The aim of this study was to analyze human CD4+ T cell responses to mammalian allergens in subjects with and without allergy using in vitro assays and novel ex vivo detection methods.

Functional and phenotypic differences were detected in allergen-specific CD4+ T cells between the study groups. Furthermore, the CD4+ T cell responses to the major dog allergen Can f 5 were analyzed for the first time in detail in this work. These results could aid the development of immunotherapy against allergy.
Human T-helper Cell Response to Mammalian Respiratory Allergens
ANSSI KAILAANMÄKI

Human T-helper Cell Response to Mammalian Respiratory Allergens

To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in the Auditorium SN201, Snellmania building at the University of Eastern Finland, Kuopio, on Friday, February 19th 2016, at 12 noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
Number 330

Department of Clinical Microbiology, Institute of Clinical Medicine, School of Medicine, Faculty of Health Sciences, University of Eastern Finland
Kuopio
2016
ABSTRACT

Sensitization to mammalian respiratory allergens is a prevalent cause for allergic disorders and asthma. Despite being among the most common and topical health problems in the Westernized world, the underlying cellular mechanisms driving the development of allergic diseases are still incompletely understood. Allergic sensitization is initiated in a complex interplay between immune cells allergen-specific T-helper cells playing an important role in the process. Therefore, understanding the T cell response is a key target in elucidating the pathogenesis of allergy. In-depth knowledge on the properties of allergenic proteins is required for developing effective immunotherapeutic tools.

The aim of this study was to analyze human CD4+ T cell responses in vitro and/or ex vivo to important mammalian allergens, cow Bos d 2, horse Equ c 1 and dog Can f 5, in subjects with and without allergy. Also, the T cell epitopes of the previously uncharacterized Can f 5 were identified. The study was conducted utilizing well-established in vitro methods of T cell analysis in conjunction with novel ex vivo methods, such as the use of HLA class II tetramers and the CD154 upregulation assay.

The frequency of allergen-specific memory CD4+ T cells in allergic subjects was found to be higher in all studies when compared to nonallergic ones. The T cell receptor avidity proved to be higher in the T cells of allergic individuals than in those of nonallergic ones. Upon allergen stimulation, CD4+ T cells of allergic subjects proliferated more strongly than the cells of nonallergic subjects. The memory CD4+ T cells of allergic subjects consistently exhibited a Th2-deviated phenotype, whereas the cells of nonallergic subjects were generally of a Th1/Th0-associated phenotype. The T cell responses were restricted by several common HLA class II molecules, a finding that can be advantageous for devising peptide-based allergen immunotherapy. Results by a novel ex vivo method based on the CD154 activation marker upregulation corroborated the view of T cell reactivity to animal allergens obtained with conventional in vitro methodology. The epitope mapping of Can f 5 revealed six distinct T cell epitopes. Peptides containing these epitopes could be useful for developing allergen-specific immunotherapy against dog allergy.


Koiran Can f 5 -allergeenin T-soluepitoopikartoitus paljasti erillistä epitooppialuetta, joista jokaiselle reagoi vähintään neljä kahdestoista allergisesta koehenkilöstä. Näiltä epitooppialueilta valittiin kokoelma peptidejä, jotka voivat tarjota lähtökohtana peptidi-immunoterapian kehittämiselle koira-allergiala kohtaan.
Ad Nemo
Acknowledgements

This study was conducted at the Department of Clinical Microbiology, Institute of Clinical Medicine, University of Eastern Finland.

I wish to express my gratitude to my supervisors, Docent Tuomas Virtanen, M.D., Ph.D. and Docent Tuure Kinnunen, M.D., Ph.D. for making this study possible. Tuomas, I truly appreciate your thorough knowledge in immunology and your endless patience with editing and revising my works to the finest detail. Tuure, thank you for your dedication to see this through. Your role in this work was central.

Likewise, I wish to thank all my current and former colleagues at the Department of Clinical Microbiology, and especially the members of the Allergy Research Group. Each of you have played some part in helping me along the way. In particular, I am grateful for the company of my fellow researchers and dear friends Aino Rönkä, M.D., Ph.D., Anne Lammi, M.D., Ph.D., Tyyne Viisanen, M.Sc. and Emmi-Leena Ihantola, M.Sc.. It has been a privilege to work with you. I want to address my special thanks to Mrs. Virpi Fisk, whose technical expertise has been involved in all parts of this work.

I am grateful to all my collaborators, in Finland and abroad. I sincerely value your contribution towards my work. I also want to show my appreciation to the official reviewers of my thesis, Professor Mikko Hurme, M.D., Ph.D. and Docent Arno Hänninen, M.D., Ph.D. for critically evaluating my work. Specifically, I want to thank Sara Wojciechowski, B.Sc. for the painstaking language revision of the text.

I want to warmly thank my parents Hannu and Leea and the rest of my family for being always there when needed. I am especially grateful to my grandparents Viljo and Irja for your tremendous support in all stages of my life. I also want to express my gratitude to my former teachers Päivi Noppari and Niilo Vartiainen. You had faith in me in times when few others did. Furthermore, you laid the very foundations upon which this work was built.

My deepest thanks go to my wife Milla. Never once did you falter by my side during this odyssey.

This study was financially supported by the Academy of Finland, Kuopio University Hospital and the Väinö and Laina Kivi foundation.

Kuopio, December 2015
Anssi Kailaanmäki
List of Original Publications

This dissertation is based on the following original publications:


The publications have been reproduced with the permission of the copyright holders. Publication I has been written under the name A. Nieminen.
# Contents

1. INTRODUCTION .................................................................................................................. 1
2. REVIEW OF THE LITERATURE ........................................................................................ 3
   2.1 Allergy.............................................................................................................................. 3
      2.1.1 General .................................................................................................................. 3
      2.1.2 Allergic sensitization ........................................................................................... 4
      2.1.3 Properties of allergens......................................................................................... 6
      2.1.4 Mammalian allergens.......................................................................................... 8
   2.2 Treatment of allergy .................................................................................................... 10
      2.2.1 General ................................................................................................................ 10
      2.2.2 Allergen immunotherapy ................................................................................. 11
      2.2.3 Peptide-based allergen immunotherapy ........................................................ 13
   2.3 T cells ............................................................................................................................. 16
      2.3.1 CD4+ and CD8+ T cell development ............................................................... 17
      2.3.2 The T cell receptor ............................................................................................. 18
      2.3.3 CD4+ T cell activation and T-helper cell subsets ........................................... 20
      2.3.4 Regulation of T cell responses.......................................................................... 22
   2.4 Detection of antigen-specific CD4+ T cells in humans ........................................ 24
      2.4.1 A needle in the haystack ................................................................................... 24
      2.4.2 Measuring antigen-specific T cell proliferation in vitro ............................... 25
      2.4.3 Direct ex vivo detection of antigen-specific CD4+ T cells ............................. 27
3. AIMS OF THE STUDY ........................................................................................................ 31
4. MATERIALS AND METHODS ........................................................................................ 32
   4.1 Subjects.......................................................................................................................... 32
   4.2 Antigens......................................................................................................................... 32
      4.2.1 Recombinant antigens (I-III). .............................................................................. 32
      4.2.2 Synthetic peptides (I-III) ................................................................................... 32
   4.3 Cell separation .............................................................................................................. 33
      4.3.1 Separation of peripheral blood mononuclear cells (I-III) .............................. 33
      4.3.2 Magnetic bead purification and the enrichment of CD4+ T cell subsets (I-III) ................................................................................................................... 33
   4.4 T Cell culture and proliferation assays ....................................................................... 33
      4.4.1 Generation of T cell lines and clones (I-III) ...................................................... 33
      4.4.2 Thymidine incorporation test (I-III) ................................................................. 35
      4.4.3 Generation of monocyte-derived dendritic cells (I) ....................................... 36
   4.5 Functional and phenotypic analyses of t cells ............................................................. 36
      4.5.1 HLA class II tetramer staining (I-II) ................................................................. 36
      4.5.2 CD154 enrichment and the ex vivo analysis of CD4+ T cells (III).................. 37
      4.5.3 Cytokine measurements (I-III) ....................................................................... 38
      4.5.4 HLA class II restriction analyses (II, III) ......................................................... 38
4.6 Prediction of the HLA class II peptide binding by bioinformatic algorithms (III) ................................................................. 39
4.7 Statistical methods ................................................................................................................................................... 39

5 RESULTS ........................................................................................................................................................................ 40

5.1 Allergen-specific naïve and memory CD4+ T cells exhibit functional and phenotypic differences between individuals with and without allergy (I) .... 40
  5.1.1 Frequency and characteristics of ex vivo-expanded Bos d 2127-142-specific naïve and memory CD4+ T cells ................................................................. 40
  5.1.2 Poor proliferation and tetramer staining of Bos d 2127-142-specific TCLs from subjects without allergy ................................................................. 41
  5.1.3 Th2 bias of Bos d 2127-142-specific TCLs derived from subjects with allergy ................................................................................................................. 42
  5.1.4 Higher CD25 and lower CXCR3 expression in Bos d 2127-142-specific TCLs from subjects with allergy ............................................................. 42

5.2 Differential CD4+ T cell responses of allergic and nonallergic subjects to the immunodominant epitope region of the horse major allergen Equ c 1 (II) ...... 43
  5.2.1 Equ c 1143-160 peptide-specific CD4+ T cell lines are obtained in similar numbers from both allergic and nonallergic subjects ........................................... 43
  5.2.2 Frequency and proliferative capacity of peripheral-blood Equ c 1-specific CD4+ T cells ........................................................................................................... 43
  5.2.3 Equ c 1 protein-specific CD4+ T cells are Th2-polarized and emerge from the memory pool ............................................................................................. 44
  5.2.4 Equ c 1-specific CD4+ T cell responses are restricted by HLA-DQ and -DR alleles ...................................................................................................... 45

5.3 Memory CD4+ T cell responses to the novel dog allergen Can f 5, prostatic kallikrein (III) .................................................................................................................. 45
  5.3.1 The frequency of Can f 5-specific memory CD4+ T cells is markedly higher in allergic than in nonallergic subjects .................................................. 45
  5.3.2 Can f 5-specific memory CD4+ T cells of allergic subjects exhibit a Th2-associated phenotype ex vivo ............................................................................. 46
  5.3.3 Can f 5 contains six T cell epitope regions that are capable of stimulating the memory CD4+ T cells of allergic subjects ........................................... 46

6 DISCUSSION ..................................................................................................................................................................... 48

6.1 Allergen-specific T cell responses in individuals with and without allergy (I-III) ................................................................................................. 48
  6.1.1 Frequencies of allergen-specific CD4+ T cells differ between allergic and nonallergic subjects .................................................................................. 48
  6.1.2 Proliferative capacity and the T cell receptor avidity of allergen-specific CD4+ T cells .......................................................................................................... 49
  6.1.3 Allergen-specific CD4+ memory T cells of allergic subjects exhibit Th2-deviated immune responses ................................................................. 50
6.2 T cell epitopes and the HLA restriction of allergenic immune responses (II, III) ................................................................. 51

6.2.1 Equ c 1 contains a distinct immunodominant epitope region, but the T cell responses to Can f 5 are dispersed in multiple sites (II, III) .................. 51

6.2.2 HLA restriction of allergen-specific T cell responses ........................................ 52

6.2.3 Bioinformatic predictions of HLA-binding motifs are not always successful in predicting experimentally identified T cell epitopes (III) .. 53

6.3 Analysis of specific CD4+ T cells in vitro and ex vivo (I-III) .......................... 54

6.3.1 Results obtained with the conventional in vitro split-well method are comparable to those obtained with direct ex vivo detection methods (I-III) ........................................................................................................ 54

6.3.2 Successes and failures in detecting allergen-specific CD4+ T cells with HLA class II tetramers (I, II) ........................................................................ 55

6.3.3 CD154-based enrichment method as an alternative approach to detect allergen-specific CD4+ T cells (III) ............................................. 56

6.4 Potential of Bos d 2, Equ c 1 and Can f 5-derived peptides for peptide-based allergen immunotherapy (I, II, III) ............................................. 58

7 CONCLUSIONS ........................................................................................................ 60

8 REFERENCES ............................................................................................................. 61
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
</tr>
<tr>
<td>AIT</td>
<td>allergen immunotherapy</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>allophtycyanin</td>
</tr>
<tr>
<td>APL</td>
<td>altered peptide ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDI</td>
<td>cell division index</td>
</tr>
<tr>
<td>CDR</td>
<td>complementary-determining region</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidl ester</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRTh2</td>
<td>chemoattractant receptor-homologous molecule expressed on Th2 cells</td>
</tr>
<tr>
<td>CTLA</td>
<td>cytotoxic T-lymphocyte-associated antigen</td>
</tr>
<tr>
<td>Cy</td>
<td>cyanine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EAACI</td>
<td>European Academy of Allergy and Clinical Immunology</td>
</tr>
<tr>
<td>EBV</td>
<td>Esptein-Barr virus</td>
</tr>
<tr>
<td>EC</td>
<td>effective concentration</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FASL</td>
<td>FAS-ligand</td>
</tr>
<tr>
<td>FcεRI</td>
<td>high-affinity IgE receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOXP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IEDB</td>
<td>Immune Epitope Database</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible T cell costimulator</td>
</tr>
</tbody>
</table>
1 Introduction

The prevalence of atopy and allergic diseases has increased dramatically in Finland and other industrialized countries (1, 2). Allergy is a tendency of the immune system to react against ubiquitous environmental agents, such as pollen or animal dust. At the systemic level, allergy can manifest itself with such symptoms as rhinitis, asthma, conjunctivitis or atopic eczema. In allergy, T-helper type 2 (Th2) cell activation leads to allergen-specific IgE antibody production by B cells. These antibodies in turn can activate mast cells to release inflammatory mediators which cause the classical allergic symptoms (3).

Mammalian allergens are important causes of indoor respiratory allergies; for example, in homes and schools, in public transport vehicles, as well as in occupational environments and recreational settings. Most known major mammalian respiratory allergens belong to the lipocalin family of proteins, with the exception of the cat allergen Fel d 1 (4). Moreover, a novel non-lipocalin dog allergen, the dog prostatic kallikrein Can f 5, has been identified recently (5).

The molecular characteristics and IgE reactivity of animal allergens have been extensively studied and the Th2-nature of the aberrant T cell response in allergic subjects is well established (6). However, the underlying mechanisms behind the activation of allergen-specific T cells remain largely obscure. Therefore, the phenotypic and functional characterization of T cells specific to important mammalian allergens is of importance for the understanding of their allergenicity.

The treatment of allergies relies almost exclusively on palliative medication that unspecifically targets the symptoms of the disease. The only curative or disease-modifying treatment currently in use is allergen immunotherapy (AIT), in which progressively larger doses of allergen extract are administered subcutaneously to induce allergen-specific tolerance. However, this immunotherapeutic treatment generally takes up to five years to complete and it contains a risk of allergen-specific IgE cross-linking on the surface of mast cells resulting in side effects. Some of these can be systemic and even life-threatening, such as anaphylaxis (7). Peptide-based immunotherapy (PIT) offers a way to circumvent these side effects. PIT employs short linear peptide fragments of the allergen that contain T cell-activating epitopes. These short peptides lack the natural three-dimensional structure of the protein and consequently fail to cross-link IgE. Therefore, PIT offers a promising alternative with increased safety and efficacy over AIT (8).

For PIT to be efficient, the characteristics of the allergen-specific T cell response need to be well understood. Moreover, information on the T cell-activating epitopes of each allergen is a necessary prerequisite for PIT.

The goal of this thesis was to comprehensively characterize human T cell responses to three important respiratory allergens of mammalian origin, cow Bos d 2, horse Equ c 1 and
dog Can f 5, in order to better understand the immunological background of sensitization and tolerance to them.
2 Review of the literature

2.1 ALLERGY

2.1.1 General
The concept of allergy was introduced over a century ago by the Viennese scientist and pediatrician Clemens von Pirquet as a general term for an immune response against an innocuous environmental substance – an allergen – that leads to host damage (9). Later, the term atopy (see below) was introduced by Coca and Cooke for a more precise definition of the inherited tendency to develop immediate-type allergic reactions (10). Finally, in 1975, the different types of hypersensitivity reactions were categorized into four subgroups by Coombs and Gell and the basis of this grouping of hypersensitivity reactions has prevailed to date (11). Since then, the term allergy has become most commonly associated with type I hypersensitivity (also known as IgE-mediated immediate-type allergy), but the concept of allergy also encompasses the T cell-mediated late-phase reactions (12,13). Typical symptoms of allergy include itchiness, rhinitis, conjunctivitis, eczema, hives, and asthma. In the most extreme cases allergy can manifest itself as a life-threatening reaction called anaphylaxis. Allergy to insect venoms and certain food substances are more commonly associated with anaphylaxis, but anaphylactic reactions may occur for other reasons as well, for example, because of certain medications. In addition to type I hypersensitivity, there are three other types of hypersensitivity: type II cytotoxic hypersensitivity is an IgG- or IgM-associated reaction to foreign antigens that leads to destruction of antigen-coated host cells; type III immune complex disease is a condition where IgG antibodies form immunocomplexes initiating inflammatory reactions often localized in joints and kidneys; and type IV delayed-type hypersensitivity that is mediated by T helper (Th) type 1 and 2 lymphocytes, and cytotoxic lymphocytes (13,14).

Atopy is a closely-related term to the immediate-type allergy. It is used to describe a usually hereditary predisposition to generate a Th2-deviated immune response towards innocuous environmental substances that leads to allergen-specific IgE production and ultimately to allergic symptoms (13,14).

Allergic sensitization and atopic tendency have become overwhelmingly common in Finland and in other industrialized countries in recent decades – so much so that it is almost considered a normal state of being and its nature as a medical condition has faded into the background (12). However, in Finland alone the estimated costs of allergy and asthma exceed 1.5 billion euros (15). Furthermore, it has recently been reported that the prevalence of allergic rhinitis in the Finnish population is 30%, conjunctivitis 15%, eczema 10-12%, and asthma 8-10% (12). It has recently been estimated that as many as 500 million people suffer from allergies worldwide and 150 million people are affected in Europe alone (16,17). In
Finland, between 150-250 people are estimated to be treated for anaphylaxis annually (12). Moreover, it has been reported that anaphylactic reactions result in 500 to 1000 deaths in the United States, 20 deaths in the United Kingdom and 15 deaths in Australia annually (18). Due to their high prevalence and chronic nature, allergic diseases inflict a heavy toll not only on a societal level, but on the individual as well. Allergy and asthma have a profound impact on the quality of life. In addition to obvious detrimental health effects, they may restrict occupational opportunities, outdoor activities, dietary options, exercise and pet ownership.

2.1.2 Allergic sensitization

There is a significant hereditary aspect in susceptibility toward atopy and allergic diseases (19,20). Studies suggest that as many as 40% of people in the Western industrialized countries display an elevated genetic predisposition towards IgE production against common environmental substances (13). This effect appears to be more prominent if inherited from the maternal side (21,22). Genes that are known or suspected to increase the risk of atopy are distributed in most human chromosomes, and most of them are linked to the control of inflammatory pathways of the innate and adaptive immune systems (19). Although many of the susceptibility genes are found in the human leukocyte antigen (HLA) system, the association is much weaker than that observed in many autoimmune diseases. Other allergy-predisposing genes affect the secretion of inflammatory mediators or cytokines or the response to them. Certain genes may affect the allergic sensitization or disease severity indirectly by modifying bodily functions affected by the inflammatory condition caused by allergy. For example, airway epithelia and smooth muscle cells are affected by allergic inflammation (19,23).

