection of 50,000 CD4 events.

### 8.5 Discussion

Cell tracking dyes have become popular in the analysis of allergen-induced proliferation, such as CFSE and PKH, to allow for detailed analysis of allergen-specific T cell frequency and cytokine expression. This approach has permitted investigations into an array of allergic sensitisations including peanut, egg, grass pollen, cow’s milk and drug allergy (80, 81, 92, 225, 226).

The purpose of this small scheme of work was to optimise a PKH assay to use in combination with intracellular cytokine staining for the analysis of allergen-stimulated proliferating CD154⁺ T cells in allergic and non-allergic individuals. Although the assay was not used for this purpose, due to time and practicality constraints, it does provide a highly reproducible protocol for future work.

PKH cells bind non-covalently to the cell “lipid bilayer” plasma membrane via its aliphatic tails; the dye is equally distributed between daughter cells during division, with low fluorescence indicating divided or proliferating cells, compared to high fluorescence representing undivided cells. PKH dyes provide advantages over CFSE methods. The staining intensity of PKH dyes is more stable compared to CFSE because the hydrophobic interactions between the dye and phospholipids of the cell membrane remain stable for longer periods. The reproducibility of PKH dyes is high, and unlike CFSE, there is no waiting period for the staining intensity to stabilise (227).

This small project sought to optimise the PKH-staining of PBMC for the investigation of allergen-induced proliferation of T helper cells in allergic and non-allergic individuals. This included research into PKH dye concentration, culture media, IL2 supplementation and cell culture techniques.
The concentration of tracking dye utilised to stain PBMC is an important factor in optimisation of proliferation assays. This study revealed a higher concentration of PKH (4µM) was appropriate in proliferation assays to achieve bright initial staining and to reduce background proliferation in unstimulated cells. PKH dyes rely on non-covalent partitioning into the cell membrane as opposed to equilibrium or covalent interactions noted in CFSE staining (227). At higher concentrations, staining intensity is brighter, but both membrane integrity and cellular viability may be reduced; in contrast, lower PKH dye concentrations may result in fluorescence spectral shifts (342) and significant reductions in staining intensity before the initial cell division (227). In addition, the staining of PBMC with PKH dyes is instantaneous, therefore poor mixing or culture techniques may result in heterogeneous and wider intensity staining. These optimisation experiments identified PKH-staining of PBMC at a slanted angle within the Falcon tube improved the reproducibility of homogenous staining.

Supplementation of cell culture media is an important factor in proliferation assays to reduce culture artefact within the dividing cells of rare T cell populations. There is little research describing the effects of various media supplementations on proliferation assays. In this optimisation study, background proliferation and culture artefact was increased in those cells cultured in FCS supplemented media compared to human AB serum or autologous serum. In addition, the use of FCS in culture media increases cost. Supplementation of culture media with 5% autologous serum reduces background PKH staining in unstimulated PBMC and is easily separated from participant peripheral blood samples, providing an advantageous and cheaper alternative to both FCS and human AB serum.

Sampling of allergen-induced proliferation at different time points allows for the detection of initial division, rates of proliferation and reduction due to cell death (227). In these optimisation experiments, proliferation was small, but present, on day 3 of allergen-stimulated culture with optimal proliferation identified following 7 days of birch allergen stimulation. On day 10, proliferation was still high; however, the frequency of dead cells (Aqvid-positive) had increased, therefore reducing the proportion of overall proliferation after this time point. Therefore, it appears a 7-day allergenic stimulation system is optimal for the detection of a PKH\textsuperscript{Low} proliferating T cell population, as confirmed and used by a number of publications (80, 81).
Although Rimaniol and colleagues investigated proliferation on day 11 of culture (226), background proliferation in unstimulated T cells was higher than in this present work. In addition, they failed to discriminate dead cells with a viability dye, perhaps overestimating the percentage of grass-induced proliferation.

The discrimination of live, dead or apoptotic cells is a crucial factor in the analysis of PKH proliferation assays. Dead and apoptotic cells release varying amounts of dye through shedding of apoptotic vesicles and loss of membrane integrity (227). Therefore, this may provide an overestimation of the frequency of proliferating T cells. Therefore, this study investigated the proportion of PKH\textsubscript{Low} dead cells following \textit{in vitro} allergen stimulation with or without supplementation with the T cell survival factor IL2. The addition of IL2 moderately improved the viability of PKH\textsubscript{Low} T cells in culture, exhibiting a reduced frequency of Aqvid-positive cells. However, addition of IL2 also caused increased background proliferation in unstimulated PBMC. The use of the Aqvid viability probe allowed for the discrimination of dead cells, therefore IL2 addition was not performed in future experiments to reduce culture artefact.

The use of PKH proliferation dyes to investigate T cell responses to allergens is rare in the literature (particularly when compared to a high number of CFSE-related publications). However, Rimaniol \textit{et al.} successfully utilised a PKH26 assay to illustrate increased proliferation to timothy grass pollen allergen in allergic individuals with seasonal rhinitis (226). Different culture steps were utilised compared to the findings of this optimisation chapter. For example, cells were stained with 2.5µM PKH26 dye, but this may simply reflect differences in dye concentrations required between PKH26 and the PKH67 used in this work. In addition, PBMC were cultured in media supplemented 10% FCS. Indeed, the background proliferation seen in the Rimaniol study is higher than that noted in this optimisation work utilising autologous serum. This work was unable to investigate the cytokine profiles of the proliferating T cells; however, it did mention the assay could be developed to accommodate this analysis. Nevertheless, this research identified precursor frequency of grass-specific T cells similar to that noted in MHC tetramer-based work, exhibiting a higher frequency of grass-specific T cells in atopic individuals compared to non-atopic controls. This finding was also confirmed in this project investigating \textit{ex vivo} CD154\textsuperscript{+} T cell responses to grass allergen (Chapter 5).
In preliminary experiments, CD154 expression was identified in mitogen-stimulated (PPD, SEB) PKH\textsuperscript{Low} cells, but not in those stimulated with birch pollen allergen. This suggests addition of autologous APCs in the restimulation culture is required for measurable cytokine responses.