Despite the evident genetic linkage, it is clear that environmental factors have a prominent role in the development of allergic diseases (12,13,19). The notable increase in the incidence of allergies during the recent decades implies that since genetic factors remain largely the same, the changes in the way of living must account for this phenomenon. This observation is backed by studies conducted among people of similar genetic background but differing environmental conditions or lifestyle. People of the eastern parts of Finland are genetically closely related to the people of Russian Karelia across the national border. Despite the similar genetic background, asthma and allergic diseases are markedly more common on the Finnish side of the border (24). Similar studies have also been carried out between the people of East and West Germany. Those in the East have a lower incidence of allergic diseases when compared to the genetically similar people of the West, and this difference has been disappearing after the reunification of Germany (25,26).

The most well-known explanation for these discrepancies is the so-called hygiene hypothesis which proposes that the Western way of life is strongly linked to the increase of allergic diseases. Especially, it is suggested that a lack of childhood exposure to certain microbes or parasites increases susceptibility to allergy by deviating the natural development
of the immune system and interfering with the normal development of tolerance and immunoregulatory components (27-29). This lack of exposure to microbes is most prominent in urban environments and hence allergic diseases are more common among people living in cities and away from farmland animals. Certain other early-life events may also alter the exposure to microbes and thus modulate the development of the immune system. Example factors that are associated with the increased incidence of allergy include: birth by caesarian section (30), lack of older siblings (31) and use of antibiotics (32) in childhood (although recent studies have cast some doubt on this last factor (33)). In addition to the hygiene hypothesis, two other lifestyle factors are linked with the elevated risk of allergic diseases, namely smoking (31,34) and obesity (35). In isolated cases, allergic sensitization can also be a result of events not linked to the person’s own immune system. It has been reported that transient allergic sensitization can occur after blood transfusion, when the IgE molecules of the donor’s blood bind to the high-affinity Fcε receptors (FcεRI) of the recipient’s mast cells (see below), triggering the allergic symptoms after subsequent allergen exposure (36). Similarly, blood marrow transplants can in rare cases result in the transfer of allergy-predisposing effector lymphocytes from donor to recipient (37).

Allergen-specific IgE is the hallmark feature of allergic sensitization. It is produced by B cells and binds to its cell surface receptors on mast cells and basophils. Upon exposure to the allergen, the cell-surface-bound IgE molecules trigger the release of inflammatory mediators that lead to allergic symptoms (38). The whole process of IgE-mediated allergic sensitization, however, begins earlier than this.

CD4-positive helper T lymphocytes, or CD4+ T cells, play a central role in orchestrating the synthesis of allergen-specific IgE (38). After gaining entry across the epithelial barriers of the human body, protein allergens are phagocytized by antigen-presenting cells (APC), such as dendritic cells (DC). DCs home in on lymph nodes, where they present the processed and fragmented allergens to CD4+ T cells. The allergen-derived peptides are non-covalently attached to type II major histocompatibility complexes (MHC; often referred to as human leucocyte antigen in humans) on the DC surface, where they are recognized by naïve CD4+ T cells with compatible T cell receptors (TCR) (13). For reasons that have remained enigmatic to date, allergen peptides induce a Th2-polarized T cell activation in susceptible individuals. One central player is the DC, which is capable of inducing Th1 vs. Th2 immune deviation (see Chapter 2.3.3). Other possible factors affecting the CD4+ T cell polarization include: the route of entry of the antigen, its physical form, associated adjuvants and the dose (38). Polarized Th2 cells, in turn, recruit B cells and other immune cells to mount an immune response by means of stimulatory cell surface receptor interactions and cytokines. Such cytokines include interleukins (IL) IL-4, IL-5, IL-9 and IL-13. Of these cytokines, IL-5 and IL-9 initiate the proliferation of eosinophils and mast cells, respectively (13,38,39).

Crucially, in terms of allergic sensitization, Th2 cells induce the activation of naïve B lymphocytes and subsequent immunoglobulin class-switching. Cell-surface interaction between CD40L on T cells and CD40 on B cells, together with the secreted cytokines IL-4 and
IL-13, instruct B cells to undergo a genetic rearrangement in the genes encoding for immunoglobulins, resulting in the isotype class switch from allergen-specific IgM to IgE. In this process, the immunoglobulin retains its specificity but attains the capacity to bind directly to FcεRI expressed on the surface of mast cells and basophils (13,40).

After subsequent exposure to the allergen, its molecules are bound by the cell-surface IgE on mast cells and basophils, resulting in FcεRI cross-linking. The cross-linking of IgE-FcεRI complexes initiates mast cell and basophil degranulation and a rapid release of inflammatory mediators. These include histamines, prostaglandins, leukotrienes and heparin. Classical allergic symptoms, such as vasodilatation and bronchial constriction, follow in a matter of minutes. The severity of the symptoms is dependent largely on the extent of allergen exposure and the amount of FcεRI-bound IgE (41,42).

Whereas the onset of the acute phase allergic response follows rapidly in the wake of allergen exposure, the late-phase response takes place hours after the initial contact with the allergen. It is characterized by a recurrent synthesis of inflammatory mediators by activated mast cells. These molecules drive the proliferation and maturation of eosinophils, basophils, macrophages and other inflammatory cells (43), which migrate to the sites of inflammation. In severe cases, this may lead to persistent proinflammatory conditions that may ultimately result in tissue damage and remodeling in vulnerable tissues, such as the lung and airway epithelium (44).

2.1.3 Properties of allergens
Known allergens are named according to the guidelines of The Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS) (45,46). The name of an allergen consists of the first three letters of the genus of the species from which the allergen is derived and the first letter of its species, followed by an Arabic number denoting the order in which the allergen has been discovered. The name of an allergen may contain a prefix indicating details about how the allergen has been produced, for example n for natural, r for recombinant or s for synthetic allergen. Similarly, isoallergens and allergen isoforms can be designated with additional suffixes (14,45,46). Isoallergens are defined as multiple molecular forms of an allergen in a species having a similar biological role and size, and the sequence identity of ≥ 67%, whereas isoforms have an identity of ≥ 90% (45). Thus, the first identified dog allergen, for example, is called Can f 1, derived from the Latin name Canis familiaris. As of March 31st, 2015, the IUIS database contains 322 animal allergens, of which 226 are derived from arthropods. There are 110 known fungal and 368 plant allergens (www.allergen.org).

The underlying reason of what makes an environmental substance an allergen remains elusive. Of the vast amounts of foreign proteins and other molecules in our environment only a limited number are allergenic (47). To be classified as an allergen, a substance must be able to bind to specific IgE and induce the onset of allergic symptoms in sensitized indi-
individuals (14). Most known allergens are proteins or glycosylated proteins and a typical allergen source often contains several IgE-binding components. Allergic individuals often have a variable pattern of IgE reactivity to allergen proteins within an allergen source. If more than 50% of subjects allergic to a given source of allergen proteins (e.g. a domestic animal, such as dog) have IgE specific to a particular protein, it is classified as a major allergen. Other allergens are considered minor allergens (45,48).

Proteins that cause allergy have a multitude of biological roles and structural forms, and it has been difficult to establish a common denominator for their capacity to elicit IgE production and allergic sensitization (49). Since allergens can be found only in a minority of all protein families, it appears that they do share common biological or physiochemical properties that direct the host immune system toward the allergy-predisposing Th2 response (47,49,50).

Despite the difficulties in finding obvious links between different allergen proteins, certain common features can be established (49,51,52). First, the allergen requires an efficient means of delivery to the immune system. Considering airborne allergens, it has been long known that the allergens need to be readily spread in the environment by wind (pollens), air currents or shedding (animal dander). Therefore, the molecular size and aerodynamics of airborne allergens need to be such that the protein effectively disperses in the environment and reaches the airways, where it encounters the cells of the immune system (53,54).

Allergens can also exhibit intrinsic enzyme activity that contributes to their allergenic potential. For example, allergens that show proteolytic activity may cleave their way through the epithelial junctions of the airway (55). The role of enzymatic activity has been extensively studied with the dust mite allergen Der p 1 (56,57). Some allergens may also directly react with B-cell (58) and T-cell (59) surface receptors or modulate dendritic cell function (60-62). The allergen proteins are rarely the only components of the allergen source and therefore other proteins associated with the allergen may be required to induce or enhance the allergic sensitization. These include adjuvant-like substances, such as those found in pollen of some plants (63,64) or microbial products (65) at certain concentrations. Allergens often contain innate immunity-stimulating substances, such as Toll-like receptor (TLR) ligands, but in markedly lower quantities compared with the levels the immune system encounters during microbial infections (47). Stimulation via TLRs can modulate the ensuing T cell response towards the Th1- or regulatory Tr1-type responses that are protective for allergy, or alternatively, towards the allergy-predisposing Th2-type response (see Chapter 2.3.3) (47,66).

Allergen concentration appears to be another distinctive factor affecting the allergic sensitization. An often-suggested postulation maintains that the risk of allergic sensitization follows a bell-shaped curve, i.e. when the allergen concentration is very low or relatively high the risk of sensitization is low (67,68). At very low concentrations there simply may not be enough allergen to induce immune activation, whereas high concentrations favor the
induction of immunological tolerance (67). The middle ground – moderate allergen concentrations at the “top of the bell” – associate with the highest risk for IgE responses. These concentrations may not be high enough to induce tolerance but not low enough to evade immune activation altogether. This phenomenon has been described in rat (67), mouse (69) and cat allergies (70,71), where at a certain level of allergen exposure the risk of IgE production peaks, and exposure at concentrations higher or lower than this reduces the risk of sensitization.

Many allergens have been shown to contain distinctive immunodominant T cell epitopes (72-76). Knowledge of these epitopes is central to devising novel immunotherapeutic approaches (see Chapter 2.2.2). There is also some evidence suggesting that immunodominance in CD4+ T cell responses is largely due to an intrinsic property of the peptide:HLA class II complexes. The kinetic stability of peptide:HLA complexes controls HLA-DM editing within the antigen-presenting cells and consequently the ensuing epitope density on antigen presenting cells, such as dendritic cells (77). However, studies suggest that the elements contributing to T cell epitope immunodominance are manifold and often dependent on the antigen in question. Factors related to the antigen processing of epitopes, such as antigen structure, proteolytic degradation, copy number and loading of peptides onto HLA molecules have been linked with immunodominance (78-80). Similarly, events related to TCR-mediated recognition of antigen, such as the precursor frequency of responding T cells or TCR affinity, have also been postulated to contribute to the ability of an epitope to elicit T cell responses (78-80).

In this context, it is of interest to note that weak TCR-mediated recognition and signaling is considered to be associated with Th2 polarization (81). As mammalian lipocalin allergens have been found to be weakly stimulatory to CD4+ T cells, weak TCR recognition can be a factor favoring sensitization to these evolutionary close proteins (4,52).

### 2.1.4 Mammalian allergens

Most of the mammalian-derived respiratory allergens belong to the lipocalin family of proteins (51,52). Lipocalins are a large family of proteins that transport small, hydrophobic molecules, such as vitamins, pheromones, steroids, bilins and lipids. Some lipocalins express alternative biological functions and have, for example, enzymatic activity or immunosuppressive properties. They range from 160 to 230 amino acids in length and have a molecular mass of approximately 20 kDa. Lipocalin proteins have been identified in dander and bodily fluids (e.g. saliva, urine and tears) of several mammalian species including humans. Certain arthropod allergens, such as the pigeon tick Arg r 1 and the cockroach Bla g 4, are also lipocalins (4,52).

Lipocalins form a major group of animal allergens. They share a considerable structural homology with one to three structurally conserved regions in their amino acid sequences. The first of these regions contains the glycine-x-tryptophan motif, which is found in more
than 90% of the presently identified lipocalins. Therefore, it is regarded as a distinctive feature of the protein family.

Perhaps the single most important non-lipocalin animal allergen is the major cat allergen Fel d 1, which is a potent sensitizer affecting over 90% of cat-allergic individuals (82). Some other mammalian allergens also fall outside the lipocalin family. However, with few exceptions, all these are considered minor allergens that have a limited impact on the allergy status of most atopic individuals. One of the exceptions along with Fel d 1 is the dog major allergen Can f 5, a prostatic kallikrein, that sensitizes up to 70% of dog-allergic subjects and is therefore one of the most important dog allergens (see below) (5).

Bos d 2, a major allergen of cow, is a 20 kDa lipocalin protein to which about 90% of farmers with bovine allergy react (83,84). Bos d 2 is suspected to be a pheromone carrier and it is secreted by the cells of the apocrine sweat glands from where it is transported onto the skin (84,85). Bos d 2, or an immunologically similar protein, is also found in the cow urine (83). Multiple isoforms of Bos d 2 exist. It exhibits a certain degree of sequence homology with several other mammalian lipocalin allergens, such as the guinea pig Cav p 2 and 3 (~40%), dog Can f 4 and 6, horse Equ c 1, Fel d 4 and Rat n 1 (~30% each). It also shares a 26% identity with the human epididymal-specific lipocalin 9 (SIB BLAST+ Network Service [SBNS], accessed August 24th 2015 at web.expasy.org/blast). The IgE binding of Bos d 2 is highly dependent on its three-dimensional structure and therefore denatured or fragmented forms of the protein readily lose their capacity to elicit IgE-dependent responses. The IgE-binding epitopes appear to be localized in the C-terminal end of Bos d 2 (4). The T cell epitopes are found between amino acids 127-142 (86). Unlike with many other allergens, the HLA associations of sensitization to Bos d 2 have been investigated in detail. Recent studies indicate that the HLA class II alleles DRB1*0101, DRB1*0404, DQB1*0302 and DQB1*0501 are associated with an elevated risk of sensitization and HLA-DRB1*0301 and DQB1*0201 with a decreased risk (87). Bos d 2 has been produced in a recombinant form and used in several allergy studies (88). Allergy to cow is an important occupational health problem in dairy and beef production. Cow dust-induced asthma is a source of disability that in severe cases can prevent a person from working with the animals (89).

Equ c 1 is the primary allergen of the horse, sensitizing 76% of horse-allergic subjects according to one study (90). Like most other major mammalian allergens, it belongs to the lipocalin family of proteins. It is a 25 kDa protein that is present in horse hair and dander (91,92). Its mRNA expression is especially high in sublingual salivary glands (91,93). As with many other lipocalin allergens, the presence of the allergen in dander extract may be partially explained by bodily excretions being transported to the skin and hair from other sources, in this instance, from saliva (91). Similar to the major cow allergen Bos d 2, Equ c 1 exhibits a high degree of amino acid identity with cat Fel d 4 (67%) and over 50% with dog Can f 6. It also has a correlative sequence homology with the human epididymal-specific lipocalin 9 as Bos d 2 (36%). Equ c 1 has been found to have surfactant-like properties, which make it capable of escaping water and being absorbed at the interface of liquid and air (92).
Studies conducted on Equ c 1 indicate that the dominant T cell epitopes are located in a restricted cluster in the molecule (75). Similarly to cow allergy, sensitization to horse poses a considerable health hazard in certain professional and recreational settings, such as in veterinarian practice or horseback riding (94).

The major dog allergen Can f 5 along with the cat Fel d 1 are notable exceptions to the dominance of lipocalin proteins as the major category of animal allergens. Approximately 40% of the Can f 5-sensitized subjects do not display IgE reactivity towards Can f 1-3 (5). Can f 5 is found in dog urine and an identical or closely related protein is detectable in dog dander. Its molecular mass is 28 kDa and it is an arginine esterase (prostatic kallikrein). The protein is specifically expressed in prostatic tissue (95,96). Therefore, it has been speculated that dog-allergic subjects might react differently to male and female dogs, and this has been noted in one case report (97). Can f 5 displays considerable identity (about 60%) with kallikreins from other mammals, including man (SBNS, accessed on June 24th, 2015). Interestingly, no other mammalian kallikrein is reported to be an allergen, except human prostatic antigen (PSA), which is associated with allergy to human semen (98-100). It is intriguing to note that in one study PSA was found to display cross-reactivity with Can f 5 and that a subject allergic to human semen did exhibit a clinically relevant reaction to both PSA and Can f 5 (101). The characteristics of mammalian allergens central to this study are summarized in Table 1.

**Table 1. Examples of important mammalian allergens**

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Animal</th>
<th>Origin</th>
<th>Amino acids</th>
<th>Molecular mass (kDa)</th>
<th>Sensitization (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bos d 2</td>
<td>Cow</td>
<td>Skin, urine</td>
<td>156</td>
<td>20</td>
<td>90%</td>
<td>83, 84</td>
</tr>
<tr>
<td>Can f 1</td>
<td>Dog</td>
<td>Saliva, dander</td>
<td>156</td>
<td>22-25</td>
<td>50-75%</td>
<td>149</td>
</tr>
<tr>
<td>Can f 4</td>
<td>Dog</td>
<td>Saliva, dander</td>
<td>158</td>
<td>16-18</td>
<td>35-60%</td>
<td>150</td>
</tr>
<tr>
<td>Can f 5</td>
<td>Dog</td>
<td>Urine, dander</td>
<td>236</td>
<td>33</td>
<td>70%</td>
<td>5</td>
</tr>
<tr>
<td>Equ c 1</td>
<td>Horse</td>
<td>Saliva, dander, hair</td>
<td>172</td>
<td>22</td>
<td>76%</td>
<td>90, 91</td>
</tr>
</tbody>
</table>

### 2.2 TREATMENT OF ALLERGY

#### 2.2.1 General

Traditionally, allergy has been regarded as an incurable condition, although it is acknowledged that the level of allergic sensitization can drastically alter during the course of the
disease. In some cases, the symptoms may either worsen or completely disappear, depending largely on incompletely known immunological and environmental factors. The treatment of allergy has traditionally relied on patient education, i.e. controlled tolerization to allergenic substances in applicable cases or avoidance of substances that cause severe allergic symptoms (102,103). For decades, the first choice of treatment was allergen avoidance, but recently the guidelines for treating allergy have been revised extensively. Current clinical practices emphasize that the patient can be symptomless and maintain normal functionality whilst being allergic at the same time (103). If patient education and lifestyle choices are insufficient in keeping the symptoms absent or tolerable, symptomatic drugs, such as antihistamines, corticosteroids and leukotriene receptor blockers, have been used to suppress the symptoms (104). However, none of these treatments offer a cure for the condition and therefore lifelong medication is needed. This is problematic, as the cumulative costs of medication and their possible side effects can be considerable in the long run (15,104). In addition, allergen avoidance may often prove impractical, as many allergens, such as pollens and animal dander, can be ubiquitous in certain environments and at certain times of the year (105,106). Exposure to some allergens, such as shellfish and nuts, may also result in a life-threatening exacerbation of symptoms, which can make complete allergen avoidance a straining endeavor.

2.2.2 Allergen immunotherapy
The only treatment in clinical use that aims for the cure or alleviation of allergy is allergen immunotherapy (AIT) (104). In AIT, gradually increasing amounts of an allergen preparation are administered, typically by subcutaneous injections, to a patient with IgE-mediated allergy (47,107,108). The goal of the treatment is to eventually tolerize the allergic individual to such a degree that the allergic symptoms become manageable or, in ideal cases, cease altogether. This is achieved through the modulation of the immune response to the allergen. The general principle of AIT has been known for a century (109,110). However, clinically controlled and standardized courses of AIT employed presently offer a more effective and safer means of treating allergy. This notwithstanding, the outcome of AIT is often uncertain, the treatment protocol requires several years to complete and is not without side effects, some of which in rare cases can result in a medical emergency (anaphylactic reactions) (17,111,112).