The PKH dye dilution technique suffers from drawbacks compared to other methods, and perhaps explains minimal use of this technique to examine allergen-specific T cell responses. Dye dilution techniques take longer to complete compared to other methods such as MHC class II tetramer analysis or cytokine capture, not to mention the \textit{ex vivo} CD154-based methods described in this project. However, this extra time required for analysis is compensated by the simpler quantification of rare T cell populations that have undergone multiple divisions to expand their cell frequency (343). However, PKH experiments also require a significantly larger number of cells. Compared to the \textit{ex vivo} CD154 technique previously described, PKH analysis requires at least double the number of PBMC. In addition, the viability and frequency of cells after PKH staining and \textit{in vitro} culture is significantly reduced, relating to the large number of centrifugation steps and transfer of cells between Falcon tubes. Nevertheless, the agreement between other assays such as tetramers and ELISPOT is good (344) and allergen proliferation studies form an important fragment of allergen-specific T cell research.

\textbf{8.6 Conclusions}

In summary, this chapter focused on the optimisation steps required to develop a PKH67 proliferation assay to investigate \textit{in vitro} proliferation following allergenic stimulation. PKH dyes have been rarely used in allergen proliferation research and to date, PKH67 has not been utilised in any allergy-related publications.

There are multiple biological applications of this PKH assay. Firstly, cytokine profiles of proliferating T cells can be analysed by restimulation with allergen on day 7 in the presence of BFA followed by intracellular cytokine staining. Further development of the assay would be required for optimal cytokine detection. For example, addition of autologous APCs may improve presentation of allergen to T cells after long-term culture, of which would also need to be PKH-stained to avoid amalgamation of fresh APCs and proliferating T cells (both PKH\textsuperscript{Low}). In addition, phenotype of proliferating T cells could be easily analysed by addition of maturity
marker antibodies CD27 and CD45RA. This would also allow for the comparison of PKH results with the *ex vivo* CD154-based detection system in terms of frequency and phenotype of allergen-specific T cells. This has recently been reviewed with CFSE and CD154 comparisons (244), but in very few participants, and no detail of phenotype or regulatory T cell function was discussed. This assay could also be used to investigate B cell involvement in allergic disease. To date, little is known about allergen-induced B cell proliferation and simple staining with CD19/CD20 and CD27 may provide interesting findings regarding differences in memory B cell activity between allergic and non-allergic individuals.

Overall, the PKH proliferation assay is a useful tool for assessing allergen-specific T cell responses and may provide complementary information to the *ex vivo* CD154-detection techniques previously described.
Chapter 9: Assessment of Sensitisation Profiles in Adults with Pollen-Food Syndrome using ISAC Microarray Technology

9.1 Introduction

Pollen-Food Syndrome (PFS) is caused by sensitisation to homologous panallergens that are common to aeroallergens and food proteins. IgE antibodies produced against one allergen can bind homologs originating from different allergens (164). This condition is estimated to affect 2% of the UK population (162). Typical symptoms are oral itching, swelling and oedema, appearing within minutes of ingesting the food. The major allergens responsible for this condition fall into three main families: the Pathogenesis-Related 10 (PR-10) protein family, Lipid Transfer Proteins (LTPs) and profilins. In Northern Europe, PFS most often affects birch pollen allergic individuals who react to PR-10 panallergens in the group of *Rosaceae* fruits, including apples, pears, apricots, cherries, peaches and plums (155).

Skin prick testing with allergen extracts has limited utility in the setting of PFS. The extracts are standardised in the context of major allergens rather than panallergens and sensitivity is poor. Furthermore, standardised sources for profilins and LTPs are difficult to source. The introduction of recombinant DNA technology has allowed for manufacture of stable and pure allergen molecules that may allow for improved sensitivity and specificity of diagnostic tests for allergic sensitisation (345). The concept has been further developed as microarray technology, allowing for analysis of large numbers of allergens in parallel and the differentiation between primary allergy and cross-reactivity, therefore assisting with the diagnosis of complex food allergies.

Experience suggests that UK patients with PFS are typically birch-sensitised, but information regarding the sensitisation profiles of individuals with PFS in the UK has been limited until recently. Detailed information on sensitisation profiles may assist in improved management and treatment of the condition as well as selection of patients for specific immunotherapy.
9.2 Aims

The aims of this study were to investigate the clinical phenotypes and sensitisation profiles of adults with PFS in a secondary care setting using microarray technology.

9.3 Study Protocol

Full information regarding contributions and protocols is located in Chapter 2: Materials and Methods.

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Table 9.1. Participant demographics

Twenty-four highly atopic adult participants with symptoms suggestive of PFS (local oro-pharyngeal symptoms upon exposure to one or more fruits, with or without systemic symptoms) were recruited from the adult allergy clinic at Brighton and Sussex University Hospitals Trust. The participants exhibited multiple allergic diseases and sensitisations (Table 9.1).