A detailed diagnosis confirming the IgE-mediated nature of the allergy and identifying the sensitizing agents is a prerequisite for AIT to be successful. Allergen immunotherapy can alleviate symptoms in various IgE-mediated forms of allergy, but it is most commonly indicated for the treatment of moderate to severe rhinoconjunctivitis and mild to moderate asthma and insect venom allergy, especially if the symptoms are insufficiently controlled with symptomatic medication (17). Allergen immunotherapy is contradicted in some instances, e.g. if the patient suffers from a malignant neoplasia, AIDS or severe uncontrolled asthma, uses β-blockers or angiotensin-converting-enzyme (ACE) inhibitors, is under 2
years old, or is pregnant at the time of the start of treatment (112). The course of treatment in AIT is long, typically lasting from three to five years. In clinical practice, AIT is administered by two methods: the traditional subcutaneous immunotherapy (SCIT), where the allergen extract is injected under the skin, and a more recent sublingual immunotherapy (SLIT), where a pill or drops of allergen are placed under the tongue (7). Both methods have proven effective in alleviating symptoms and reducing the use of medications (7,108). These forms of AIT have also been shown to induce long-term tolerance as well as preventing new sensitizations. Recent follow-up studies indicate that the tolerance induced by AIT may last even up to 12 years (7). A third way to administer immunotherapy is an experimental approach called intralymphatic immunotherapy (ILIT), in which the allergen extract is delivered by injection into lymph nodes, typically inguinal lymph nodes, under ultrasonic guidance (113,114). Relevant immune cells, such as CD4+ T lymphocytes and dendritic cells, are concentrated in lymph nodes, and therefore ILIT facilitates the efficient uptake and recognition of allergens by these cells. Consequently, as few as three injections are required for tolerance to occur (113,115). ILIT comes with the added benefit that lymph nodes contain few basophils and mast cells, reducing the possibility of adverse immunological reactions. Technically, ILIT does not require specialized instruments or non-standard procedures, since intralymphatic injections are routinely administered e.g. in radiology as a means of delivering imaging agents. Despite the promising results that have even exceeded those obtained with SCIT and SLIT (115), ILIT has not found its way into widespread clinical practice, perhaps partly because it requires a specialized physician familiar with intralymphatic injections. From a patient’s vantage point, it may also be potentially intimidating. Some researchers also consider oral immunotherapy (OIT) for food allergens as a form of AIT. OIT comprises the daily consumption of milligram to gram amounts of allergen preparation, typically mixed with food, with incremental increases in dosage over a period of weeks or months (116). A needle-free approach, transcutaneous immunotherapy, based on skin-permeating nanoparticles has also recently been investigated as a treatment option for allergies, such as the Japanese cedar pollinosis (117).

Each of the above mentioned forms of AIT is associated with several potential problems. First, with the exception of ILIT, the treatment takes several years to complete: in SCIT and SLIT the time frame ranges from three to five years, with frequent administrations of the allergen preparation (118). With SCIT, this means weekly or monthly appointments with a physician for the injections. SLIT has been devised partially to overcome this inconvenience, since after the initial treatments performed under the supervision of a medical practitioner, the patient can easily administer the required dose at home. A second important issue with the current forms of AIT is that allergen extracts are often incompletely characterized and contain components, which may predispose the patient to new allergic sensitizations (119-121). Third, AIT can instigate systemic side effects ranging from asthma attacks to rare but potentially life-threatening anaphylactic reactions through IgE cross-linking caused by the allergen (111). Yet another concern associated with AIT is that in several controlled trials of
OIT and SLIT the therapeutic effect remained modest for a considerable number of patients with outcomes largely lacking clinical tolerance. Moreover, patients with severe allergy were often completely omitted from the studies, even though they would have been the target group most in need for an effective treatment (116). Finally, troubling figures were observed regarding patient compliance and persistence among subjects undergoing the AIT treatment, as it was discovered that as few as 23% of patients receiving SCIT and 7% of those receiving SLIT reached the minimally required duration of 3 years of treatment (122).

Allergen immunotherapy-related risks can be reduced by producing allergen preparations in a highly standardized form as recombinant proteins. In this way, every component and its concentration in the final therapeutic product can be subjected to the Good Laboratory Practice (GLP)-level quality control and screening (123). In addition to the benefit of knowing the exact specifications of the final product, recombinant protein technologies offer a way to tailor the proteins in such a way that they contain a reduced amount of IgE-binding sites (so-called hypoallergens) while still containing the required T cell epitopes, reducing the occurrence of untoward IgE reactions (124,125).

Mechanisms of tolerance induced by AIT remain incompletely known, but they are thought to be largely mediated by a shift in the balance of T-helper cell responses away from the Th2-type response. Several studies have identified possible mechanisms for this, which include the deletion or the induction of anergy of allergen-specific Th2 cells, induction of populations of regulatory T cells and the induction of allergen-specific IgG4 production by B cells (47,107,116,123,126). IgG4 is thought to exert its protective role by blocking the IgE binding of allergens and the IgE-mediated antigen presentation by B cells (127). A recent study suggested that AIT should primarily focus on supporting the generation of regulatory T cells (Tregs) by utilizing Treg-inducing adjuvants in AIT preparations rather than striving for a shift in the Th1/Th2 ratio (128). In addition to effector T cells, the function of dendritic cells has been shown to be modified by Tregs (129).

2.2.3 Peptide-based allergen immunotherapy
To further reduce the risk of adverse effects related to AIT and to improve the standardization and efficacy of immunotherapeutic agents, an alternative approach called peptide-based allergen immunotherapy (PIT) has been studied intensively. Short synthetic peptides containing the relevant T cell epitopes typically lack the capacity to bind allergen-specific IgE and cross-link the FcεRI receptors on mast cell surfaces but still retain the T cell reactivity required for immunomodulation to take place (104). T cell epitope-mediated anergy was first reported by O’Hehir et al. in 1991 (130) and the capacity of allergen-derived T cell epitopes to induce tolerance to a whole allergen molecule was first discovered in 1993 by Briner et al. (131) for cat allergen Fel d 1 and by Hoyne et al. (132) for dust mite allergen Der p 1. Other preclinical studies conducted in mice with allergens, such as the mite Der p 2 (132), birch Bet v 1 (133), and the bee venom phospholipase A2 (PLA2) (134), similarly displayed some success in modifying the ensuing immune response towards a non-pathogenic
phenotype. Mechanistically, these studies reported suppressed T cell proliferation and decreased production of the cytokines IL-13, IFN-γ, and especially the Th2-associated IL-4. Furthermore, a decrease in the levels of allergen-specific IgE and increase in the levels of protective IgG4 were observed.

The first clinical trials were performed in humans with cat Fel d 1 soon after the initial murine studies. These trials employed a vaccine consisting of equimolar amounts of two 27-amino acid peptides of Fel d 1 at high concentrations (up to 750 µg). They managed to alleviate the pulmonary function and improve the overall status of cat-allergic patients, although the effect in general remained modest (135-137). Moreover, the success was hampered by frequently occurring immediate side effects, such as nasal congestion, flushing, pruritus and chest tightness, which were most likely due to the cross-linking of IgE by the long peptides. Delayed adverse reactions, such as asthmatic responses in subjects without underlying asthma, were later attributed to cytokine release by T cells that were activated by the peptides (138). Subsequent trials employing shorter Fel d 1 peptides, 13 to 17 amino acids in length, at roughly ten-fold lower concentrations reported a clear decrease in the frequency of aforementioned adverse effects (138,139). Another early trial, conducted with the bee venom allergen PLA2, utilizing peptides of 10 to 17 amino acids in length, proved safe and generally effective. Adverse side effects to the peptide preparation were absent in three of the five test subjects and the remaining two had mild local reactions. Furthermore, three of the test subjects displayed improved clinical tolerance, but in two the effect remained modest (140). It was suggested that the mixed results were due to an incomplete HLA coverage of the peptides which lead to a failure to induce T cell responses in subjects with certain HLA phenotypes. This hypothesis gained support when a subsequent study, using peptides covering the whole PLA2 sequence, displayed an improved clinical outcome (141). However, this study demonstrated once again the elevated tendency of long peptides to elicit adverse side effects. In the trial, long > 40-mer peptides were applied, and a substantial portion of the test subjects became skin prick test-positive to the peptides shortly after the injections. Furthermore, some subjects exhibited generalized symptoms, but these remained mild (141).

After these initial studies, Fel d 1 and PLA2 PITs have been examined in additional clinical trials in different settings. For example, in a very recent clinical phase III trial, Fel d 1-derived synthetic peptide immuno-regulatory epitopes (SPIREs) were utilized with promising results (142,143). In parallel to human studies, murine models of PIT have been developed for multiple additional allergens, such as Japanese cedar (144), Timothy grass pollen (145), olive pollen (146), egg-white (147) and hornet venom (148). Furthermore, peptides potentially suitable for PIT have been identified in recent years for several mammalian allergens, such as the horse Equ c 1 (75), cow Bos d 2 (86) and dog allergens Can f 1 (149) and Can f 4 (150) to which no PIT trials have been conducted as of yet.
The mechanisms of tolerance in PIT appear to overlap at least in part with those observed in conventional AIT, as anergy, immune deviation and the deletion and induction of regulatory T cells all appear to be contributing factors in successful outcomes (104,139,140,151,152). IL-10-producing Type 1 regulatory cells (Tr1 cells, see below in Chapter 2.3.4) appear to be the dominant regulatory T cell subtype induced by PIT (153,154). Specific IgG4 apparently has a lesser role in PIT than in conventional AIT, since short peptides are less potent in stimulating antibody production (104). Furthermore, the immunomodulatory mechanisms of PIT appear to include the inhibition of allergen-specific T cell proliferation, the reduction of both Th2 (IL-4 and IL-13) and Th1 (IFN-γ) cytokine production, and the increase of IL-10 production, a cytokine with immunosuppressive properties (139,141,155). Studies indicate that the presence of IL-10 is required for inducing Tr1 cells (see 2.3.4) (152), although a novel subset called human tolerogenic dendritic cells or DC-10s may be involved as well (156). In addition to Tr1 cells, another subset of IL-10-producing lymphocytes, regulatory B (Br1) cells, has recently been described and it may play a role in successful immunotherapy (126,157). In addition to IL-10, Br1 cells produce IgG4, which is thought to reduce allergen-specific IgE formation (158).

An alternative version of peptide immunotherapy, utilizing altered peptide ligands (APLs), has been studied in several immunological settings ranging from infectious diseases and cancer immunology to allergy and autoimmunity. It has been studied most extensively in the context of autoimmune diseases where it has been investigated in the animal models of type 1 diabetes (159), multiple sclerosis (160,161) and arthritis (162,163). APLs take the concept of PIT a step further through the introduction of amino acid substitutions to the native peptide sequences. The basic concept of APLs was described already several decades ago (164,165). Mechanistically, APLs may convey antagonistic, partially agonistic or superagonistic signals via the peptide:HLA-TCR interface and thereby modify the ensuing T cell response depending on the altered signal (166-168). Antagonistic ligands typically engage the TCR without activating it and block the action of agonistic signals, inhibiting the activation of the antigen-specific T cell (165,167). Partially agonistic ligands may induce activating signals of varying intensity depending on the degree of TCR affinity they exhibit. They may, for example, activate only a certain subpopulation of antigen-specific cells or induce only some of their effector functions (164,168). Superagonistic peptides, as the name implies, exert a more robust signal and are therefore capable of inducing T cell activation at lower concentrations than the natural ligand (166).

Studies of APLs (159-163,169,170) in autoimmune diseases have demonstrated that therapeutically they may exploit several mechanisms that have potential to improve the outcome of the disease. Antagonistic APLs may, for example, block the activation of the disease-associated effector cells, whereas superagonistic ligands have a potential to induce activation-induced cell death in their targets. In both scenarios, APLs act by impeding the pathogenic function of the autoimmune cells. Another mechanism of action of APLs could
be the induction of a new T-helper cell population that can modulate the existing pathogenic T cells through a phenomenon called bystander suppression (169,171).

Despite the promising theoretical immunomodulatory mechanisms, APLs have been employed scarcely in clinical trials and the limited number of trials that have been conducted have largely been marred with persistent setbacks, such as adverse side effects or meager immunoregulatory results (172-174). For example, subcutaneous injections of an altered peptide ligand NBI-6024 of the insulin β-chain amino acids 9-23 epitope did not improve or maintain β-cell function in type 1 diabetic patients (174). So far, no clinical trials of APLs have been performed in allergic diseases. Different types of allergen immunotherapy are summarized in Table 2.

**Table 2. Modes of allergen immunotherapy for respiratory allergies**

<table>
<thead>
<tr>
<th>Type</th>
<th>Active substance</th>
<th>Benefits</th>
<th>Disadvantages</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergen-specific immunotherapy(a)</td>
<td>Whole allergen preparations</td>
<td>Contains a wide range of T cell epitopes, proven efficient</td>
<td>Difficult to standardize, risk of adverse reactions, normally takes 3 to 5 years</td>
<td>In clinical practice</td>
</tr>
<tr>
<td>Peptide-based immunotherapy(b)</td>
<td>Synthetic peptides containing identified T cell epitopes (from c. 10 up to 60 amino acids)</td>
<td>Well-defined antigen preparations, reduced risk of IgE-mediated adverse reactions</td>
<td>Long peptides may still cause adverse reactions, limited number of clinical trials</td>
<td>Not in clinical practice, phase III trials</td>
</tr>
<tr>
<td>Immunotherapy with altered peptide ligands(c)</td>
<td>Synthetic peptides containing amino acid substitutions in order to enhance efficacy</td>
<td>Well-defined antigen preparations, potential for more robust immunomodulatory responses</td>
<td>Meager results in clinical trials of autoimmune diseases, adverse reactions</td>
<td>No clinical trials in allergy</td>
</tr>
</tbody>
</table>

Key references: \(a\)107, 108; \(b\)151, 153; \(c\)172, 173

**2.3 T CELLS**

T cells have a central role in the adaptive immune response (175,176). T cells with unique functions are divided into several subtypes. The two most prominent types of T cells are those that express either the CD4 (CD4+ T cells) or CD8 (CD8+ T cells) cell surface molecules. These cells together make up the majority of all T cells (13). Other minor T cell subsets include invariant natural killer T (iNKT) cells and γδ+ T cells that express characteristics for both innate and adaptive immunity (177). CD4+ and CD8+ T cells can be further divided into various phenotypic subpopulations (see Chapter 2.3.3). With the exception of γδ+ T cells and their unique T cell receptor (TCR), all T cells are characterized by TCRs composed of disulfide-linked α and β chains. They are cell surface molecules, the function of which is to recognize antigens presented in association with the major histocompatibility complex (HLA) molecules (see Chapter 2.3.2).
Of the two major subsets of T cells, CD8+ T cells are termed as cytotoxic T cells. Their function is to recognize cells infected by intracellular pathogens and, upon recognition, facilitate apoptosis of the infected cell by a direct cell-to-cell interaction. The recognition takes place through the TCR-peptide:HLA class I interaction, when the infected cell presents microbial peptides associated with its HLA class I surface molecules. In addition to intracellular pathogens, such as viruses, CD8+ T cells survey the host body for malignant cells and facilitate their eradication. Unlike CD8+ T cells, CD4+ T cells recognize the antigens of extracellular origin in the context of HLA class II molecules on the surface of professional antigen-presenting cells (such as DCs and B cells), and they exert their characteristic function through the secretion of activating or inhibitory cytokines and cell contact-mediated signals (see Chapter 2.3.2).

2.3.1 CD4+ and CD8+ T cell development
The earliest T cell progenitor cells migrate from the bone marrow to the perimedullary cortex region in the thymus where they are called thymocytes. As the thymocytes mature, they migrate to the subcapsular area of the thymic cortex and, finally, enter the medulla of the thymus from which they are ultimately released into circulation (13,175,177,178). At the time of entry to the thymus, the T cells lack the CD4 and CD8 surface molecules and their T cell receptor genes have not undergone genetic rearrangement. During this phase of their development, these cells are called double-negative (DN). As the DN cells mature, their TCR β-chain genes are rearranged first, after which the chain is coupled with the CD3 molecule complex and an invariant pre-TCR α-chain (179). Simultaneously, the thymocytes enter an extensive proliferative phase. These events induce the expression of CD4 and CD8 coreceptors and the cells progress to the double-positive (DP) stage. The DP cells then proceed to rearrange the α-chain of their TCR and subsequently recognize self-peptide:HLA complexes with their newly formed TCRs. During the thymic education, self-peptides are presented to DP cells by specialized cells called cortical epithelial cells (177,178). A majority of the DP cells fail to bind to the peptide:HLA complexes with sufficient avidity and they undergo apoptosis, with only some 10% of T cell precursors being capable of binding to the self-peptide:HLA complex with sufficient avidity to block the apoptotic pathway (13). This phase is called positive selection, which also determines the lineage of the DP cells. If the thymocyte recognizes the peptide presented by an HLA class I molecule, it commits itself to the CD8 lineage, downregulating the expression of CD4 and becoming single-positive (SP). A peptide recognized in the context of an HLA class II molecule induces an opposite event, i.e. CD4 upregulation and the loss of CD8. During their time in the medulla, resident thymocytes undergo a series of phenotypically and functionally distinct maturation stages, e.g. downregulation of signal transducer CD24 and L-selectin (CD62L) (177). Finally, in vitro studies with CD8+ T cells suggest that in addition to appropriate TCR stimulation, cytokine receptor signals are required for the production of functionally mature cells (180).
The final phase of thymic maturation of T cells consists of negative selection of the cells in the medulla of the thymus, where DCs and macrophages derived from the bone marrow present to the SP thymocytes HLA molecules loaded with peptides originating from self-antigens (13). If the developing T cells bind the self-peptides with high affinity they receive a signal from the APCs to undergo apoptosis. This process eliminates most of those T cells that could induce an autoimmune response. As a result of the thymic selection process, the prospective T cells possess low but sufficient avidity to self-peptide:HLA complexes. The thymic selection is an austere tribulation for the thymocytes, since up to 99% of the thymocytes that develop in the thymus also die in the thymus, while only a small fraction of the T cell candidates survive positive and negative selection, entering the pool of circulating T cells as naïve CD4+ and CD8+ lymphocytes (179,181).

Upon entering the periphery, naïve T cells (or so-called recent thymic emigrants) migrate to the peripheral lymphoid organs, such as lymph nodes, spleen and the mucosa-associated lymphoid tissues, where adaptive immune responses are initiated (182). For a T cell immune response to be induced by an external agent, such as a microbe or allergen, the naïve T cells specific for the appropriate antigens must encounter DCs presenting those antigens in a peripheral lymphoid tissue. As foreign antigens can enter the body anywhere, they must be brought from the site of entry to the peripheral lymphoid organs by migrating DCs, where they present the antigens on their HLA molecules. Certain soluble antigens can also reach the lymph nodes independently via blood circulation and lymphatic fluid, where they are taken up and presented to the T cells by APCs residing in the lymphatic tissue. In this way, a naïve T cell traveling through lymphoid tissues can be in contact with thousands of DCs every day and hence greatly improve the chances of encountering the antigens derived from the site of their entry. If the TCR-peptide:HLA interaction is of high avidity, the T cell activates (see Chapters 2.3.2 and 2.3.3). If they fail to encounter their specific antigen, naïve T cells return to the bloodstream and continue recirculating (13,182).

### 2.3.2 The T cell receptor

T cell receptors are cell-surface heterodimers composed of disulfide-linked α and β chains or, in some cases, of γ and δ chains (13,183). T cells with the conventional α and β TCR chains form the bulk of the lymphocyte population and they are responsible for recognizing peptide antigens presented in association of HLA molecules on antigen-presenting cells (13,183). The chain genes contain distinct segments called variable (V), diversity (D), joining (J) and constant (C), although the D segment is lacking in the α chain. They are fused together by somatic recombination as the T cell develops. The genetic diversity of the TCR genes is remarkable and it results in a myriad repertoire of TCRs with differing specificities and affinities. It has been estimated that in the mouse T lymphocytes are able to assemble about $10^{15}$ different TCR variable regions (175,184). This is largely achieved by employing a set of no more than circa 400 genes by particular recombination processes that cut, splice and rearrange variable-region genes.
The binding site of the TCR is composed of three complementary-determining regions (CDR1-3), which together participate in the recognition of the peptide:HLA complex. The hypervariable CDR3 loop of α and β chains is composed of chains that consist of an amino-terminal variable (V) region and a carboxyterminal constant (C) region, as well as diversity (D, heavy β chains only) and joining (J) regions. (35,181). It is located centrally within the TCR contact surface, interacting with the antigenic peptide placed in the peptide-binding groove of an HLA molecule. The less-variable CDR1 and CDR2 loops mainly interact with the HLA molecule itself. Therefore, the TCR recognition is determined by both the peptide and the HLA molecule compatibility – a feature that is called HLA restriction (13). The TCR engagement alone is not sufficient to carry out the signal required for T cell activation. Additional costimulatory signals are provided by receptor interactions with cytokines and APC costimulatory cell-surface molecules. The downstream TCR signaling is conveyed through the T cell coreceptor CD3 complex (see Chapter 2.3.3) (185).

The major histocompatibility complex (MHC; i.e. HLA in humans) is a set of genes whose equivalent is found in all mammals (13). As is with the TCR, the HLA complex displays remarkable genetic variability and it is responsible for a great degree for controlling the immune response in health and disease. Hence, it is involved in a multitude of roles, being expressed in nearly every nucleated cell of the human body. In humans, the bulk of the HLA complex is located on chromosome six and it contains over 200 genes (186). Some HLA-related genes are found on different chromosomes altogether, e.g. β2-microglobulin on chromosome 15 (187). Many of the genes within the HLA locus, such as TAP, LMP and TNF, are involved in antigen processing or presentation, or are otherwise related to innate or adaptive immune responses (13). The HLA genes exhibit extensive polymorphism and there are multiple alleles of each gene in human populations. For example, the most multiform loci in humans, namely HLA-A, HLA-B and HLA-DRB1, contain 673, 1077 and 669 known alleles, respectively, according to a summary compiled in 2008, and additional alleles have been identified since then (186). Despite the central role of the HLA complex in immune regulation, many of the HLA-associated genes are not related to immune function.

HLA genes involved in immune recognition are normally divided into two subgroups based on their structure and immunological role. The molecules encoded by HLA class I genes are ubiquitously expressed in virtually all cells of the body, except erythrocytes. Their function is to present degraded fragments of cytosolic proteins to CD8+ T lymphocytes, also known as cytotoxic T cells. This process constitutes an integral part of immunity, for example, to viral infections and cancer (175). The HLA class I molecule is composed of a 43 kDa α-chain and a 12 kDa β2-microglobulin. These subunits are synthesized separately in the cytosol and assembled in the endoplasmic reticulum (ER) where also a peptide fragment of an enzymatically spliced endogenous protein is attached in the peptide-binding groove between the HLA molecule subunits. This groove has a cleft that can accommodate peptides 8 to 10 amino acids in length. Finally, the functional HLA I:peptide complex is transported to the cytoplasmic membrane (188).
In regard to allergy, the HLA class II molecules play a more substantial role. They are expressed on the surface of specialized APCs, such as dendritic cells, thymic epithelial cells, monocytes and B cells (13). These cells actively survey their surroundings for foreign antigens and internalize them via endocytosis. Intracellularly degraded peptide chains of exogenous antigens are then presented in the context of the HLA class II molecules to another major subset of T lymphocytes, the CD4+ T cells. The HLA class II molecule contains two membrane-spanning, non-covalently linked components, the α-chain (34 kDa) and the β-chain (29 kDa). When emerging from the ER they also contain a third subunit, an invariant chain that prevents the loading of endogenous peptides in the peptide-binding groove. The three-subunit HLA class II molecule is transported into endosomes, where the extracellular proteins, as well as the invariant chains, undergo proteolysis. Peptides of 13 to 17 amino acids in length originating from extracellular proteins are loaded in the peptide-binding groove of the HLA class II molecule and the HLA II:peptide complexes are transferred to the cell surface where they are presented to CD4+ T lymphocytes (13).

2.3.3 CD4+ T cell activation and T-helper cell subsets
Successful engagement of the T cell receptor to its cognate peptide:HLA complex on the APC surface is a prerequisite for the activation and differentiation of T cells, but is not sufficient alone to initiate the subsequent activation cascade (189). Instead, the TCR is coupled with the non-polymorphic CD3 complex that transmits the downstream TCR signal. This begins with the phosphorylation of the cytoplasmic ends of the CD3 molecule, and the ensuing signaling cascade results in the activation of transcription factors (such as AP-1 and NF-κB) that lead to the expression of multiple genes required for T cell proliferation, differentiation and effector functions (185). Furthermore, the T cell coreceptors CD8 and CD4 interact with the non-polymorphic surfaces of the membrane-proximal domains of the HLA class I or II molecules, respectively. The role of these receptors is to stabilize the TCR-HLA complex and enhance the TCR signaling (190).

However, for T cells to activate properly, additional signals are required, mediated by costimulatory molecules as well as cytokines. The most important costimulatory molecules are the glycoproteins B7-1 (CD80) and B7-2 (CD86) that are expressed by APCs. They interact with the CD28 protein on the surface of T cells (191). Of these glycoproteins, B7-2 is constitutively expressed on the surface of APCs and plays a major role in T cell activation, whereas B7-1 is only upregulated in the event of APC activation through B7-2 (192). The interaction of CD28 with its ligands is especially important for the activation of naïve T cells. Inhibition of CD28 signaling results in impaired T cell activation (192).

Upon T cell activation, the signal conveyed through CD28 gradually upregulates the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on T cells. This molecule in turn binds to the same B7 molecules as does CD28 but with increased affinity, thus inhibiting the CD28-B7 interaction and relaying an inhibitory signal to the T cell (191). This, in concert with other
regulatory elements, such as the molecules ICOS, 4-1BB, CD30 and OX40, helps to maintain T cell homeostasis (193).

The ligation of CD28 and subsequent T cell activation also induce the production of IL-2, which is an important mediator of T cell proliferation and differentiation (194). As the circulating naïve CD4+ T cells encounter their specific antigen presented by an APC, and if the proximal milieu is favorable for T cell activation, clonal expansion and differentiation into effector cells are initiated.

Several CD4+ T cell subsets exists (Figure 1), of which the well-established T-helper 1 (Th1) and T-helper 2 (Th2) cells were identified nearly three decades ago (195). A third major subset, Th17 cells, was identified more recently (196). The Th1-type immune responses activate macrophages, promote the IgG isotype class-switching of B cells and initiate the cytotoxic CD8+ T cell effector functions (13,197). These events help the immune system to fight intracellular threats, which normally include viruses and certain intracellular bacteria, but also malignant cells (198). The classical Th1-associated cytokines include interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNFα) (199). Factors promoting the Th1 differentiation of naïve T cells include IL-12 and IL-27, and the subsequent activation of the transcription factor T-bet via the signal transducer and activator of transcription (STAT) 1 pathway (200). IL-27 also blocks the transcription factor GATA-3, which is characteristic for Th2 cells (200).

The role of Th2 cells is to promote the immune response against certain parasitic infections, especially helminths and to induce humoral immune responses (198). Th2 cells also play a crucial role in allergic diseases (see Chapter 2.1.2). Central elements in the Th2-type responses are eosinophil and mast cell activation, and B cell class-switching to the IgE isotype (126). Th2 cells are characterized by the secretion of cytokines IL-4, IL-5 and IL-13 (199). Their activation is driven by IL-4, which causes the upregulation of GATA-3 by STAT6. Weak stimulation through the TCR is also a factor that is known to polarize T cells towards the Th2 phenotype (201,202).

The role of IL-17-producing Th17 cells is to support the immune system to eradicate extracellular bacteria and pathogenic fungi (203). Factors driving the differentiation of Th17 cells include the cytokines TGF-β in conjunction with IL-6. The hallmark transcription factor for Th17 cells is RORγ, which is activated by the STAT3 pathway.

Several other CD4+ T cell populations have also been discovered in recent years, but their precise roles in health and disease are still unknown. These include follicular helper (Tfh) (204-207), Th9 (208) and Th22 (209-211) T cells. Whether each of these cell types constitute their own distinct population also remains an open question (199), since recent studies indicate that T cell phenotypes display a considerable degree of plasticity, rather than being permanently committed, as previously thought (212,213). Finally, CD4+ T cells include several subpopulations with immunoregulatory functions (see Chapter 2.3.4).
Figure 1. Antigen presentation by DCs to naïve T cells and other factors (innate immune response substances, vitamins, cytokines in the environment) induces the T cells to produce ILs and differentiate into Th1, Th2, Th9, Th17, Th22, or follicular Th (Tfh) cells. These T cell subsets can promote different types of inflammatory responses on the basis of their respective cytokine profiles, responses to chemokines, and interactions with other cells. Reprinted by permission from Elsevier B.V.: Journal of Allergy and Clinical Immunology (127), copyright 2015.

2.3.4 Regulation of T cell responses

T cell responses are regulated at multiple levels, which demonstrates the importance of maintaining a balance between the purposeful immune activation against pathogens and the harmful activation against self-antigens or harmless external substances, such as normal bacterial flora (13, 214, 215). The primary means of establishing tolerance to self-antigens is the deletion of autoreactive T cells in the thymus (known as central tolerance, see Chapter 2.3.1). Various additional mechanisms are operative in the peripheral lymphoid organs, mucous membranes and blood, collectively known as peripheral tolerance (216). Unwanted T cell activation can be avoided altogether by means of immunological ignorance, where the threshold of activation against self-antigens or harmless environmental substances is not reached, e.g. through low levels of antigen (214) or restricted anatomical access by the immune cells (215). Similarly, anergy, as first described by Jenkins and Schwartz almost 30 years ago, can occur when T cell activation fails to take place, for example, following a lack of required costimulatory factors. T cells become subsequently anergic, i.e. they fail to acti-
vate after subsequent exposure to their cognate antigen (217). T cells may also undergo activation-induced cell death (AICD) by means of apoptosis. In Th1 cells, AICD is initiated by signaling through the FAS ligand (FASL); Th17 cells with substantially lower FASL expression are more resistant to it. According to a recent study, the resistance to cell death by Th17 cells could contribute to the pathology of certain immunological diseases, such as multiple sclerosis, where their numbers have been shown to be significantly elevated (218).

Peripheral tolerance is primarily maintained by various subsets of regulatory T cells (Tregs). Of these, two of the most studied ones are the T-regulatory type 1 (Tr1) cells and the forkhead box protein 3 transcription factor-positive Tregs (FOXP3+ Tregs) (219). Both subtypes are capable of suppressing effector T cell proliferation and cytokine production, and they both express CTLA-4 and the glucocorticoid-induced TNFR family-related molecule (GITR), which have immunosuppressive and inhibitory functions (220, 221).

Tr1 cells are an inducible subset of regulatory T cells that play a crucial role in promoting and maintaining immunological tolerance. The primary mechanisms by which Tr1 cells exert their control over immune responses are the secretion of IL-10 (222) and the induction of apoptosis in cells of myeloid lineage (e.g. DCs) by means of granzyme B (223, 224). However, a defined cell surface signature for Tr1 cells has not yet been established. Their identification relies on their unique cytokine profile, which, in addition to the production of high levels of IL-10, is characterized by minimal amounts of IL-4 and IL-17, which separates them from Th2 and Th17 cells (219). They also secrete low levels of IL-2 and may secrete IFN-γ in certain instances. Like other regulatory T cell subsets, activated Tr1 cells may transiently express FOXP3, but its expression is not constitutive and not on such a high level as is seen in CD25+Foxp3+ regulatory T cells. Tr1 cells are induced in peripheral tissues from naïve CD4+ T cells by tolerogenic antigen-presenting cells in the presence of IL-10, and their activation is not dependent on the expression of FOXP3 (219). Upon encountering their specific antigen in the context of peptide:HLA class II complex, Tr1 cells secreting IL-10 may also induce bystander suppression in T cells specific to different antigens. Another characteristic mediator of Tr1 function is the transforming growth factor (TGF)-β (225), which is also the primary suppressive cytokine of a subset of T cells called Th3 cells. However, a definite surface marker for Tr1 has not been identified, despite the extensive efforts by the scientific community (220). Recently, the surface molecules CD49b and LAG-3, as well as the transcription factors cMaf, Blimp-1, and AhR, have been associated with this subset of T cells (226).

Another major subset of regulatory T cells consists of FOXP3+ Tregs that were originally identified by Sakaguchi et al. in 1995 by their constitutive expression of CD25, a subunit of the IL-2 receptor (227). Later, in 2003, the FOXP3 transcription factor was identified as both a marker and a lineage-determining factor for CD4+CD25+ Tregs (228, 229). FOXP3+ Tregs are produced in the thymus, from where they emerge as functional regulatory T cells (central or natural (n) Tregs), or they can differentiate from peripheral-blood naïve CD4+ T cells (inducible or peripheral FOXP3+ Tregs). Functionally, these Tregs of different origin cannot be distinguished from one another, but they both mediate their suppressive capabilities
mostly through contact-dependent inhibition, according to in vitro studies (13,230). In addition, they have been reported to produce immunoregulatory cytokines IL-10 and TGF-β in vivo (230). Unlike the other abovementioned Treg subsets, nTregs are potentially self-reactive T cells that appear to be selected in the thymus based on their high-affinity binding to HLA molecules loaded with self peptides (13).

Perhaps the most striking example of the importance of uncompromised regulatory T cell function is the so-called Immune, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, which is caused by deleterious mutations in the FOXP3 gene (231). Patients that suffer from this disease are typically males, who often succumb to insurmountable systemic autoimmunity within the first year of life. A number of other mutations associated with the regulatory T cell function, such as loss-of-function mutations in CD25, signal transducer and activator of transcription 5B (STAT5B) and the ubiquitin-conjugating enzyme-encoding gene ITCH, result in the IPEX-like phenotypes. On the other hand, excessive proliferation or inappropriately high suppressive activity by Tregs is linked with medical conditions associated with impaired effector T cell function. For example, elevated numbers of Tregs are associated with poorer prognosis in several cancers, such as breast and ovarian carcinomas (232,233). Along with autoimmunity, insufficient function of Tregs is suspected to play a role in the pathogenesis of allergic diseases and the restoration of their appropriate function may offer a way to treat these conditions (154,234).

2.4 DETECTION OF ANTIGEN-SPECIFIC CD4+ T CELLS IN HUMANS

2.4.1 A needle in the haystack
The human T cell repertoire consists of multiple phenotypically distinct subsets of T cells with a staggering number of different antigen specificities (see Chapter 2.3.2). T cells specific to certain antigens, such as autoantigens, tumor antigens and environmental antigens, are among the rarest cell types in the peripheral blood (235). In particular, the repertoire of naïve T cells with almost an unlimited variety of T cell receptors is so large that there is a T cell specific for almost any conceivable antigenic epitope (13). The overwhelming majority of these naïve T cells has no relevance in the context of any immunological disease (175). Therefore, the cells of interest are often those that have already encountered their specific antigen and expanded clonally in vivo, i.e. memory T cells. Moreover, the low number of antigen-specific naïve T cells makes their detection a daunting challenge (235).

Even the CD4+ T cells specific to potent immunogenic antigens of microbial origin are rare, especially within the naïve T cell repertoire where clonal expansion has not taken place. This frequency is estimated to be in the order of 0.2 to 60 specific CD4+ T cells per 106 naïve peripheral blood T cells (235-241). Repeatedly boosted responses by strongly immunogenic antigens, such as those from tetanus toxin or the influenza virus, can display transiently high numbers of antigen-specific cells in the memory pool, up to 1000 per 106 CD4+ T cells. However, in the absence of recent antigen challenge, the frequencies are in the range of 10-
50 per 10⁶ cells (241). Animal allergen-specific CD4+ T cells tend to be considerably more rare, their frequencies ranging from 1 to 10 specific cells per 10⁶ memory T cells in allergic individuals and even less in non-atopic ones (149,150,242). Autoantigen-specific T cells, such as those encountered in type 1 diabetes or celiac disease, appear to be fewer still, being in the range of one in a million (241).

The rarity of T cells specific for a given antigen sets considerable restraints for their direct detection. Direct detection is often achieved by means of flow cytometry, but rare events are easily lost in the background noise (235). Therefore, the overall cell numbers that need to be analyzed to detect a rare cell population often become unfeasibly large, costing time and valuable reagents (235,243). However, modern flow cytometers capable of high throughput of millions of cells combined with increasingly affordable, yet highly-specific labeling methods have significantly improved the situation. The characteristics of three central antigen-specific CD4+ T cell detection methods are summarized in Table 3.

Table 3. Detection of antigen-specific CD4+ T cells

<table>
<thead>
<tr>
<th>Method</th>
<th>Duration</th>
<th>Parameter</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Split-well method⁴</td>
<td>2-4 weeks of in vitro culture</td>
<td>Cell proliferation</td>
<td>Technically straightforward, permits natural processing of antigens by APCs</td>
<td>Long duration, dependant on proliferative capacity of T cells, changes in phenotype possible</td>
</tr>
<tr>
<td>HLA class II tetramers⁵</td>
<td>With initial in vitro stimulation: one to two weeks; Direct ex vivo detection with magnetic enrichment: 3 to 6 hours</td>
<td>Binding to T-cell receptor</td>
<td>Fast, sensitive, allows ex vivo phenotyping and single-cell sorting</td>
<td>Requires previous knowledge of T cell epitopes and HLA restriction, affinity, availability of tetramers</td>
</tr>
<tr>
<td>Activation markers⁶</td>
<td>3 to 16 hours initial antigen stimulation + enrichment/staining</td>
<td>Expression of CD154, CD137, CD69 etc., or combination thereof</td>
<td>Fast, sensitive, not limited by HLA restriction or knowledge of T cell epitopes, ex vivo phenotyping and single-cell sorting</td>
<td>Enrichment for rare T cell populations required, shortest stimulation times suitable for peptide antigens only</td>
</tr>
</tbody>
</table>

Key references: ⁴245; ⁵241, 255-257; ⁶235, 243

2.4.2 Measuring antigen-specific T cell proliferation in vitro

Since the limiting factor in detecting antigen-specific T cells is often the extreme rarity of the cells of interest, direct detection has traditionally been difficult and impractical. Therefore, traditional methods have typically utilized an initial in vitro stimulation step to induce the proliferation of the cells of interest in order to expand them sufficiently for detection. Since the activated cells proliferate exponentially, even a single cell can give rise to a great number of progenitors in a short time frame of few days up to two weeks (72,244).
The so-called split-well method (245), for example, relies on antigen-specific proliferation. If the cells are seeded in low enough numbers in cell culture plates (typically a 96-well plate), statistically only one cell or less of the specificity of interest ends up in each well. The cells are then stimulated with the antigen of interest, such as an allergen, and the cells are cultured for a sufficiently long time, typically up to two weeks. The contents of the wells are then divided into two daughter plates (keeping part of the cells in the original plate), of which one is restimulated with the antigen and the other serves as a control. If the antigen-specific cells have expanded in the well, the reactivation of these cells can be measured as proliferative activity. A widely-utilized method for this is the radiolabeled thymidine incorporation test (245), where \([^{3}H]\)-labeled thymidine is added to the culture medium of proliferating cells. Then, the radioactivity of the stimulated cells is compared to that in the non-stimulated control plate to identify the cultures with elevated proliferative activity.