Participants were skin prick tested according to clinical protocols for the following extracts: early pollinating tree mix, mid pollinating tree mix, grass pollen, birch pollen, cat dander and house dust mite (ALK-Abello, UK). A wheal and flare response greater than 3mm compared to a negative control solution was categorised as positive.

The classification of food allergy in each participant was analysed using a standardised physician-administered food allergy questionnaire including analysis of 38 plant-derived foods implicated in PFS. Participants were categorised into three
groups for each food item: [1] allergic (clinical symptoms upon exposure), [2] non-allergic (consistently tolerates the food without symptoms) and [3] not known.

IgE sensitisation profiles were determined using ISAC ImmunoCAP microarray technology involving semi-quantitative measurement of serum IgE antibodies directed against over 100 allergen components. Briefly, ISAC microarray slides were washed and placed into a humidity chamber. 20µl of participant serum sample was placed on each allergen microarray chip (four chips to one slide). The slides were incubated for 2 hours before washing and addition of the IgE detection antibody for 60 minutes. Slides were rewashed and allowed to completely dry before analysis on the Genepix 4000B microarray reader with optimised settings (532nm, 100% power, and PMT gain 590). Results were expressed as ISAC standardised units (ISU/L) and results greater than 0.3ISU/L were categorised as positive. Microarray images were exported to the MIA software to generate sensitisation reports. Statistical comparisons were performed using the student’s t test with p values < 0.05 deemed significant.

9.1 Results

9.1.1 Implicated Foods and Reported Symptoms

As expected, the most implicated foods causing local oro-pharyngeal symptoms were those of the *Rosaceae* fruit family (Figure 9.1). On average, the mean number of foods reported by patients to cause symptoms was 7.5. Allergic symptoms to raw potato, kiwi fruit, carrots and peanuts were also common. Very few participants reported allergic symptoms to citrus fruits, banana or cucumber.

All participants reported local oro-pharyngeal symptoms typical of PFS, such as itching and tingling of the lips, mouth and throat. However, eight (33%) participants also reported severe symptoms associated with some foods. Ingestion of tree nuts and peanuts caused breathing difficulties in seven participants and one participant reported similar symptoms upon raw carrot ingestion. Apple, pear, banana, melon and tomato induced diarrhoea soon after ingestion in four subjects. In addition, two participants reported facial angioedema after ingestion of peach and raw apple. These symptoms were self-limiting and further medical attention was not required. There were no reports of symptoms describing systemic anaphylaxis upon ingestion of the foods.
Unfortunately, the results for individual allergens were clearly not a good guide of allergy or tolerance to the particular allergen in the context of PR-10 sensitisation, although this was not formally analysed.
Figure 9.1. Food reported to cause local oro-pharyngeal symptoms in participants with PFS
9.1.1 Cross-Reactive Components

All twenty-four participants were sensitised to the Bet v 1/PR-10 protein cluster indicated by the detection of IgE specific for Bet v 1, the major birch pollen allergen, and other homologous PR-10 proteins in foods. Interestingly, four participants did not report spring rhinitis despite sensitisation to the Bet v 1/PR-10 protein cluster. Seventeen participants (71%) were monosensitised to the PR-10 protein family with the remaining participants co-sensitised to PR-10 and either LTPs or profilin. Sensitisations to the non-cross-reactive aeroallergens is summarised in Figure 9.2.

Four participants were co-sensitised to the PR-10 protein family and profilin protein cluster demonstrating specific IgE to both Bet v 1 and Phl p 2 (timothy grass). All profilin-sensitised individuals were allergic to grass pollen with seasonal rhinitis in the summer.

Three participants were co-sensitised to the PR-10 protein family and LTPs with positive results for Pru p 3 (peach) and Art v 3 (mugwort). Co-sensitisation to both profilin and LTPs was not observed.
Figure 9.2. Specific IgE directed against non-cross-reactive Aeroallergen molecules. Both the allergen and allergen component are described.
9.1.1 Co-Sensitisation to Profilin

Participants with co-sensitisation to the PR-10 protein and profilin clusters reported allergic reactions to a significantly larger range of foods compared to individuals monosensitised to PR-10 proteins \((p=0.0036, \text{ Figure 9.3})\). Although allergic symptoms to melon, papaya, banana and citrus fruit were rare, reactions to these foods were predominantly seen in individuals with sensitisation to profilin.

![Figure 9.3](image.png)

**Figure 9.3.** Mean number of reported foods causing oro-pharyngeal symptoms in participants either PR-10 monosensitised or co-sensitised to profilin

9.2 Discussion

Data relating to the sensitisation profiles of adults with PFS has been lacking in the UK, until recently. This study aimed to define patterns of sensitisation using microarray analysis and component-resolved diagnosis. This study is complementary to larger ongoing studies investigating adult sensitisation profiles within UK and European populations (162, 267).

Oro-pharyngeal symptoms were reported in all subjects of the study cohort, with the *Rosaceae* fruits and nuts reported as the most common food allergens. Other common food allergens eliciting symptoms included potato (when peeling the raw product), kiwi fruit and tomato. Most of the participants illustrated allergies to multiple foods. The foods reported were comparable to the previous work of Skypala.
and colleagues, who described reported symptoms to apple, peach, pear and other *Rosaceae* fruits in a larger UK population (162). In addition, the EuroPREVALL study demonstrated high sensitisation rates and reported symptoms to hazelnut, apple and peach (267).