Cell proliferation can be measured by other means as well. One such method that has a long history in immunological research is the carboxyfluorescein succinimidyl ester (CFSE) dilution assay (246). CFSE is an intracellular fluorescent dye. Prior to the initial antigen stimulation, the cells are incubated with the dye, which is retained within the cells. As the antigen-specific T cells divide upon exposure to the antigen of interest the amount of dye within each dividing cell is halved. Because of this, the dividing cells can be detected with a flow cytometer based on the dilution of the CFSE dye. The flow-cytometric analysis of CFSE-low cells has an additional benefit, as it permits the single-cell sorting of the dividing cells. The CFSE method itself is known to be prone to the problem of bystander activation of non-specific cells (235). They are inevitably present in cultures that have been initiated from a polyclonal pool of T cells, and therefore each single-cell sorted clone needs to be screened for antigen specificity (247). Furthermore, CFSE labeling can be used to monitor cell division and migration \textit{in vivo} in animal models, as it allows the detection of cell migration and proliferation in tissues (246).

In addition to measuring T cell division rates directly, cell activation can also be measured by means of cytokine assays. As subsets of T cells encounter their specific antigen, they produce characteristic cytokines upon activation (13,200). The pattern of secreted cytokines is typical for each T cell subset (see Chapter 2.3.3). The culture medium can be sampled and analyzed directly for a wide array of cytokines with immunochemical methods, such as ELISA (149,150,242). Secreted cytokine levels correlate with the number and activity of proliferating cells, although this gives only a rough estimation at best. A greatly improved sensitivity can be conveniently achieved by the enzyme-linked immunospot (ELISPOT) assay, which yields both quantitative and qualitative information about antigen-specific T cells within a given cell population (248,249). Cytokine production-based cell enumeration can also be achieved by flow cytometry, either by cell-surface bound capture antibodies (235) or by trapping the cytokines in the cytosol by means of secretion inhibitors, such as Brefeldin A or Monensin (250,251). The flow-cytometric visualization methods of cytokine production...
have the added benefit of permitting other phenotypic characteristics of the cells of interest to be analyzed simultaneously (252).

2.4.3 Direct *ex vivo* detection of antigen-specific CD4+ T cells

Until recent years, it has been challenging to detect antigen-specific CD4+ T cells directly *ex vivo*, as the sensitivity of most direct detection methods has not been adequate or reliable enough to identify the cells of interest. Moreover, the capacity of flow cytometers has been insufficient to process large cell numbers required to obtain a high enough number of specific events. This problem has been especially evident for the rarest subpopulations of antigen-specific T cells, such as those specific to allergens and autoantigens (235). While certain detection methods, such as ELISPOT, can achieve a theoretical resolution of one antigen-specific T cell per million total T cells, its reliance on a fixed parameter (e.g. IFN-γ production) to detect cell activation, makes it prone to miss the cells of differing functionalities, *i.e.* cells that do not produce IFN-γ upon activation in this case (241).

One approach for directly identifying antigen-specific T cells is to use a method that specifically targets the TCRs of the cells of interest (*i.e.* peptide:HLA multimers, such as tetramers; see below). Alternatively, activated cells can be detected upon exposure to the antigen of interest (*i.e.* detection T cell activation markers, such as costimulatory molecules or cytokines released upon activation; see below) (235). Even with these tools, many antigen-specific T cells are so rare that, if unprocessed, an additional problem is that these cells can easily be lost in the background noise caused by non-specific binding of reagents or non-specifically activated cells (235).

The resolution of *ex vivo* detection assays can be significantly improved and the amount of cells that need to be analyzed lowered by subjecting the PBMC preparation to preanalysis purification and enrichment (243). The general idea behind the enrichment approach is to eliminate most of the cells that are of no interest in the context of antigen-specific CD4+ T cells. These steps routinely include the purification of CD4+ T cells from other blood cells. T cells can be either directly isolated based on suitable surface markers (positive selection) or alternatively all other blood cells can be depleted from the cell preparation (negative selection) (235). Most purification methods employ a combination of antibodies attached to magnetic beads. The beads bind the labeled cells to a magnetic substrate, such as a purification column, through which the cells are run. The labeled cells are retained within the matrix. Alternatively, the bead-labeled cells can be magnetically attracted to the bottom of a test tube and non-labeled cells can then be washed out.

Even with purified CD4+ T cells, the number of cells with irrelevant specificities is so high that the limitations listed above usually impede the reliable detection of antigen-specific cells of interest (235,243). By combining four or more fluorochrome-labeled antigen peptide:HLA complexes together to form a tetramer the overall avidity of the T cell-binding peptide:HLA complexes increases so that it becomes stably attached on a T cell (253). However, designing functional tetramers can be a laborious process, since in addition to the
knowledge of the T cell epitopes of the target antigen, also their restricting HLA alleles must be known (241). A technique called tetramer-guided epitope mapping (TGEM) has been devised to address this problem (254). In TGEM, large peptide libraries are loaded onto HLA molecules, and pools of peptide-loaded HLA tetramers are then used to probe the T cell population of interest by flow cytometry. The pool displaying a positive signal is then dismantled by separately testing the individual peptides to identify the correct peptide:HLA combination (255). Another problem with HLA tetramers is that the stability of the peptide:HLA complex may vary greatly (256). Some tetramer complexes remain inherently unstable and may degrade in a matter of days, which requires using a fresh batch in each experiment (257). Moreover, HLA tetramers may fail to detect important T cell specificities that express TCRs with a lower affinity to the peptide:HLA complex (235). This is an especially important consideration when detecting cells related to autoimmune diseases and cancer (257). To overcome these limitations, multiple ways to improve the staining conditions and the affinity threshold have been described, including the use of additional antibodies targeting the tetramer itself and the use of protein kinase inhibitors (257).

Functional tetramers loaded with T cell epitope-containing peptides and labeled with a fluorochrome, often phycoerythrin (PE), can be used to detect antigen-specific T cells from antigen-stimulated and expanded T cell cultures (243). The extent of this analysis can be further increased by analyzing simultaneously other phenotypic markers of interest, such as chemokine receptors or cytokine production (258). Tetramers can also be used in detecting antigen-specific T cells directly after the *ex vivo* enrichment of tetramer-positive cells (see below) and in the *in situ* analysis combined with immunohistochemistry (259).

Combining HLA tetramer staining with a magnetic pre-enrichment step can offer a fast and efficient way to study antigen-specific T cell populations *ex vivo*. When compared to the CFSE dilution assay that requires one to two weeks of culture, or to the split-well method that can take up to one month or more, tetramer-based assays combined with an initial enrichment step often require less than a working day to complete (235). This short time frame, in turn, enables investigators to analyze the T cells of interest without affecting the phenotype or hindering the cell-division capacity of the cells that potentially take place after a long *in vitro* culture (241). Additionally, a short antigen-induced stimulation prior to the analysis can be used to facilitate additional phenotypic analyses, such as cytokine secretion (72,258,260). In the case of very rare antigen-specific CD4+ T cells, however, expansion of the cells *in vitro* may be required (235).

The tetramer-based approach is also extremely specific, exhibiting minimal non-specific binding in well-optimized conditions (247). As previously mentioned, this is a prerequisite for analyzing very rare events, where even the smallest background signal may have a profound impact on the reliability of the results. Furthermore, the successful cloning of rare T cells by means of single-cell sorting is highly dependent on the specific staining and low background (235,261,262). Tetramers conjugated with several fluorochromes, or with different combinations of them, can also be used to detect CD4+ T cells of different specificities in
a single sample (263,264). Finally, the tetramer approach does not require the target cells to be able to divide or even undergo antigen-specific activation, thus enabling tetramers to identify also non-proliferating, inactive or anergic antigen-specific T cells of certain subtypes, which could be missed by methods relying on the antigen-specific activation of the cells (235).

An alternative approach to the detection of antigen-specific cells is to use antibodies that bind to T cell activation markers and identify these by a flow cytometer. Numerous activation markers have been used for this purpose, either alone or in tandem with other such markers. The following markers among others have been proposed for detecting activated T cells: CD69, CD25, CD71, HLA-DR, CD134, CRTAM, CD137 and CD154 (CD40L) (235,265-270). However, susceptibility to bystander activation limits the use of several of these markers (e.g. CD25, CD69) but they may be used to increase the resolution of a flow-cytometric analysis in conjunction with other markers. Some markers also have a high level of constitutive expression in certain T cell subsets (CD25, CD69, CRTAM) or are expressed late during the activation process (HLA-DR, CD71, CD134) (235,271). Interestingly, with low doses of antigen several activation markers become expressed without the cells undergoing cell division. This can be regarded as an indication of high sensitivity of certain activation marker-based methods. It also follows that methods relying solely on cell proliferation, such as the split-well assay or the CFSE dilution assay, may sometimes miss the antigen-specific T cells that do not achieve the threshold required for mitosis (271).

CD69 is a widely used marker suitable for the identification of both activated CD4+ and CD8+ T cells. It is an early indicator of T cell activation, as its expression is detectable 3 to 15 hours after the antigen challenge (271). It is also expressed on B cells and NK cells (272). However, as mentioned above, CD69 is expressed by a variable amount of non-activated cells and is prone to upregulation as a consequence of bystander activation. Therefore, it can only be employed as an auxiliary marker in most applications. In flow cytometry, it can increase the sensitivity and optical resolution of the method (235).

Especially in the context of CD4+ T cells, an early activation marker CD154 (CD40 ligand or CD40L), a member of the tumor necrosis factor (TNF) superfamily, has been shown to be a highly specific activation marker of antigen-specific T cells (265,266,269). CD154 upregulation takes place rapidly and it is detectable as early as 3 hours post stimulation (273). It is expressed by all functionally activated CD4+ T cells regardless of their phenotypic or differentiation status. In addition to CD4+ T cells, it is expressed by a subset of CD8+ T cells, but this is easily overcome by excluding CD8+ cells from the flow-cytometric analysis. CD154 also has a particularly low background \textit{ex vivo}, which is an important feature when detecting extremely rare cells. The low background is likely to result from the rapid internalization and degradation of the molecule following its engagement with its reciprocal receptor, CD40. However, it follows that the internalization or degradation of CD154 must be blocked
in order to detect it on antigen-activated cells, for example, by anti-CD40 monoclonal antibodies. Furthermore, CD154 staining can be combined with the magnetic pre-enrichment to detect rare CD4+ T cells (see Chapter 4.3.2).

Yet another member of the TNF superfamily, CD137 (also known as 4-1BB or ILA) is similarly considered a marker of sufficiently high specificity and sensitivity for analyzing rare T cells (268,274,275). It is expressed by both CD4+ and CD8+ cells, as well as by γδ+ T cells and its upregulation is detectable 16 to 24 h after stimulation. In addition, it has been shown to be expressed by CD4+ FOXP3+ regulatory T cells (276), making it a feasible alternative for Treg analysis.
3 Aims of the study

The aims of the study were

- To analyze the DRB1*0401-restricted responses of peripheral blood-derived memory and naïve CD4+ T cells from subjects with or without allergy against the immunodominant epitope of the major cow dander allergen Bos d 2 by HLA class II tetramers \textit{in vitro} (I).

- To explore the functional and phenotypic properties of CD4+ T cells of horse-allergic and healthy subjects specific to the immunodominant epitope region of the major horse allergen Equ c 1 and to assess \textit{in vitro} its potential for peptide-based allergen immunotherapy (II).

- To investigate in detail the human memory CD4+ T cell responses to the major dog allergen Can f 5, a kallikrein protein, and to evaluate its properties \textit{in vitro} in the context of peptide-based allergen immunotherapy (III).

- To compare and analyze conventional \textit{in vitro} and novel \textit{ex vivo} methods for detecting allergen-specific CD4+ T cells (I-III)
4 Materials and methods

4.1 SUBJECTS

Five HLA-DRB1*0401-positive cow-allergic subjects and five HLA-DRB1*0401-positive control subjects with no history of atopy (I), fourteen clinically diagnosed horse-allergic subjects and nine horse dust-exposed nonatopic control subjects (II) and twelve dog-allergic subjects and twelve dog dust-exposed nonatopic control subjects (III) were recruited for the studies. The allergic subjects were characterized at the Pulmonary and Dermatology Clinic of Kuopio University Hospital (277). They had positive skin prick test (SPT) reactions (≥ 3 mm) against commercial allergen extracts (ALK Abellô, Hørsholm, Denmark) or allergen-specific UniCAP results (FEIA; Pharmacia, Uppsala, Sweden; > 0.7 kU/l) against the allergen relevant for the study they took part in. They reported the exacerbation of their allergic symptoms, such as asthma and rhinoconjunctivitis, upon exposure to the allergen. The control subjects reported no history of atopy or allergic symptoms and had negative SPTs (I, II) or a negative allergen-specific IgE result (III). Heparinized venous blood samples and serum samples were collected from all subjects. The HLA-DQ and -DR genotypes were determined at the Clinical Laboratory of Finnish Red Cross Blood Service (Helsinki, Finland) or at the Immunogenetics Laboratory of University of Turku (Turku, Finland). The study was approved by the Ethics Committee of Kuopio University Hospital and a written informed consent was disclosed by all participants.

4.2 ANTIGENS

4.2.1 Recombinant antigens (I-III)

The recombinant (r) cow allergen rBos d 2 (I) (278) and the horse allergen rEqu c 1 (II) (75) were produced in Pichia pastoris. The recombinant dog allergen rCan f 5 was provided by Phadia Laboratory Systems (Uppsala, Sweden) (5).

4.2.2 Synthetic peptides (I-III)

The immunodominant epitope-containing 16-mer Bos d 2127-142 peptide (I) (EL-EKYQQLNSERGVPN) was produced using the PerSeptive 9050 Plus automated peptide synthesizer (Millipore, MA, USA) with the Fmoc method (76). The immunodominant region-containing 18mer Equ c 1143-160 peptide (II) (GIVKENIDLTIDRCFQ) and 74 three amino acid-overlapping 16-mer Can f 5 peptides covering the whole protein sequence (III) were synthesized by GL Biochem (Shanghai, China) at a minimum of 80% purity. All peptides were purified by high-performance liquid chromatography and the purity was veri-
fied by mass spectrometry. The peptides were dissolved in water with the exception of water-insoluble peptides which were dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, Waltham, MA, USA).

For the direct *ex vivo* analyses utilizing the CD154 enrichment method (III) 27 Can f 5 peptides (III, Table S1) that were verified to stimulate Can f 5-specific CD4+ T cells in epitope mapping (see Chapter 4.4.1) were pooled together and used at a final concentration of 1 µg/ml per peptide.

### 4.3 CELL SEPARATION

#### 4.3.1 Separation of peripheral blood mononuclear cells (I-III)
Peripheral-blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. The blood samples were let to temper for 30 minutes at room temperature (RT) before diluting 1:1 with phosphate-buffered saline (PBS). Diluted blood was then carefully layered on top of 10 to 15 ml of the Ficoll-Paque Plus solution in a conical 50 ml tube. After centrifugation, the PBMC layer was collected and washed twice to reduce platelet contamination and plasma protein amounts. The cells were counted with a hemocytometer and cell viability was assessed by Erythrocin B staining (Sigma-Aldrich Chemie, Steinheim, Germany).

#### 4.3.2 Magnetic bead purification and the enrichment of CD4+ T cell subsets (I-III)
Naïve (CD45RA+CD45RO-) and memory (CD45RA-CD45RO+) CD4+ T cells were isolated from freshly purified PBMCs with the no-touch naïve or memory CD4+ T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). The mean purity for naïve cells was 94% (I, II) and for memory cells 97% (I, II) or ≥98% (III), as determined by flow-cytometric analyses. In brief, freshly isolated PBMCs were stained with a biotinylated antibody cocktail included in the separation kit. It labels all unwanted cells. After incubation, streptavidin-conjugated magnetic beads were added in the suspension. The cells were then placed in a magnetic column where the antibody/magnetic bead-labeled cells were retained. The column was washed several times to elute the unbound cell fraction (naïve or memory CD4+ T cells). Finally, the column was removed from the magnetic stand and the remaining cells were flushed out and used after irradiation (3000 rad) as APCs.

### 4.4 T CELL CULTURE AND PROLIFERATION ASSAYS

#### 4.4.1 Generation of T cell lines and clones (I-III)
T cells were cultured in complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 µM 2-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino acids (all from Lonza, Verviers, Belgium)
and 5% inactivated human AB serum (Sigma-Aldrich, St. Louis, MO, USA) in +37°C and 5% of CO₂.

T cell lines (TCLs) and clones (TCCs) were generated from freshly isolated PBMCs (II) or purified CD45RA+CD45RO- naïve (I) and CD45RA-CD45RO+ memory CD4+ T cells (I, III) as follows:

The split-well method was used to generate mono-/oligoclonal long-term TCLs specific to Bos d 2127-142 peptide (I). 2.5 x 10⁴ naïve or memory CD4+ T cells were stimulated with 5 x 10³ peptide-pulsed (10 µM) autologous monocyte-derived DCs (see Chapter 4.4.3) in 96-well plates (20-30 replicate wells). After 4 days, the cultures were supplemented with 10 IU/ml of rIL-2 (Strathmann Biotech, Hannover, Germany). After 10 days, the cultures were restimulated with autologous peptide-pulsed γ-irradiated (3000 rad) PBMCs. On day 20, half of the T cells in each well were split into two new daughter plates, and stimulated with γ-irradiated (6000 rad) T2.DR4 cells (1 x 10⁴ per well) pulsed for 4 h with or without Bos d 2127-142. After 3 days, cell proliferation was measured by the thymidine incorporation assay (see chapter 4.4.2).

To expand cells for T cell cloning (I), purified naïve or memory CD4+ T cells (10⁶ per well) were stimulated with 2 x 10⁵ autologous monocyte-derived DCs pulsed with 0.1 or 10 mM of the Bos d 2127-142 peptide in 48-well plates in a volume of 1 ml. On days 7 and 10, fresh medium supplemented with rIL-2 (25 IU/ml) and rIL-15 (10 ng/ml, Strathmann Biotech) was added to the cultures. On day 14, the T cells were stained with tetramers (see Chapter 4.5.1) and analyzed. For the generation of TCCs, tetramer-positive CD4+ T cells were single-cell sorted with an EPICS Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA, USA) into 96-well round-bottomed plates and expanded in the presence of 10⁵ allogeneic γ-irradiated PBMCs, 5 x 10³ γ-irradiated EBV-transformed B cells (both irradiated at 6000 rad), 1 mg/ml of phytohaemagglutinin (PHA) and rIL-2 (25 IU/ml).