Eight participants reported serious symptoms upon ingestion of foods including difficulty breathing and facial angioedema, but no subjects described symptoms consistent with systemic anaphylaxis. The more severe reactions related to tree nuts and peanuts in the context of sensitisation to PR-10 proteins. Severe reactions were also described with both raw and cooked peanuts, confirming the findings of Mittag *et al.* that the PR-10 protein Ara h 8 is stable and not as susceptible to heat degradation compared to other PR-10 proteins (346).

Sensitisation to the PR-10 protein family was reported in all participants, suggesting PFS relates mostly to birch pollen allergy in this small population. The results are consistent with other European countries with similar geographical climate and flora (347-349). For example, Wensing and colleagues identified 85% of participants were sensitised to PR-10 proteins from a Dutch population associated with IgE against apple, hazelnut and peach (349). Similarly, Hansen *et al.* demonstrated 75% of individuals were sensitised to Cor a 1, the major hazelnut allergen and Bet v 1 homolog, within a Northern European population (Switzerland, Denmark) using similar ImmunoCAP ISAC microarray technology (350). A systematic review of European sensitisation patterns by Schmidt-Andersen and colleagues also established PR-10 protein sensitisation and associated birch pollen allergy was common in PFS patients in Italy, Austria, Switzerland, Germany and the Netherlands, although this meta-analysis is confounded by differences of methodology and sample size between the studies (155). The results of the present study appear to conform to patterns of sensitisation reported in Northern and Central Europe.

LTPs were first described by Fernandez-Rivas and colleagues; sensitisation to LTPs in Mediterranean countries is a major cause of local and systemic reactions to *Rosaceae* fruits (351, 352). LTPs are present in a variety of pollen sources, particularly those native to the Mediterranean, such as mugwort (Art v 3), in addition to many plant-derived foods, most notably peach (Pru p 3). The present work illustrated co-sensitisation to PR-10 proteins and LTPs in a small proportion of the
study population. However, the clinical relevance of co-sensitisation to LTPs in this small cohort is doubtful: two of three sensitised individuals reacted to peach, but none described systemic reactions to peach or any other plant-derived foods. The clinical relevance of LTPs remains unknown; however, recent publications have highlighted a possible role for LTPs outside of the Mediterranean context. Recent literature has demonstrated allergy to LTPs in peach-allergic Austrian individuals is associated with a strong risk for systemic reactions (353). Furthermore, allergy to the hazelnut LTP allergen Cor a 8 caused severe reactions in allergic participants in the Netherlands (354). In addition, Skypala et al. illustrated 36% of individuals with severe reactions to plant-derived foods were sensitised to LTPs and reported allergies to tree nuts and peanuts (355). High rates of LTP sensitisation to hazelnut Cor a 8 were noted in a Spanish population (350) however in this study, severe reactions were associated with PR-10 sensitisation compared to LTPs. Larger populations need to be studied to further investigate the role of sensitisation to LTPs in PFS in the UK.

Although all patients were skin prick test positive to birch extract and tested positive for IgE directed against the PR-10 protein cluster, four participants had no history (past or present) of spring rhinitis. Two of these participants had symptoms restricted to the summer months only, one participant had chronic symptoms relating to house dust mite allergy, and one had no history of rhinitis at all. This observation highlights the need to investigate sensitisation to panallergens in patients presenting with symptoms related to plant-derived foods, regardless of their rhinitis history.

In this study, all individuals with profilin sensitisation were allergic to grass pollen and had allergic rhinitis in the summer months. This confirms the findings of previous literature illustrating an association between grass pollen allergy and profilin sensitisation (167, 168). In addition, those individuals sensitised to profilin also reported a significantly larger number of food allergies compared to participants who were monosensitised to PR-10 proteins. This finding has been replicated in previous reports (167, 168, 349) and suggests profilin sensitisation is clinically important in some PFS patients within the UK. Similarly, allergies to citrus fruit, melon and banana were also reported in the present study, although at a lower incidence, in keeping with a previous study identifying profilin sensitisation in the Mediterranean (168). This study did not identify individuals monosensitised to
profilins, perhaps suggesting this situation is rare in the UK (although sample size was small).

The clinical relevance of sensitisation to profilins is under debate due to discrepancies in the findings of previous reports. For example, numerous studies have illustrated profilins have little or no clinical relevance (349, 356) whereas other studies have demonstrated it is clinically relevant (168, 351, 352). It is possible these differences relate to population selection criteria within each study (168) as well as geographical variations in the prevalence of profilin sensitisations.

This study successfully investigated the sensitisation profiles of adults with PFS in a small UK population using microarray technology and component resolved diagnosis. This method allows for the analysis of large numbers of allergens in parallel and enables the differentiation between primary allergy and cross-reactivity, therefore assisting in the diagnosis of complex food allergies. The use of microarray technology in the diagnosis of allergic disease may lead to improvements and development of more successfully targeted immunotherapy as well as determining individuals most suited to this line of treatment.

However, in the context of PR-10 sensitisation, the method had poor predictive value on an allergen-source basis. This is in keeping with the results of Ebo et al, who demonstrated that ISAC technology could not discriminate between sensitisation and true allergy (357). Despite this, the assay is clearly able to define patterns of sensitisation to panallergens in addition to type I food allergens and therefore represents a useful research tool.

Component resolved diagnosis and microarray technology allows accurate identification of the specific molecules causing disease by analysis of multiple allergens in a simple and fast technique. Moreover, only small amounts of patient serum are required, providing advantages in the analysis of IgE profiles in those individuals unable to provide sufficient serum, such as young children with early onset allergies. In addition, the microarray chips are easily adapted to increase the number of allergen components as more are manufactured. This technique may also prove useful in the analysis of sensitisation profiles before, during and after treatment as well as providing possible applications in autoimmunity and infectious disease.
9.3 Conclusions

In conclusion, this study cohort conforms to the Northern European pattern of birch-pollen associated PFS with cross-reactivity between the major birch pollen allergen Bet v 1 and homologues in food proteins. Sensitisation to LTPs and profilins was also noted, but the clinical relevance of these remains to be elucidated. Profilin sensitisation was associated with reactions to a significantly larger number of foods compared to PR-10 monosensitised individuals. Peanuts and tree nuts were reported as the highest risk foods although symptoms consistent with systemic anaphylaxis were not described. Overall, the microarray technology is a useful tool for distinguishing primary allergy and cross-reactivity and may aid in the determination of patients most suited to specific immunotherapy.
Chapter 10: Concluding Remarks

10.1 CD154 as a Marker of Allergen-Specific T Cells

There is considerable evidence of a role of T helper cells in the initiation and maintenance of allergic disease. The investigation of these allergen-specific T cells at the single cell level is important for a better understanding of tolerance, sensitisation and desensitisation to common environmental allergens.

CD154 is a T cell activation marker that is transiently expressed upon ligation of the T cell receptor, therefore providing direct access to an antigen-specific population following ex vivo stimulation. This method provides significant advantages for the detection of allergen-specific T cells, particularly the ability to phenotype responding cells directly ex vivo and the simplicity of the protocol. Using a modified approach, the method is compatible with cell sorting to provide viable allergen-specific T cells for functional studies.

The aim of this PhD project was to optimise an ex vivo allergen stimulation system followed by detection of responding CD154⁺ T cells, and to apply the method to study these allergen-specific CD154⁺ T cells in allergic and non-allergic individuals. This is the first report of detailed phenotyping in allergic disease using this method.

We were able to resolve CD154⁺ T helper cell populations in both allergic and non-allergic subjects following ex vivo stimulation with cat allergen, grass pollen allergen and birch allergen. The frequency of responding cells was greater in allergic compared to non-allergic participants with respect to grass pollen – a true seasonal allergen – in keeping with previous reports (79). For cat allergen (a true perennial allergen) and birch pollen (a seasonal allergen with perennial characteristics in our assay system) this difference was not evident. The frequency of responding cells was far higher than estimates derived from tetramer-based research (91). Tetramers only detect a proportion of the allergen-specific response, but it is possible that the CD154⁺ population identified in our assay was enriched rather than specific for allergen-specific T cells. This may be particularly evident in the case of birch pollen allergen, where the response is likely to include T cells responding to plant-derived PR-10 homologs.
In this regard, it should be noted that we have not formally demonstrated the CD154$^+$ population identified was truly allergen-specific. Functional studies to demonstrate allergen-specificity were beyond the scope of this project but will be required if the method is to gain wider acceptance in allergy research. Nevertheless, the concordance between our findings and previous literature is encouraging, and the CD154 assay provides significant advantages over previous techniques, with potential clinical and biological applications.

10.2 Allergen-Specific T Cell Cytokine Responses

Substantial evidence now suggests that tolerance to airborne allergens is an active process dependent upon a balanced T cell response that may include T$_{H1}$, T$_{H2}$ and regulatory T cell responses. However, the exact nature of the relationship between these three T cell subsets in allergy and health remains unclear. A significant aim of this PhD project was to characterise and phenotype allergen-specific T cells following \textit{ex vivo} allergen stimulation based on CD154 expression.

This project consistently identified T$_{H2}$ polarised allergen-specific responses in allergic individuals, consistent with previous literature (77, 144, 153). There was no difference in the frequency of T$_{H1}$ cells in allergy and health, indicating a mixed cytokine profile as described for a number of allergens (90, 225). The predominant response in tolerant individuals consisted of a CD154$^+$IL10$^+$ T$_R1$-like and/or T$_{H1}$ cell population. It seems the relationship between these T cell subsets rather than their absolute numbers distinguishes allergy from health, as T$_{H2}$ responses were also seen in non-atopic participants but appeared to be counterbalanced by increased T$_{H1}$ and T$_R1$-like responses. Individuals who were atopic, but not sensitised to the test allergen, and individuals who were asymptomatic but sensitised to the test allergen mounted a similar T cell response to those with true clinical allergy. Therefore, although the method could accurately discriminate between non-atopic individuals and allergic individuals, it was not able to discriminate between those with true clinical allergy and those who are sensitised or even atopic but not sensitised to the test allergen. It might be possible to do so with the addition of extra markers to the panel, but alternatively the determinants of allergic disease as opposed to the presence of sensitisation may be more downstream and not detectable using T cell-
based assays; meanwhile, the observation remains interesting as it suggests an intrinsic tendency to allergen-specific T\(_H\)2 cell involvement in the atopic phenotype.

This thesis also demonstrates a significant and positive correlation between the concentration of circulating birch-specific IgE and the frequency of T\(_H\)2 cells, rather than the T\(_H\)2:T\(_H\)1 ratio, suggesting the absolute T\(_H\)2 cell frequency represents an important parameter in allergic sensitisation. It seems likely that the advent of more sensitive T cell assays will improve understanding of the relationship between humoral and T cell responses to allergens. To further refine this relationship, future work will include the analysis of grass and cat-specific IgE and IgG4 levels for comparison with T\(_H\)2 cell frequency.