To obtain Equ c 1143-160-specific TCLs (II), PBMCs of each donor were cultured in 30 wells of 96-well round-bottomed plates at a density of 2 x 10⁵ cells per well in 150 µl of medium with Equ c 1p143-160 (10 µg/ml). On day 5, 50 µl of fresh medium was added together with rIL-2 (10 IU/ml). On day 10, the cells were restimulated with Equ c 1p143-160 (10 µg/ml) along with 1.5 x 10⁵ γ-irradiated (3000 rad) autologous PBMCs as APCs and rIL-2 (10 IU/ml) in a total volume of 150 µl. On day 15, 50 µl of fresh medium was added together with rIL-2 (10 IU/ml). Finally, on day 20, the wells were split to create two replicate plates by transferring 50 µl of cell suspension per well to new 96-well daughter plates. Cultures in one of the daughter plates were stimulated with Equ c 1p143-160 (10 µg/ml) and the other served as a control plate. Proliferation was measured by the thymidine incorporation assay. Positive cultures were transferred into a 48-well plate and restimulated with Equ c 1p143-160 (10 µg/ml) and rIL-2 (25 IU/ml) in the presence of 10⁶ γ-irradiated autologous PBMCs as APCs. The cell lines were incubated for 14 days and supplemented with fresh medium and rIL-2 (25 IU/ml) every 2-3 days before analyses.
In order to create Equ c 1-specific TCCs (II), naïve or memory CD4+ T cells were isolated from freshly purified PBMCs with the no-touch naïve or memory CD4+ T cell isolation kits (Miltenyi Biotec). Non-CD4+ T cells retained in the separation column were used as γ-irradiated APCs (3000 rad). One million naïve or memory T cells were labeled with 1 µM carboxyfluorescein succinimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit, Invitrogen, Eugene, OR, USA), according to the manufacturer’s instructions, and expanded in a 24-well plate along with 3 x 10^6 γ-irradiated APCs and Equ c 1_p143-160 (10 µg/ml) at +37°C. On day 7, half of the cells were analyzed for CFSE fluorescence intensity. Cell division index (CDI) was calculated by dividing the number of CFSE_nlow cells in the stimulated sample by the number of CFSE_nlow cells in the unstimulated sample, and CDI > 2 was considered a positive proliferative response. For the rest of the cells, half of the volume was replaced with fresh medium supplemented with rIL-2 (25 IU/ml). On day 14, the CFSE-labeled TCLs were analyzed again for CFSE fluorescence intensity. Dividing cells were then single-cell sorted into 96-well plates containing 5 x 10^4 γ-irradiated PBMCs, 2.5 x 10^3 γ-irradiated EBV-transformed B cells (both 6000 rad), 1 µg/ml of PHA, and 25 IU/ml of rIL-2 using the EPICS Elite ESP flow cytometer (Beckman Coulter). The clonality of the sorted T cells was verified by flow-cytometric TCR Vβ-chain analysis, as described previously (279).

For the generation of Can f 5-specific TCLs, memory CD4+ T cells of each donor were isolated as described in Chapter 4.3.2. The PBMCs depleted of memory CD4+ T cells that were retained in the separation column were flushed out and 1.5 x 10^5 cells per well were used as APCs after γ-irradiation (3000 rad). 5 x 10^4 memory T cells per well were seeded into 50 wells on a 96-well plate for stimulation with rCan f 5 (10 µg/ml). Cells in 10 wells were stimulated with tuberculin purified protein derivate (PPD, 1 µg/ml; Statens Serum Institut, Copenhagen, Denmark) to serve as positive controls. The cell cultures were initialized in 100 µl of medium, and on day 5, 50 µl of fresh culture medium and rIL-2 (25 IU/ml) were added in the wells. On day 10, the cells were restimulated with Can f 5 (10 µg/ml) and rIL-2 (25 IU/ml) along with 1.5 x 10^5 autologous, γ-irradiated PBMCs as APCs in a total volume of 200 µl per well. On day 15, 50 µl of medium was replaced with fresh medium and rIL-2 (25 IU/ml). Finally, on day 20, the wells were split, essentially as described above, to detect Can f 5-specific TCLs. Cell proliferation was measured by the thymidine incorporation assay. Positive wells were transferred into a 48-well plate and restimulated with Can f 5 (10 µg/ml) and rIL-2 (50 IU/ml) along with 10^6 autologous, γ-irradiated PBMCs as APCs. The cell lines were cultivated for 14 days and supplemented with fresh medium and rIL-2 (50 IU/ml) every 2 to 3 days until further analyses.

4.4.2 Thymidine incorporation test (I-III)

For the thymidine incorporation test, [3H]thymidine was added to the cultures (1 µCi per well; GE Healthcare, Little Chalfont, UK) and, following an additional 16 h incubation, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactive decay was
measured by liquid scintillation counting (Wallac Micro Beta 1450). The results were expressed as counts per minute (CPM). Stimulation index (SI) was calculated in a following manner: CPM of a stimulated culture divided by CPM of a corresponding unstimulated culture. Criteria for positive cultures were: I: SI > 2 and ∆CPM > 1000; II: SI > 2; III: SI > 5 and ∆CPM > 1000. The positive cultures were deemed antigen-specific and collected for further analyses. In brief, the cells were transferred to 48-well plates and re-stimulated with the relevant antigen in the presence of IL-2 to facilitate further expansion. After two weeks of in vitro expansion, the cells were collected, counted and, depending on the study, used in subsequent proliferation tests, phenotypic analyses or peptide screening, as described in detail in the corresponding chapters.

4.4.3 Generation of monocyte-derived dendritic cells (I)

Monocyte-derived mature dendritic cells (DCs) were cultured according to a protocol described previously (244). In brief, PBMCs were incubated for 2 h in serum-free AIM-V medium (Life Technologies, Paisley, UK) in 6-well plates to facilitate adherence. Non-adherent cells were washed out, and the adherent cells were cultured for 2 days in AIM-V supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng/ml; Strathmann Biotech, Hannover, Germany). 24 hours after starting the culture, PolyI:C (25 µg/ml; Sigma, St Louis, MO, USA) was added. The purity of the DC cultures was determined to be ≥80% by flow cytometry (BD FACSCanto II) after staining the cells with FITC-labeled anti-CD14, anti-CD86, and anti-CD83 mAbs (BD Biosciences, San Jose, CA, USA).

4.5 FUNCTIONAL AND PHENOTYPIC ANALYSES OF T CELLS

4.5.1 HLA class II tetramer staining (I-II)

The DRB1*0401:Bos d 2127-142 and DRB1*0401:PSA64-78 tetramers (I) were generated, as previously described (280). Briefly, the chimeric cDNA were transfected into Schneider cells by standard calcium phosphate transfection techniques, and cells expressing the transfected HLA class II molecule were selected for purification by G418 screening. The DR*0401 molecules were made soluble with CuSO4 and purified by affinity chromatography. The HLA molecules were biotinylated by the Bir A enzyme and loaded with the relevant peptide by incubating for 72 h at +37°C, and then further incubating overnight at room temperature with phycoerythrin (PE)-streptavidin.

Tetramer staining was performed by incubating T cell cultures with 0.5 µg of phycoerythrin (PE)-labeled tetramers in 50 µl of culture medium for 2 h at +37°C. Then, the cells were incubated for an additional 20 min at +4°C with anti-CD4 FITC, anti-CD19 PerCP-Cy5.5 and anti-CD14 PerCP-Cy5.5 (all from BD Biosciences, San Jose, CA). Next, the cells
were washed twice and further incubated for 10 min with the Via-Probe reagent (BD Biosciences) at +4°C, and analyzed with a BD FACSCanto II flow cytometer. Dead cells, CD19+ B cells and CD14+ monocytes were excluded from the analyses.

The DRB4*0101:Equ c 1143–160 tetramer and the control tetramer DRB4*0101:GAD65555–567 (II) were generated, as described elsewhere (281). The staining was performed by incubating T cell cultures with 0.5 µg of the PE-labelled tetramers in 50 µl of culture medium for 2 h at +37°C. Thereafter, anti-CD4 FITC was added and the cells were incubated for a further 20 min at +4°C. Finally, the cells were washed twice and analyzed with the BD FACSCanto II flow cytometer, essentially as described above.

4.5.2 CD154 enrichment and the ex vivo analysis of CD4+ T cells (III)

The ex vivo analysis of allergen-specific CD4+ T cells was initialized by stimulating 5-7.5 x 10^7 PBMCs from seven allergic patients and four nonallergic control subjects with a pool of Can f 5 peptides (see below) in 6-well plates with 2.5 x 10^7 PBMCs per well in 2.5 ml of cell culture medium. Furthermore, 5 x 10^6 PBMCs were seeded in 48-well plates in 1 ml of medium with PPD (10 µg/ml) or Staphylococcus enterotoxin B (SEB, 1 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) as memory T cell-specific and non-specific positive controls, respectively. An equal number of PBMCs as the stimulation with peptides was seeded on identical plates with no antigen to serve as negative controls. Also, 10 µg/ml of anti-CD40 (BioLegend, clone HB14) was added to each well and the cells were incubated for 16 h at +37°C before magnetic purification.

A total of 27 peptides (III, Table S1) from the epitope mapping of Can f 5 (III, Figure 5), causing a response by the memory CD4+ T cells of one or more of the 12 allergic patients examined, were pooled to yield a final concentration of 1 µg/ml for each peptide in the stimulation. The peptides were dissolved in DMSO, resulting in a 0.27% (vol/vol) final DMSO concentration in the cultures. DMSO was included in the control wells at the same concentration.

The Can f 5 peptide-specific T cells were enriched based on the expression of the CD154 activation marker with the Miltenyi Biotec anti-CD154-biotin and anti-biotin microbeads kit, according to the manufacturer’s instructions. Briefly, the cells were first collected, washed, and suspended in the cell separation buffer. After this, the cells were incubated with biotinylated anti-CD154 antibodies and anti-biotin microbeads, causing the activated CD154-positive cells to be retained in the magnetic column. Prior to adding the cells in the column, a 1/100 fraction of stimulated and negative control cells was collected to determine the pre-column state. The column was then washed and the column-bound cells were eluted outside the magnet to collect the CD154-enriched fraction of cells. Before the flow-cytometric analysis, the CD154-positive fraction, pre-column cells, as well as PPD and SEB controls, were incubated for 15 min at RT with the following antibody cocktail (all from BioLegend): anti-CD14-BV510, anti-CD19-BV510, anti-CD69-PerCP-Cy5.5, anti-CD4-APC-H7, anti-
CD45RA-FITC, anti-CRTh2-BV421, anti-CXCR3-Alexa647, anti-CCR6-PECy7, and anti-biotin-PE (for CD154). For the PPD and SEB-stimulated cell samples, the anti-biotin-PE was replaced with anti-CD154-PE (Miltenyi Biotec). Counting beads (Sphero AccuCount Fluorescent Particles 7.7 µm, Spherotech Inc., Lake Forest, IL, USA) were added to each sample tube prior to the analyses.

Live lymphocytes were gated based on their forward (FSC) and side scatter (SSC) properties, and doublets were gated out by SSC-A vs. SSC-W and FSC-A vs. FSC-H comparison. CD14+ and CD19+ events were also gated out. Memory CD4+ T cells were gated as CD4+CD45RA-, from which antigen-activated cells were detected by their CD69+CD154+ expression. For the chemokine receptor analysis, unstimulated memory cells were used to determine the expression of the receptors on polyclonal memory CD4+ T cells and their frequencies were compared to those of Can f 5 and PPD-specific CD4+ memory T cells. Cell frequencies were calculated with the aid of counting beads by using the following formula: n/(N x 100), where n is the total number of CD4+CD45RA-CD69+CD154+ events in the enriched fraction and N is the total number of CD4+CD45RA- memory T cells in the pre-enriched sample.

4.5.3 Cytokine measurements (I-III)
Concentrations of the cytokines IL-4, IL-5, IL-10 and IFN-γ produced by antigen-stimulated TCLs (Bos d 2127-142, 10 µM; Equ c 1143-160 and Can f 5, each 10 µg/ml) were determined in duplicate by the Luminex method (Bioplex; Bio-Rad, Hercules, CA; II) or by ELISA (Duoset kits; R&D Systems, Minneapolis, MN; I, III), according to the manufacturers’ instructions. Supernatants (100 µl per well) for the assays were collected from the proliferation test plates 3 days after the stimulation. The supernatants were stored at -80°C until analyzed.

4.5.4 HLA class II restriction analyses (II, III)
In studies II and III, the HLA class II restriction of Equ c 1- and Can f 5-specific TCLs and TCCs was identified by inhibiting their proliferative responses with monoclonal antibodies (1 µg/ml) to HLA-DP (clone B7/21, III), HLA-DQ (clone SPVL3, II-III) and HLA-DR (clone L243, II-III), essentially as described previously (282). In addition, allogeneic, partially HLA-matched PBMCs from a donor expressing only one shared allele with the subject whom the TCL was derived from was used in a proliferation test to further determine the restricting HLA allele (II).
4.6 PREDICTION OF THE HLA CLASS II PEPTIDE BINDING BY BIOINFORMATIC ALGORITHMS (III)

The HLA-DP, -DQ and -DR peptide binding motifs of Can f 5 were predicted with the Immune Epitope Database Analysis Resource tool (283,284). The alleles were chosen to match the study population. The top 3% binding motifs for each locus were considered as the predicted high-binding epitopes.

4.7 STATISTICAL METHODS

All statistical analyses were performed with the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The Mann-Whitney $U$ test was used to compare the non-Gaussian data of non-related study populations in such cases as cell frequencies (I-III), tetramer staining intensities (I), cell proliferation (I) and cytokine phenotypes (I-III). For paired samples, the Wilcoxon signed rank test was used (III). The Fisher’s exact test was used for contingency analyses. The Grubb’s test was used to detect outliers in study populations (II). The Spearman correlation test was used to calculate the correlation of CD4+ T cell frequencies obtained by the split-well and the CD154 enrichment assays, as well as that of the allergen-specific IgE levels and CD4+ T cell frequencies (III). $p$ values of $\leq 0.05$ were considered statistically significant.
5 Results

5.1 ALLERGEN-SPECIFIC NAIÝVE AND MEMORY CD4+ T CELLS EXHIBIT FUNCTIONAL AND PHENOTYPIC DIFFERENCES BETWEEN INDIVIDUALS WITH AND WITHOUT ALLERGY (I)

5.1.1 Frequency and characteristics of ex vivo-expanded Bos d 2127-142-specific naïve and memory CD4+ T cells

Purified naïve (CD45RA+CD45RO−) and memory (CD45RA−CD45RO+) CD4+ T cells from the PBMCs of five cow-asthmatic and five nonallergic subjects were stimulated for 14 days with Bos d 2127-142 and stained with DRB1*0401:Bos d 2127-142 tetramers in order to detect specific T cells (I, Figure 1A-1D). Bos d 2127-142 tetramer-positive cells were detected in the memory T cell cultures in 4 out of 5 subjects with allergy, but only in 2 out of 5 of nonallergic subjects (I, Table 1). The mean (± SEM) frequency of tetramer-positive cells was 0.38 ± 0.19% and 0.18 ± 0.16% for subjects with and without allergy, respectively. According to previous studies, CD4+ T cells are estimated to undergo at least 10–12 divisions in 14 days (72,244). Based on this rate of cell division, it can be estimated that the frequency of Bos d 2127-142-specific memory T cells in the peripheral blood of subjects with allergy is low, in the range of 1 per 10^5 to 10^6 circulating CD4+ T cells. Tetramer-positive responses were undetectable in the cultures of most of the subjects without allergy, reaffirming the notion that the number of Bos d 2127-142-specific memory T cells in the peripheral blood of subjects with allergy is higher than that of subjects without allergy.

To enable the expansion of both high- and low-avidity naïve Bos d 2127-142-specific T cells (285-287), naïve CD4+ T cells were stimulated with two peptide concentrations (10 and 0.1 μM). With this approach, Bos d 2127-142 tetramer-positive cells were detected in the T cell cultures of naïve origin of all the subjects studied (I, Table 1). Although the overall frequency of the tetramer-positive cells appeared to be lower after the expansion of naïve T cells at the lower (0.1 μM) antigen concentration (I, Table 1), the phenomenon was less apparent in subjects with allergy, as two of them (A-2 and A-4) showed higher numbers of tetramer-positive T cells after expansion at the lower concentration. Nonetheless, when the cultures with the highest frequencies of tetramer-positive cells from each individual were included in the analysis, the mean frequency of Bos d 2127-142 tetramer-positive cells in the naïve T-cell-derived cultures of allergic and nonallergic subjects was largely the same, 0.81 ± 0.38% and 0.61 ± 0.35%, respectively (I, Figure 2A). Based on this, it can be estimated that the frequency of Bos d 2127-142-specific naïve T cells in the peripheral blood appears to be within the same range as that of memory T cells.

The intensity of tetramer staining of the ex vivo-expanded Bos d 2127-142-specific CD4+ T cells was higher in the T cell cultures of naïve origin from allergic than nonallergic subjects; a statistically significant difference was observed in the mean fluorescence intensity (MFI)
of tetramer staining (I, Figure 2B; \( p = 0.02 \), Mann-Whitney U test). Notably, previous reports have demonstrated that the binding ability of HLA class II tetramers to T cells is directly proportional with the TCR avidity of the cells (280,288,289). In line with these findings, our data suggest that despite the largely comparable precursor frequencies (I, Figure 2A), the Bos d 2127-142-specific CD4+ T cells of naïve origin from subjects with allergy display higher TCR avidity to the peptide than those from nonallergic subjects (I, Figure 2B).

To ensure that the \textit{ex vivo}-cultured Bos d 2127-142 tetramer-positive T cells are specific to the Bos d 2 allergen and its naturally processed T cell epitope, T cell clones obtained by single-cell sorting of tetramer-positive cells after the \textit{ex vivo} culture of naïve and memory CD4+ T cells were analyzed (I, Figure 3). All clones stained positively with the Bos d 2127-142 tetramer and proliferated when stimulated by both the Bos d 2127-142 peptide and recombinant Bos d 2, verifying their capacity to recognize the naturally processed epitope of Bos d 2 (I, Figure 3A). Furthermore, in response to the peptide the memory cell-derived clones from subjects with allergy produced IL-4 but not IFN-\( \gamma \) nor IL-10, indicating a Th2 phenotype (see Chapter 2.3.3). In contrast, the naïve cell-derived clones produced low levels of both IL-4 and IFN-\( \gamma \), consistent with an undifferentiated Th0 phenotype.

5.1.2 Poor proliferation and tetramer staining of Bos d 2127-142-specific TCLs from subjects without allergy

To further investigate the functional and phenotypic characteristics of circulating Bos d 2127-142-specific T cells, the split-well method was used to generate mono-/oligoclonal DRB1*0401-restricted long-term TCLs from peripheral-blood naïve and memory CD4+ T cells (244).