Further analysis revealed significant correlations between T\(_H\)2, T\(_H\)1 and T\(_R\)1-like responses, such that in health, the frequencies of these T cell subsets increased together. By contrast, the relationship between these T cell subsets was dysregulated in allergy with abrogation of the IL10 and/or T\(_H\)1 responses and a significantly higher T\(_H\)2:T\(_H\)1 ratio. This further supports the fundamental work of Akdis et al. describing a “fine balance between T\(_H\)1, T\(_H\)2 and T\(_R\)1-like responses” (77) with a switch in this cytokine profile playing an integral role in the allergic phenotype.

### 10.3 Maturation Phenotype of Allergen-Specific T Cells

This thesis also describes the phenotype of responding CD154\(^+\), T\(_H\)1 and T\(_H\)2 T cell populations following \textit{ex vivo} allergenic stimulation. This type of investigation has not been performed in such detail and information regarding T\(_H\)1 and T\(_H\)2 cell phenotype is a novel aspect of this work.

T cell responses to perennial allergens have been described as early differentiated in both allergic and non-allergic individuals (90), consistent with the findings of this project. In keeping with tetramer-based studies investigating seasonal allergens (78), responding grass and birch-specific T cells were predominantly CD45RA-negative, CD27-positive, consistent with an early-differentiated memory phenotype, with a particularly significant proportion of effector memory T\(_H\)2 cells noted in grass pollinosis. Consequently, the frequency of memory T\(_H\)2 cells was higher for seasonal allergens compared to the perennial cat allergen. Research into more extensive
memory marker panels, including chemokine receptors, may provide more detailed and accurate insights into the phenotype of these allergen-induced T cells.

10.4 Seasonal Allergen Exposure and Immunotherapy
This thesis also presented pilot data regarding modulation of T cell responses during the pollen season and following allergoid immunotherapy. The results were inconclusive with current participant numbers, not helped by low birch pollen counts in the 2012 season. Although consistent changes in the frequency of Th1 and Th2 cells were not observed during the season, it is possible that seasonal birch pollen exposure increased the Th2:Th1 ratio in allergic individuals; in contrast, this ratio remained low in non-allergic subjects both in and out of season. This is in keeping with previous work and demonstrates the utility of the assay in research monitoring.

Only three patients undergoing allergoid immunotherapy were studied. We observed marked reductions in the Th2:Th1 ratio, although an increase in the IL10 response was not consistently observed.

10.5 T cell Responses to the Staphylococcal Superantigen SEB
Colonisation with superantigen-producing staphylococci is common in atopic diseases and evidence increasingly favours an important role for superantigens as a co-factor in the pathogenesis of allergic diseases. However, the susceptibility factors regarding these increased colonisation rates remain unclear. In addition, little is known of T cell responses to superantigens in allergy and health.

This thesis demonstrated a significant reduction in Th17 responses in highly atopic polysensitised individuals compared to healthy non-atopic controls that does not reflect differences in regulatory T cell frequency or TCRVβ3 specificity. Although the findings are preliminary, this reduction in the Th17 response may provide an alternative explanation for increased bacterial colonisation in atopic diseases. If confirmed, this could have potentially important implications for the pathogenesis and treatment of atopic diseases.

10.6 Pollen-Food Syndrome
The sensitisation profile of patients with PFS in the UK is likely to be similar to the rest of Northern Europe, but in 2009 there was little formal data. In collaboration
with Dr Rabia Rashid (academic trainee), we reported the clinical and sensitisation profile of a small group of adults with PFS using microarray technology.

The findings of this study conformed to the Northern European pattern of birch-pollen associated PFS with cross-reactivity between the major birch pollen allergen Bet v 1 and homologues in plant-derived foods. Co-sensitisation to lipid transfer proteins was also evident in some patients, but their clinical phenotype was no different to the birch-monosensitised cohort. The significance of LTP sensitisation in the UK remains an active research area.

10.7 Future Work

In summary, this project has illustrated an important relationship between T\textsubscript{H2}, T\textsubscript{H1} and T\textsubscript{R1}-like responses in allergy and tolerance. However, allergen-specificity was not formally demonstrated. It is likely the CD154\textsuperscript{+} T cell population is enriched for allergen-specific T cells; the frequency of CD154\textsuperscript{+} T cells, expressing either IL4, IFN\textgamma or IL10, related to humoral responses and were consistent with previous reports. However, it would be of benefit to formally demonstrate allergen-specificity of these T cell responses. In this instance, the CD154\textsuperscript{+} T cells could be separated via magnetic-activated cell sorting (MACS) for re-stimulation experiments with the index allergen and alternative antigens.

It would be interesting to compare the frequency, cytokine profile and phenotype of \textit{ex vivo} CD154\textsuperscript{+} responding T cells with the \textit{in vitro} PKH\textsuperscript{Low} allergen-stimulated T cells. The CD154 assay was recently evaluated alongside a CFSE dye dilution technique, in which prolonged culture abrogated the specificity of CD154 expression, but this was analysed in very few participants and no detail or phenotype of regulatory T cell function was discussed (244).

It would be interesting to analyse the modulation of T cell responses during seasonal allergen exposure in a larger group of participants and in a better pollen season. This work is currently under investigation by Dr Nicola Gray. In addition, it would be beneficial to investigate T cell responses following immunotherapy in more detail using the CD154 assay.

The similarities between allergic and sensitised individuals suggest T cell responses are not responsible for the manifestation of clinical symptoms. It would be
interesting to further examine the differences in these participant groups, perhaps with the investigation of B cell function and regulatory mechanisms in mast cell and eosinophil function. It would also be interesting to investigate a larger T\(_H2\) cytokine repertoire, such as IL5 and IL13, to compare the findings more directly with previous literature. Additional T cell subsets, such as T\(_H17\) cells, could easily be investigated.

There are multiple avenues for improvement and further analysis regarding T cell responses to superantigens in allergy and health. An interesting finding of this PhD was the unexpected reduction in T\(_H17\) cells in highly allergic individuals following stimulation with the superantigen SEB. T\(_H17\) cells are known to up-regulate skin β-defensins and protect the skin from bacterial infection. Reduced T\(_H17\) responses to superantigens may therefore explain increased staphylococcal colonisation in eczema, with implications for the pathogenesis and treatment. I have been awarded an MRC Centenary Award to investigate this T\(_H17\) reduction in eczema and its biological relevance to the production of anti-microbial peptides in the skin.

10.8 Final Remarks

This project firmly illustrates an important balance between T\(_H2\), T\(_H1\) and T\(_R1\)-like immune responses to allergens in allergy and health, supporting the work of Akdis and colleagues and providing complementary and novel data in the field of allergen-specific T cells. The T\(_H2\):T\(_H1\) ratio may be of clinical relevance in food and drug sensitivity and the CD154 assay illustrates success in tracking changes during immunotherapy and seasonal allergen exposure. Moreover, allergic individuals illustrate significant differences in T\(_H17\) responses to superantigens, perhaps reflecting increased colonisation rates in these individuals.
Chapter 11: Posters, Presentations and Publications

11.1 Posters


“Atopic donors illustrate reduced *ex vivo* T cell responses to superantigens”, BSACI Annual Conference 2011, Nottingham, UK

“T cell biology in allergic disease” BSMS Postgraduate Research Symposium 2011, Brighton, UK

“Pollen-Food Syndrome in Sussex, United Kingdom: clinical phenotype and component-resolved diagnosis in secondary care”, BSI Annual Conference 2010, Liverpool, UK

11.2 Oral Presentations

“Reduced T_h17 responses to the superantigen Staphylococcal Enterotoxin B (SEB) in chronic rhinosinusitis”, BSACI Annual Conference 2012, Nottingham, UK

“Ex *vivo* analysis of CD154+ T helper cells in atopic birch-allergic individuals compared to non-allergic controls, American Academy of Allergy, Asthma and Immunology (AAAAI) Annual International Conference 2012, Florida, USA

“Atopic individuals illustrate reduced *ex vivo* T cell responses to superantigen”, European Academy of Allergy and Clinical Immunology (EAACI) Allergy Summer School 2011, Edinburgh, UK

11.3 Publications


Chapter 12: Appendices

12.1 BD Biosciences LSR II Flow Cytometer Settings

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Table 12.1. Maximum absorption and emission spectra for commonly used fluorochromes

12.2 FMO Controls

12.2.1 Overnight Allergen Stimulation (OAS) Panel

Antibodies:
- CD3-AlexaFluor700
- CD4-PerCP
- CD25-APC-Cy7
- CD27-FITC
- CD45RA-ECD
- CD154-PB
- IFNγ-PeCy7
- IL4:PE
- IL10-APC
Figure 12.1. FMO controls for the OAS antibody panel (Page 1 of 5)
FMO controls for the OAS antibody panel (Page 2 of 5)
FMO controls for the OAS antibody panel (Page 3 of 5)
FMO controls for the OAS antibody panel (Page 4 of 5)
FMO controls for the OAS antibody panel (Page 5 of 5)
12.2.1 *SEB Panel*

Antibodies:
- CD3-AlexaFluor700
- CD4-ECD
- CD8-PerCP
- CD154-PB
- IFNγ-PeCy7
- IL4-PE
- IL10-APC
- IL17-FITC
Figure 12.2. FMO controls for the SEB antibody panel (Page 1 of 5)
FMO controls for the SEB antibody panel (Page 2 of 5)
FMO controls for the SEB antibody panel (Page 3 of 5)
FMO controls for the SEB antibody panel (Page 4 of 5)
FMO controls for the SEB antibody panel (Page 5 of 5)
Glossary

Allergy
An IgE-mediated inappropriate immune response to common environmental allergens such as food proteins and pollens

Anaphylaxis
An IgE-mediated generalised allergic reaction causing both respiratory and cardiovascular compromise

Antigen Presenting Cell (APC)
A cell of which the main function is to present antigen to T cells via MHC:peptide complexes

Asthma
IgE-mediated inflammatory disease of the lower respiratory tract

Atopic March
The sequential development of clinical symptoms and multiple allergic conditions during infancy and childhood normally in the order of eczema, asthma and allergic rhinitis

Atopy
A genetic predisposition to produce IgE antibodies against environmental proteins: these individuals may develop multiple allergies, will have high eosinophil counts and high IgE levels in circulation

B Cell
A lymphocyte of the immune system with principle functions in the humoral immune response including acting as an APC and production of antibodies

CD3
A protein complex (cluster of differentiation) forming part of the T cell complex expressed on all T cells

CD4
A glycoprotein co-receptor expressed on T helper cells that recognises MHC class II molecules

CD8
A glycoprotein co-receptor expressed on T cytotoxic cells that recognises MHC class I molecules

CD25
A protein complex, the alpha chain of the IL2 receptor, expressed on activated T cells and used as a marker to identify regulatory T cells in conjunction with FOXP3

CD27
A receptor important for the maintenance and maturity of T and B cells: present on early-differentiated T cells