Employing this technique, allergen-specific TCLs were generated from allergic and nonallergic subjects. The number of the memory cell-derived TCLs obtained from the subjects with allergy was significantly higher than that from the nonallergic subjects (I, Table 2; 7.1 and 2.0% of seeded wells, respectively, \( p < 0.05 \), Fisher’s exact test). This finding supports the concept that the frequency of peripheral-blood Bos d 2127-142-specific memory T cells of subjects with allergy is higher than that of nonallergic subjects. Likewise, presuming that each TCL represents a monoclonal expansion of Bos d 2127-142-specific memory T cells of subjects with allergy produced IL-4 but not IFN-\( \gamma \) nor IL-10, indicating a Th2 phenotype (see Chapter 2.3.3). In contrast, the naïve cell-derived clones produced low levels of both IL-4 and IFN-\( \gamma \), consistent with an undifferentiated Th0 phenotype.
the memory cell-derived TCLs from both study groups were compared (I, Figure 4A), although the limited number of memory cell-derived TCLs obtained from subjects without allergy precludes a definite conclusion.

To further elucidate this phenomenon, the generated TCLs were stained with DRB1*0401:Bos d 2127-142 tetramers (I, Figure 4B). Fourteen of the 17 TCLs of naïve or memory origin (82%) analyzed from subjects with allergy stained with the specific tetramers, whereas only one of the five TCLs (20%) from nonallergic subjects showed a positive staining \((p = 0.02, \text{Fisher’s exact test})\). Thus, the diminished proliferative capacity of the Bos d 2127-142-specific TCLs from the control group seems to be linked to their weaker tetramer binding, which in turn reflects a weaker TCR avidity, as mentioned above.

### 5.1.3 Th2 bias of Bos d 2127-142-specific TCLs derived from subjects with allergy

The cytokine profile of Bos d 2127-142-specific TCLs was determined by measuring IFN-γ, IL-4 and IL-10 in the culture supernatants (I, Figure 5A-C). Memory cell-derived TCLs from allergic subjects produced predominantly IL-4 and lesser amounts of IL-10, whereas the cytokine production of respective TCLs from nonallergic subjects, and naïve TCLs from both groups, was generally weak. The memory cell-derived TCLs from subjects with allergy were thus predominantly Th2-deviated, whereas almost all memory cell-derived TCLs from the nonallergic subjects, and naïve cell-derived TCLs from both groups were of an undifferentiated Th0 phenotype (I, Figure 5D). However, a low level of IL-4 production was detected in 45% of TCLs of naïve origin from subjects with allergy (I, Figure 5B). In contrast, none of the TCLs of naïve origin from subjects without allergy produced detectable levels of IL-4. This finding suggests a subtle Th2 bias in TCLs of naïve origin from subjects with allergy when compared to those without allergy.

### 5.1.4 Higher CD25 and lower CXCR3 expression in Bos d 2127-142-specific TCLs from subjects with allergy

Finally, the expression of phenotypic cell-surface markers on long-term Bos d 2127-142-specific TCLs was analyzed by flow cytometry (I, Figure 6). As a result of long \textit{in vitro} culture, both memory and naïve cell-derived TCLs from allergic and control subjects exhibited an effector memory phenotype (CD45RO+CD45RA-CD62L-CCR7-). Nonetheless, in line with previous studies (285,290), a low residual level of CD45RA expression was observed in naïve cell-derived TCLs (CD45RA expression 4.4 ± 1.7% vs. 0.7 ± 0.4% for TCLs of naïve and memory origin, respectively; \(p = 0.004, \text{Mann-Whitney } U \text{ test})\). No expression of CTLA-4, CCR4 or CCR6 was observed in the TCLs, while the majority of the TCLs expressed CD25 and CXCR3 at variable levels. The expression of these markers was comparable between memory and naïve cell-derived TCLs within the allergic (I, Figure 6A) and nonallergic (I, Figure 6B) subject groups. However, both memory and naïve cell-derived TCLs from allergic subjects expressed significantly higher levels of CD25 (I, Figure 6C; \(p = 0.001 \text{ Mann-Whitney } U \text{ test})\) but lower levels of CXCR3 (I, Figure 6D; \(p = 0.006\) than the TCLs from control subjects. The
elevated expression of the T cell activation marker CD25, the IL-2 receptor α-chain, by TCLs from subjects with allergy is likely associated with their increased capacity to expand in vitro (I, Figure 4A). Furthermore, the lower levels of the Th1-associated chemokine receptor CXCR3 (291) may be linked to the detected Th2 bias in TCLs of both memory and naïve origin from subjects with allergy (I, Figure 5).

5.2 DIFFERENTIAL CD4+ T CELL RESPONSES OF ALLERGIC AND NONALLERGIC SUBJECTS TO THE IMMUNODOMINANT EPITOPE REGION OF THE HORSE MAJOR ALLERGEN EQU C 1 (II)

5.2.1 Equ c 1143-160 peptide-specific CD4+ T cell lines are obtained in similar numbers from both allergic and nonallergic subjects

The split-well method was used in order to evaluate the frequency of peripheral blood CD4+ T cells of horse-allergic and healthy subjects specific to the immunodominant epitope region of the horse major allergen Equ c 1 (75). Fifty-two and thirty Equ c 1143-160-specific TCLs were generated from the PBMCs of allergic and nonallergic subjects, respectively (II, Figure 1). When the frequency of Equ c 1143-160-specific TCLs was analyzed per person, there was no significant difference between the subject groups (3.7 ± 0.6 [mean ± SEM] and 3.3 ± 1.1 TCLs, respectively; \( p > 0.05 \), Fisher’s exact test). Likewise, when the Equ c 1143-160-specific TCLs that were also specific to the Equ c 1 protein (protein-specific TCLs) were compared there was no statistically significant difference, albeit the number of TCLs showed some tendency for difference between the groups (2.1 ± 0.6 and 1.3 ± 0.9 TCLs per person, \( p = 0.19 \); II, Figure 1: black columns). However, when one nonallergic subject out of nine (subject Q, II, Figure 1; Grubb’s test for outliers \( p < 0.01 \) = significant outlier) with an exceptionally high number of protein-specific TCLs (eight; the next largest number for a nonallergic individual was two, II, Figure 1) was excluded from the analysis, the difference was statistically highly significant (0.5 ± 0.3 TCLs per nonallergic person, \( p < 0.001 \)). Therefore, this finding indicates that the recognition of the naturally processed epitope of Equ c 1 by CD4+ T cells may be a distinctive factor between the patients with allergy and most of the healthy subjects.

5.2.2 Frequency and proliferative capacity of peripheral-blood Equ c 1-specific CD4+ T cells

The frequency of Equ c 1143-160-specific CD4+ T cells was estimated by the number of antigen-specific cultures obtained by the split-well method. A total of six million PBMCs were plated per person (30 wells, \( 2 \times 10^5 \) PBMCs per well). Therefore, assuming that each Equ c 1143-160-specific well represents a monoclonal TCL, the mean frequency of Equ c 1143-160-specific T cells of subjects with allergy was 0.63 per \( 10^6 \) and that of nonallergic subjects was 0.56 per \( 10^6 \) PBMCs. Assuming that PBMCs contain around 30% of CD4+ T cells, it can be calculated that there are approximately 2.10 and 1.85 per \( 10^6 \) Equ c 1143-160-specific cells in the peripheral
CD4+ T-cell pool of allergic and nonallergic subjects respectively. Extending the estimation to the CD4+ cells that were also Equ c 1 protein-specific, the numbers of specific cells were even lower, around 1.18 per 10^6 CD4+ cells for allergic and 0.74 per 10^6 for nonallergic subjects. Again, if the eight protein-specific TCLs obtained from the nonallergic subject Q were excluded from the calculations, the protein-specific CD4+ T cells were detected extremely rarely in most nonallergic subjects (0.28 per 10^6).

It has been previously observed that although T cell responses to lipocalin allergens are generally weak (75,278,279), allergen-specific TCLs from allergic subjects proliferate more effectively than TCLs from nonallergic subjects (149). This was also the case in study I (see above). Similarly, the results of study II are in line with these findings, as the Equ c 1 protein-specific TCLs from allergic subjects proliferated significantly more strongly when stimulated with the Equ c 1143-160 peptide than those from non-sensitized subjects (p < 0.01, Mann-Whitney U test; II, Figure 2). Moreover, the protein-specific TCLs obtained from allergic subjects exhibited significantly stronger proliferative responses than the TCLs that recognized the Equ c 1143-160 peptide (p < 0.01, II, Figure 2) only. This observation may reflect the higher TCR avidity of the Equ c 1 protein-specific TCLs and further implies that the T cells specific to the naturally processed epitope are the cells associated with allergy.

5.2.3 Equ c 1 protein-specific CD4+ T cells are Th2-polarized and emerge from the memory pool

The cytokine profiles of the Equ c 1 protein-specific TCLs were determined by measuring the concentrations of IL-4, IL-5, IL-10 and IFN-γ in the cell culture supernatants (II, Figure 3). The TCLs from subjects with allergy produced markedly higher levels of the Th2 cytokines IL-4 and IL-5 when compared to the TCLs from nonallergic subjects (p < 0.01 and p < 0.05, respectively, Mann-Whitney U test; II, Figure 3). No statistically significant difference was observed in the IL-10 and IFN-γ production (p > 0.05; II, Figure 3). These findings corroborate previous observations (72,262,292-294), indicating that allergen-specific CD4+ T cell responses in allergic subjects are Th2-biased compared with those in subjects without allergy.

In order to determine whether the Equ c 1 peptide-specific responses emerge from the naïve or memory T cell pool, short-term T cell cultures were generated from naïve (CD4+CD45RA+) and memory (CD4+CD45RO+) T cells isolated from PBMCs of eight allergic and six nonallergic subjects and analyzed using the CFSE dilution method. Specific T cell expansion with Equ c 1143-160 was detected in the memory T cell-derived cultures of five allergic subjects out of eight (63%), whereas no responses were observed in the memory T cell-derived cultures of the six nonallergic subjects studied (p < 0.05, Fisher’s exact test; II, Fig 4B). All the peptide-specific T cell proliferation of the nonallergic subjects was detected in the cultures derived from naïve cells (II, Figure 4B), notably including the response of the nonallergic subject Q (CFSE analysis shown in II, Figure 4A) that had an abnormally high frequency of Equ c 1-specific T cells (II, Figure 1). To confirm that the ex vivo-expanded
CFSE$_{\text{low}}$ T cells were specific to the Equ c 1$_{143-160}$ and the Equ c 1 protein, TCCs obtained by cloning the expanded T cells were tested with both the peptide and the protein. The positive results of five memory TCCs from allergic subjects and two naïve TCCs from a nonallergic subject are shown in Figure 5A. Collectively, these results indicate that the Equ c 1-specific CD4+ T cell responses of allergic subjects originate from memory cells while those of subjects without allergy derive from the naïve T cell pool.

5.2.4 Equ c 1-specific CD4+ T cell responses are restricted by HLA-DQ and -DR alleles

The HLA class II restriction of Equ c 1 protein-specific TCLs and TCCs from subjects with allergy was determined by blocking their proliferative responses with anti-HLA-DQ and -DR antibodies (representative examples shown in Figure 5B). As shown in Table 1, three HLA-DQ and six HLA-DR-restricted TCLs were detected. Similarly, both HLA-DQ and -DR restrictions were observed with the seven Equ c 1 protein-reactive TCCs from five different subjects (Figure 5B and Table 1). Further tests using partially HLA-matched allogeneic PBMCs as APCs revealed that two of the DQ-restricted TCLs were restricted by DQB1*0501 and one by DQB1*0602 and both of the DQ-restricted TCCs were restricted by DQB1*0603 (Table 1). Interestingly, it was observed that five of the six DR-restricted TCLs and all of the five DR-restricted TCCs were restricted by either DRB1*0404 or DRB4*0101 (one TCL was not determined). As the DRB1*0404 and DRB4*0101 restrictions could not be distinguished with partially HLA-matched PBMCs in this experimental setting because of the linkage disequilibrium between these two alleles, we stained one monoclonal and one oligoclonal TCL from a DRB1*0404/DRB4*0101-positive horse-allergic subject with the DRB4*0101:Equ c 1$_{143-160}$ HLA class II tetramer (Figure 6). Positive tetramer staining verified that the DRB4*0101 allele is involved in restricting the CD4+ T cell response to Equ c 1$_{143-160}$. Collectively, these findings indicate that a wide array of HLA class II alleles, including DRB4*0101, is able to bind and present the immunodominant epitope region of Equ c 1.

5.3 MEMORY CD4+ T CELL RESPONSES TO THE NOVEL DOG ALLERGEN CAN F 5, PROSTATIC KALLIKREIN (III)

5.3.1 The frequency of Can f 5-specific memory CD4+ T cells is markedly higher in allergic than in nonallergic subjects

The frequency of Can f 5-specific memory CD4+ T cells in peripheral blood was evaluated by two complementary methods. As a first approach, the split-well method was employed (150,242) to generate TCLs specific to the Can f 5 protein from highly purified peripheral blood CD4+CD45RO+ T cells. Can f 5-specific TCLs were obtained from all (100%) of the 12 Can f 5-allergic but only from 7 (58%) of the 12 nonallergic subjects studied ($p = 0.04$; Fischer’s exact test). Furthermore, the calculated frequency of memory CD4+ T cells was approximately 10-fold higher in allergic than in nonallergic subjects ($7.9 \pm 7.4$ vs $0.8 \pm 0.8$ per
10^6 memory CD4+ T cells, respectively; III, Figure 1A). The Can f 5-specific TCLs from allergic subjects also exhibited a higher functional TCR avidity and a Th2-biased cytokine secretion profile compared to the TCLs from nonallergic subjects (III, Figure 2). These findings are consistent with results obtained with Bos d 2 and Equ c 1 in studies I and II.

In order to further validate the data obtained, memory CD4+ T cells were first stimulated with a pool of 27 Can f 5 peptides (III, Table 1). These peptides were selected based on the T cell epitope mapping (5.3.3) exploiting the aforementioned Can f 5 protein-specific TCLs. Then, the novel CD154 enrichment method (243) was employed to detect Can f 5-specific memory CD4+ T cells directly ex vivo by flow cytometry. One allergic subject (A-5) had an exceptionally high (469 per 10^6) numbers of Can f 5-reactive memory CD4+ T cells in peripheral blood, permitting us to directly affirm that the frequency and phenotype of Can f 5-specific T cells was similar before and after the CD154 enrichment (III, Figure S1). Using the ex vivo method, Can f 5-specific memory CD4+ T cell frequencies varied between 1 and 469 per 10^6 cells and were significantly higher in allergic than in nonallergic subjects (III, Figure 3B). Notably, the frequencies of Can f 5-specific T cells extrapolated from the split-well data (III, Figure 1A) correlated well \( r = 0.87, p < 0.001 \) with the frequencies detected ex vivo with the CD154 enrichment method (III, Figure 3B), although the frequencies obtained with the latter approach were systematically slightly higher (III, Figure 3C). The ex vivo frequency of tuberculin purified protein derivate (PPD)-specific memory CD4+ T cells was similar between allergic and nonallergic subjects (III, Fig 3B). The serum levels of Can f 5-specific IgE did not correlate with the frequencies of memory CD4+ T cells determined with either of the two methods (unpublished data).

5.3.2 Can f 5-specific memory CD4+ T cells of allergic subjects exhibit a Th2-associated phenotype ex vivo

The phenotype of the CD154-enriched CD4+ memory T cells of allergic subjects was analyzed ex vivo by measuring the expression levels of CCR6, CXCR3 and CRTh2 (III, Figures 4 and S3). The Can f 5-specific CD4+ memory T cells were concentrated in the CCR6-CXCR3-subset, which mostly contains Th2-type memory T cells (183). Additionally, Can f 5-specific memory T cells displayed elevated expression of CRTh2, a more specific marker of Th2-polarized cells (295). Contrary to Can f 5, PPD-specific memory T cells were distinctively concentrated in the CCR6+CXCR3- and CCR6+CXCR3+ subsets that contain memory T cells polarized towards Th17 and Th1/17 phenotypes, respectively (183). These observations confirm the Th2-polarized nature of Can f 5-specific CD4+ memory T cells already observed with long-term TCLs (5.3.1.)

5.3.3 Can f 5 contains six T cell epitope regions that are capable of stimulating the memory CD4+ T cells of allergic subjects

The T cell epitopes of Can f 5 were identified by assessing the reactivity of 52 TCLs specific to the Can f 5 protein (5.3.1) from the 12 allergic subjects to overlapping 16-mer peptides
covering the complete Can f 5 amino acid sequence. With this approach, a mean of 4 (range 1-6) epitopes were recognized per allergic subject (III, Figures 5 and S4). Six evident epitope regions that were recognized by at least 4 of the 12 allergic subjects were identified (III, Figure 5). The most distinct one, located between the amino acids 163-184, was recognized by 7/12 (58%) of the subjects. T cells of all the 12 allergic subjects recognized at least one of the six epitope regions. The HLA restriction of 38 TCLs was identified by blocking their proliferative responses to Can f 5 peptides by HLA-specific antibodies (III, Figure S5A) (150). 47% of the Can f 5-specific T cell responses were restricted by HLA-DR, 39% by HLA-DQ and 13% by HLA-DP (III, Figure S5B). Finally, bioinformatic algorithms were used to determine whether the obtained T cell epitope data correlated with the computational predictions of HLA class II peptide-binding motifs in Can f 5 (III, Figure S4). Interestingly, only 55% of in silico-predicted epitopes were confirmed as T cell epitopes by the experimental approach used in this study. Moreover, 50% of the confirmed epitopes were not included in the predicted ones.
6 Discussion

6.1 ALLERGEN-SPECIFIC T CELL RESPONSES IN INDIVIDUALS WITH AND WITHOUT ALLERGY (I-III)

6.1.1 Frequencies of allergen-specific CD4+ T cells differ between allergic and nonallergic subjects
In-depth knowledge of the nature of allergen-specific CD4+ T cell responses in allergic and nonallergic subjects is a prerequisite for understanding the pathogenesis of allergy. Likewise, meticulous scrutiny of the healthy immune response towards allergens in nonallergic subjects may help in developing specific immunotherapeutic tools for treating allergy. With this in mind, the functional and phenotypic characteristics of CD4+ T cells specific to important mammalian allergens cow Bos d 2, horse Equ c 1 and dog Can f 5 were analyzed in studies I-III.

Recent research has pointed out that the frequencies of allergen-specific memory CD4+ T cells are higher in allergic subjects than in nonallergic subjects (150,242,260,261,293,296). Comparable findings have also been presented in organ-specific autoimmune diseases with autoantigens when diseased and healthy subjects have been examined (297,298). The results from studies I-III unanimously support this notion, as elevated memory CD4+ T cell frequencies were consistently detected in allergic subjects. However, study II demonstrated that this phenomenon is only evident when allergen-specific memory and naïve CD4+ T cell responses are analyzed separately, as no difference in the frequency of allergen-specific CD4+ T cells between allergic and nonallergic subjects was noticed when the whole CD4+ T cell pool was studied. Similar findings have been reported in studies with other allergens: only marginal differences in the allergen-specific CD4+ T cell frequencies between allergic and nonallergic subjects were detected when unpurified cell populations were examined (299-302). This phenomenon is likely attributable to the similar frequencies of allergen-specific naïve CD4+ T cells in the peripheral blood of allergic and nonallergic subjects: the proliferation of naïve cells upon stimulation in vitro could mask the memory CD4+ T cell responses.