CD45RA
A receptor important for the maintenance and maturity of T cells: present on naive T cells

CD154
T cell activation marker transiently expressed on CD4 T cells following TCR ligation

Cytokine
Signalling molecule secreted by a cell for regulation and intercellular communication

Cytokine Capture
Cytokine capture assays involve the secretion and subsequent capture of cytokines from activated T cells and detection by addition of fluorochrome-labelled secondary antibodies specific to the cytokine of interest
Early Phase Response

Allergen cross-links the FceRI receptor-bound IgE leading to mast cell degranulation and release of preformed biological mediators

Eczema

Prolonged IgE-mediated inflammatory response in the skin typically manifesting as itchy and swollen rashes and lesions

Eosinophil

Granulocyte of the innate immune system predominantly residing in the gastrointestinal tract (and tissues), producing biological mediators including major basic protein, eosinophilic cationic protein, neurotoxins, eosinophil peroxidase, cytokines, platelet-activating factor and CLC protein

Filagrin

An epidermal barrier protein of which its mutations are associated with atopic dermatitis

Flow Cytometry

Measurement of cells in a flow system designed to pass cells through a laser emitting a beam of monochromatic light in a single stream

Food Allergy

Development of an IgE-mediated immune response to a variety of food proteins; a more generalised or systemic allergic reaction with symptoms such as angioedema, urticaria and compromised respiratory and cardiovascular systems

FOXP3

Transcription factor forkhead box P3 involved in the regulation and development of regulatory T cells

Histamine
Biological mediator released from pre-formed granules in mast cells associated with the early phase allergic response

**Hygiene Hypothesis**

Reduced exposure to infectious disease in childhood through increased vaccinations, use of antibiotics and increased hygiene, leads to an insufficient transfer from T\(_{H2}\) to T\(_{H1}\) immune responses explaining the increased prevalence of allergy in Western populations

**IFN\(\gamma\)**

Cytokine secreted predominantly from T\(_{H1}\) cells during immune responses against extracellular pathogens and bacterial infection of macrophages

**IgE**

Immunoglobulin E class of antibody seen at elevated concentrations in atopic individuals

**IgG**

Immunoglobulin G class of antibody involved predominantly in the immune response to pathogens

**IL4**

Cytokine typically secreted from T\(_{H2}\) cells

**IL10**

Cytokine secreted from a variety of cells typically associated with regulatory function

**IL17**

Cytokine typically secreted from T\(_{H17}\) cells

**Late Phase Response**

Recruitment and migration of cells, notably T\(_{H2}\) cells and eosinophils, to the site of allergic inflammation
Leukotrienes

Secondary mediator synthesised and secreted from activated mast cells

Lipid Transfer Proteins (LTPs)

A group of heat-stable panallergens present in a number of pollens and Rosaceae fruits

Mast Cell

A cell of the innate immune system containing granules rich in histamine important in early phase reactions to allergens

MHC Molecules

A cell surface molecule enabling T cells to recognise processed peptides of antigens and to discriminate self-antigens

MHC Tetramers

A complex of four MHC molecules related to a specific peptide bound to fluorescently labelled streptavidin

Neutrophil

Most abundant type of white blood cell involved in phagocytosis in conjunction with degranulation and release of biological mediators

Pathogenesis-Relation 10 Proteins (PR-10)

Plant panallergen present within the group of Rosaceae fruits and the major birch pollen allergen Bet v 1, also termed the Bet v 1/PR-10 cluster

Perennial Rhinitis

IgE-mediated inflammatory disease of the nasal mucosa, associated with sneezing, itching and conjunctivitis, present all year round. Causative allergens include pet dander and house dust mite.

Pollen Food Syndrome
Sensitisation to homologous panallergens within aeroallergens and food proteins associated with oral symptoms such as itching and swelling of the mouth, lips and throat

**Profilin**

Actin-binding protein and panallergen associated with the pollen-food syndrome

**Prostaglandin**

Secondary mediator released during mast cell degranulation responsible for bronchoconstriction and increased vascular permeability

**Seasonal Rhinitis**

IgE-mediated inflammatory disease of the nasal mucosa associated with the pollen seasons, e.g. birch and grass allergy

**Sensitisation Phase**

On initial exposure to allergen, $T_{h}2$ cells are activated leading to production of IgE by B cells; IgE binds to mast cells, subsequently termed as “sensitised”

**Superantigens**

Family of bacterial exotoxins able to potently stimulate T cells causing mass cytokine production, proliferation, apoptosis and generalised immunosuppression

**Staphylococcal Enterotoxin B (SEB)**

Superantigen secreted by *Staphylococcus aureus*

**T Cell**

A lymphocyte of the immune system with principle functions in adaptive immunity including cytokine production, cytotoxic activity and regulatory function

**T Cell Receptor (TCR)**

Molecule on the surface of T cells that functions in the recognition of MHC:peptide complexes
$\text{T}_{\text{H}1}$
Subset of T helper cell involved in the immune response to extracellular pathogens and bacterial infection, through production of IFN$\gamma$

$\text{T}_{\text{H}2}$
Subset of T helper cell involved in the immune response to parasitic infection and allergy, through production of IL3, IL4, IL5, IL9 and IL13

$\text{T}_{\text{H}17}$
Subset of T helper cell involved in immune responses to extracellular bacterial and fungal infections, through production of IL17, IL21, IL22 and TNF$\alpha$

T Regulatory Cell
Subset of T cell with regulatory and suppressive function falling into the categories of naturally-occurring and inducible regulatory T cells
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