The average frequency of allergen-specific cells in the total pool of CD4+ T cells was estimated to be low, around 1 per 10⁶ cells with no significant difference between allergic and nonallergic donors. When purified memory CD4+ T cells alone were analyzed with the split-well method (I, Table 1; III, Figure 1A), the frequency of allergen-specific cells was in the range of 1 to 30 per 10⁶ cells for allergic subjects. In nonallergic subjects, the frequency was lower, approximately 1 to 10⁶ (III, Figure 1A). Moreover, in some individuals, responses often remained undetectable indicating an even lower overall frequency. In study III, a tenfold difference in the frequency of Can f 5-specific memory CD4+ T cells was observed be-
tween allergic and nonallergic subjects with the split-well method. These results were cor-
roborated by another approach, the flow-cytometric analysis of the upregulation of the T
cell activation marker CD154 \textit{ex vivo} (III, Figure 3B and C).
Collectively, our results indicate that in nonallergic subjects allergen-specific CD4+ T cells
fail to activate and/or expand \textit{in vivo}. The possible factors behind this phenomenon are dis-
cussed below.

6.1.2 Proliferative capacity and the T cell receptor avidity of allergen-specific CD4+ T
cells
The precise mechanisms leading to the observed differential frequencies of allergen-specific
memory CD4+ T cells in allergic and nonallergic subjects remain to be clarified. Studies I
and II identified higher \textit{in vitro} proliferative responses in Bos d 2 and Equ c 1-specific CD4+
T cells obtained from allergic subjects than in those from nonallergic subjects. However, it
remains unclear whether the robust expansion of allergen-specific cells in allergic individu-
als is due to the intrinsic characteristics of their CD4+ T cells and/or APCs, or whether it is
due to the inadequate peripheral regulation of the responses in atopic individuals (303).

The avidity of the T cell receptor (TCR) to its cognate antigen is a well-defined factor
affecting the outcome of T cell activation (201,286,304-306). Interestingly, in study III, a sig-
nificant difference was detected in the functional avidity (EC$_{50}$ values) between the T cells
of allergic and nonallergic subjects: the TCLs of allergic donors appeared to have higher
avidity to Can f 5 (III, Figure 1B). Similar findings have previously been observed with two
other allergens Can f 1 (242) and Can f 4 (150). Furthermore, in study I, the Bos d 2-specific
T cells from allergic subjects exhibited higher intensity of peptide:HLA tetramer staining, a
phenomenon linked with higher TCR affinity to the MHC-peptide complex (285-287), than
those from control subjects. Based on these observations, there is mounting evidence that
the allergen-specific memory CD4+ T cells of allergic persons are of higher avidity than their
counterparts in healthy individuals. However, the aforementioned findings do not exclude
the possibility that high-avidity CD4+ T cells are still present in healthy individuals, but are
not able to expand. Therefore, the apparent preferential expansion of high-avidity CD4+ T
cells in allergic patients could also be a result of poor peripheral regulation (see below).

T cell expansion is, in addition to the TCR avidity, driven by signals through costimula-
tory molecules and the prevailing cytokine milieu (191,193). Therefore, these factors can also
play a part in controlling the expansion of allergen-specific CD4+ T cells. The role of IL-10
and the function and frequency of peripheral-blood regulatory T cells have been investi-
gated as possible immunoregulatory elements that could be responsible for the differential
expansion of allergen-specific CD4+ T cells between allergic and healthy individuals. An
early study suggested that the frequency of IL-10-producing Tr1 cells is increased in nonal-
lergic subjects, and that these would suppress the activation of pathogenic Th2 cells (307).
However, more recent studies have failed to corroborate these findings, demonstrating no
difference in the frequency and cytokine production capacity of IL-10-producing T cells between allergic and nonallergic subjects (308,309). Moreover, no change in the proliferative responses of allergen-specific CD4+ T cells was observed in our own earlier study when either IL-10 was blocked by mAbs or CD4+CD25+ regulatory T cells were depleted from the cultures (242).

The development and maturation of T cells takes place in the thymus. The recognition of self-antigens presented by APCs, such as thymic epithelial cells and DCs, shapes a unique T cell repertoire for each individual. Indirect evidence suggests that thymic selection could favor the generation of Th2-biased T cell responses to animal allergens in susceptible individuals. Many important lipocalin allergens display considerable sequential homology with endogenous human lipocalins (4). For example, Can f 1 and the human tear lipocalin share a 60% amino acid identity (4,149), and a similar degree of homology has been recently reported between the kallikrein allergen Can f 5 and human PSA (5,101). Sequential homology to self-antigens could lead to the selective depletion of high-avidity allergen-specific T cells from the T cell repertoire (52,149). Since weak stimulation through the TCR is known to favor Th2 differentiation (see Chapter 2.3.3), the allergen-specific T cell repertoire purged of T cells with high-avidity TCRs could be inherently biased towards Th2-type responses. This idea is supported by the findings that allergen-specific CD4+ T cells, despite having higher avidity and proliferation capability in allergic than in nonallergic subjects, generally have substantially weaker functional avidity and proliferative capacity than do T cells specific to microbial antigens, such as PPD and influenza virus haemagglutinin (241,242). Some studies have also found that mammalian allergens stimulate T cells in a suboptimal way, i.e. there are heteroclitic ligands that are capable of eliciting stronger response than the natural ligand (52,76,282).

6.1.3 Allergen-specific CD4+ memory T cells of allergic subjects exhibit Th2-deviated immune responses

Along with the increased frequencies of allergen-specific memory CD4+ T cells in allergic subjects, another feature of the allergenic immune response is the overwhelmingly Th2-biased phenotype of effector cells compared to that of healthy controls (72,149,150,242,260,310). This discrepancy was evident when the in vitro production of the major Th2-associated cytokines IL-4 (I, Figure 5; II, Figure 3) and IL-5 (II, Figure 3; III, Figure 2A) was measured. In studies I-III, the Th2-associated cytokines were repeatedly secreted in higher quantities by the allergen-specific CD4+ T cells of allergic subjects upon stimulation than by those of nonallergic subjects. The production of the cytokines IL-10 and IFN-γ, associated with Tr1 and Th1 cells (183), respectively, was more similar between the groups, but there was some tendency of higher IFN-γ production among nonallergic subjects (III, Figure 2A).

A common criticism regarding the measurement of cytokines in the cell culture supernatants of long-term TCLs is that the prolonged in vitro cultivation might affect the phenotype
of the cells (235,243). However, memory CD4+ T cells appear to retain their original phenotype at least partially. For example, the measurements of Th1 and Th2-associated cytokines of in vitro-cultured memory T cells have repeatedly been reported to yield similar results as methods utilizing shorter culture times or a direct ex vivo approach (72,150,242,260,266,310). In study III, this concern was also addressed by exploiting the recently described CD154 enrichment method, which allows a direct ex vivo characterization of antigen-specific T cells (243,265,266). Since the analysis can be carried out within hours following the antigen exposure, the short period of in vitro-stimulation ensures that the cytokine phenotype and the surface molecule expression of antigen-reactive T cells reflects their status in vivo (see Chapter 2.4.3). In study III, the expression of the chemokine receptors CCR6, CXCR3 and CRTh2 on Can f 5-specific and the microbial antigen PPD-specific memory CD4+ T cells was analyzed from 6 allergic donors (III, Figure 4). Based on the low expression of CCR6 and CXCR3, the Can f 5-specific cells could be considered Th2-biased (183). This notion is further supported by the elevated levels of CRTh2 expression, which is a more specific marker for Th2 cells (295). To the contrary, memory CD4+ T cells specific to PPD were distinctly concentrated within the CCR6+CXCR3- and CCR6+CXCR3+ populations, which contain memory T cells polarized towards Th17 and Th1/17 phenotypes, respectively (183).

6.2 T CELL EPITOPEs AND THE HLA RESTRICTION OF ALLERGENIC IMMUNE RESPONSES (II, III)

6.2.1 Equ c 1 contains a distinct immunodominant epitope region, but the T cell responses to Can f 5 are dispersed in multiple sites (II, III)

For the purpose of devising safer and more efficient immunotherapeutic products, the characteristics of the T cell epitopes of allergens need be carefully evaluated and identified (104,301,311). For example, peptide immunotherapy relies on the selection of peptides that are known to elicit the response of allergen-specific T cells, while also covering a wide repertoire of restricting HLA alleles expressed within the target population. In a previous study, Equ c 1 was shown to contain an immunodominant T cell epitope region with two distinct epitopes within the amino acids 143-160. Almost all Equ c 1-sensitized individuals evoke a robust T cell response to the region (75). Therefore, it was chosen as an ideal target for further studies focusing on the immunological characteristics of allergen-specific T cell responses in horse allergic and nonallergic subjects. In study II, the immunogenicity of the Equ c 1143-160 peptide was confirmed, as specific CD4+ T cell responses were detected in all allergic and also in 75% of nonallergic subjects. However, the results indicated that a substantial proportion of the TCLs generated with the immunodominant peptide failed to recognize the whole allergen protein. Notably, TCLs specific to the native protein were extremely rare in all nonallergic subjects except one, whereas the TCLs of allergic subjects recognized the whole allergen more often. This observation might be explained by the ex-
istence of Equ c 1143-160 peptide-specific naïve CD4+ T cells in the peripheral blood of nonallergic donors that were reactive to the peptide only (II, Figure 4A and B). The peptide-only specific T cells from both study groups, as well as protein-specific TCLs of nonallergic subjects, also displayed considerably lower proliferative responses than the Equ c 1 protein-specific TCLs of allergic subjects. That probably reflects their low avidity for the peptide. The protein-specific responses of allergic persons were overwhelmingly prevalent in the memory CD4+ T cell pool, indicating a previous in vivo activation upon contact with the allergen. So it appears evident that the Equ c 1143-160 is capable of stimulating Equ c 1-specific memory CD4+ T cells in the peripheral blood of allergic individuals, but a similar response is absent in the peripheral blood of nonallergic persons, likely due to the absence of allergen-specific memory CD4+ T cells.

In addition to Equ c 1, T cell responses against several other lipocalin allergens, such as cow Bos d 2, dog Can f 1 and Can f 4 as well as rat Rat n 1 (74,76,149,150), have been reported to be localized around distinct regions that contain one or more immunologically dominant epitopes. These T cell epitopes are an ideal starting point for developing peptide-based immunotherapy. In study III, the T cell epitopes of the non-lipocalin allergen Can f 5 were examined by mapping the whole protein sequence with 74 overlapping 16-mer peptides. Somewhat surprisingly, the memory CD4+ T cell responses to Can f 5 were evenly spread out along much of the length of the protein sequence, with responses detected to a total of 43 different peptides (III, Figures 5 and S4). However, the vast majority of cell responses from T cells targeted six distinct regions, to each of which at least 4/12 of allergic subjects reacted. These regions, with the most prominent one located between the amino acids 163-184, covered all allergic subjects in the study. The dispersed nature of the Can f 5 epitopes could have implications for PIT, as a pool of the aforementioned immunogenic peptides is likely required to obtain a complete coverage for a target population (see Chapter 6.4).

6.2.2 HLA restriction of allergen-specific T cell responses
In autoimmune diseases, it is well known that certain HLA alleles increase the risk of a disease, while others confer protection from it (312). Such diseases include type 1 diabetes (313), multiple sclerosis (314), systemic lupus erythematosus (315,316) and coeliac disease (317). However, in allergic diseases a similar linkage seems less clear. Despite the limited number of known predisposing HLA alleles, atopy and allergic diseases contain a strong genetic element, as described in Chapter 2.1.1 and (318). However, not all restricting HLA alleles are equally represented in the studied populations of allergic and nonallergic individuals. HLA-associations have been identified, for example, with the mugwort allergen Art v1 (73) and cow Bos d 2 (87). This notwithstanding, most allergens contain multiple epitopes, the T cell responses to which are restricted by several HLA alleles. This has implications for the design of potential immunotherapeutic products (see Chapter 6.4). For PIT to be successful, the peptides chosen for the product should ideally contain all the important T cell epitopes.
within the allergen and cover a wide array of HLA alleles expressed within the population in question (107).

The majority of both Equ c 1 (II, Figure 5B) and Can f 5 (III, Figure S5) -specific responses were HLA-DR-restricted. Studies with other allergens, such as those from ragweed (319) and Timothy grass (294), have described similar findings, with HLA-DR as the dominant restricting element. Similarly in study III, bioinformatically predicted HLA-binding motifs were overwhelmingly HLA-DR-biased (see Chapter 6.2.3). This could be partially explained by the fact that DR alleles are the most extensively studied of HLA alleles. HLA-DQ was also frequently identified as a restrictive element with both Equ c 1 and Can f 5-specific responses, with as many as 39% of the analyzed Can f 5-specific TCLs exhibiting restriction. Furthermore, 13% of the responses of Can f 5-specific TCLs were restricted by HLA-DP. The overall dominance of HLA-DR as a restrictive element is not unexpected, since it is the most copiously expressed HLA class II molecule on APC surfaces (320).

Taken together, the above results, and results reported with other allergens, indicate that the immunodominant epitopes of allergens are frequently presented by several HLA class II alleles. Understanding the HLA restriction of the specific T cell responses to major allergens can offer important insights to allergenic processes. This may contribute to the design of immunotherapeutic allergen preparations.

6.2.3 Bioinformatic predictions of HLA-binding motifs are not always successful in predicting experimentally identified T cell epitopes (III)

The whole Can f 5 sequence was analyzed with the IEDB prediction tool (283,284). The predicted HLA-binding motifs were compared with the epitopes identified by an experimental approach. Previously, bioinformatic algorithms have been utilized in predicting T cell epitopes with a 50 to 75% success rate (150,294,321). T cell response was predicted to be restricted to a total of 31 epitopes by DR for Can f 5, along with only 1 epitope for DP and DQ each. Fifty-eight percent of the epitope-specific T cell responses predicted to be restricted by HLA-DR were also identified with the experimental approach adopted (III, Figure S4). No experimental verification for the predicted HLA-DP and -DQ motifs could be obtained. Importantly, half of the experimentally verified epitopes were not among the predicted ones. The variation in the successful prediction of HLA-binding peptide motifs between different studies may be attributed to differences in the chosen threshold for the motifs regarded as potential binders. A threshold of the highest 3% binding was chosen in study III. That was the same as in the previous study with Can f 4 (150). However, setting the threshold at 1 or 5% did not significantly affect the results (data not shown). Additionally, differences in the experimental in vitro study designs may contribute to the relatively wide range observed in the prediction successes.

Based on the above results and the results of other studies, it seems that bioinformatical prediction algorithms alone are not sufficient to reliably identify all relevant CD4+ T cell epitopes within an allergen (150,294,321). However, they can be a useful tool for screening
potential T cell epitopes, even though the predicted epitopes should be verified with experimental methods. These findings underline the notion that HLA binding is most probably not the sole defining factor in determining the strength of T cell response to a given epitope, since responses to epitopes predicted not to bind efficiently to HLA molecules could still be experimentally detected. One way to verify the actual binding capacity of an HLA allele to a peptide is to measure it in vitro (150,282). Moreover, other factors, such as the efficiency of processing and presentation of peptides by APCs, probably influence the T cell epitope repertoire (77,322).

6.3 ANALYSIS OF SPECIFIC CD4+ T CELLS IN VITRO AND EX VIVO (I-III)

6.3.1 Results obtained with the conventional in vitro split-well method are comparable to those obtained with direct ex vivo detection methods (I-III)

In studies I-III, the split-well method (see Chapter 4.4.1) was used to detect allergen-specific cells in total PBMCs and in purified naïve and memory CD4+ T cells. As a straightforward approach for analyzing antigen-specific T cells in humans, it has been one of the methods of choice for decades (323). Employing Equ c 1143-160 peptides in study II, specific T cell responses were detected in all but two of the 23 subjects studied (II, Figure 1). Most of these responses likely originated from the naïve CD4+ T cells, as further analyses employing purified CD45RA+ and CD45RO+ CD4+ T cells indicated that all detectable responses in nonallergic subjects emerged from the naïve pool (II, Figure 4B). In line with this notion, several studies with autoantigens have demonstrated that differences in the T cell reactivity between subjects with and without an autoimmune disease can only be demonstrated by analyzing the memory T cell compartment (298,324,325). To gain a better understanding of the underlying nature of T cell responses in allergy, highly purified CD45RA+ naïve and CD45RO+ memory CD4+ T cells were used for the split-well approach in studies I and III. Utilizing this methodology it was indeed apparent that allergen-specific memory CD4+ T cell responses were extremely rarely detected in nonallergic subjects (I, III).

The estimated frequencies of allergen-specific CD4+ T cells in peripheral blood based on the split-well assay generally agreed well with the estimates derived by other methods, such as the direct ex vivo HLA class II tetramer assay (260,261,310) and the CD154-based enrichment and detection (study III). This is also the case in other studies with different allergens (260,273,292). Similarly, most of the phenotypic parameters, such as the production of cytokines IL-4, IL-5 IL-10 and IFN-γ (I-III) or the expression of chemokine receptors CCR6, CXCR3 and CRTh2 (III), pointed to a Th2-biased phenotype in T cells and TCLs derived from allergic subjects and Th0/Th1-biased phenotype in nonallergic subjects. However, the long in vitro stimulation prior to the split-well analysis was likely to result in the leveling of possible differences in the expression of cell surface molecules, such as CCR4, CCR6 and CCR7, as observed in study I (Figure 6A and B). Despite the prolonged in vitro cultivation
with the split-well approach, differences were still seen in some of the measured parameters, such as the Th1-associated receptor CXCR3 (I, Figure 6A and B), indicating that the Th1/Th2 dichotomy in vivo remains detectable in vitro (291). Indeed, similar results were obtained in another study, where the T cells of nonallergic subjects were shown to express higher levels of CXCR3 than those of allergic subjects after extended in vitro culture (72). Furthermore, when the peripheral blood Th1, Th17 and Th2 subsets of CD4+ T cells were purified ex vivo by flow-cytometric cell sorting and then stimulated in vitro for extended time, their original phenotypes remained clearly detectable (Ihantola et al., unpublished results).

Taken together, it can be concluded that the split-well can be reliably applied to estimate the frequency of allergen-specific CD4+ T cells, at least when naïve and memory pools are separated from one another. However, its usefulness may be limited to certain Th1/Th2-associated phenotypic parameters, such as the production of cytokines IL-4, IL-5, IL-10 and IFN-γ, and cell surface molecules, such as CXCR3. The expression of other parameters, unless otherwise shown, may be altered during the prolonged in vitro cultivation, obscuring the nature of the original in vivo status. Overall, prolonged in vitro culture comprises a multitude of factors potentially affecting the survival and immune characteristics of cells. These include the phenotypic status of the cell in question and its neighboring cells, variances in the quality and quantity of APCs and the local cytokine milieu (235). Finally, most protocols employing in vitro stimulation require time up to several weeks, which is a disadvantage in itself. To address these issues, more applicable methods for detecting and analyzing antigen-specific CD4+ T cells have been devised (see below).

6.3.2 Successes and failures in detecting allergen-specific CD4+ T cells with HLA class II tetramers (I, II)

During recent years, the HLA class II tetramer-based assays have been a tool of choice for detecting antigen-specific CD4+ T cells. In study I, DRB1*0401:Bos d 2127-142 tetramers were used to evaluate the frequencies and functional properties of naïve and memory T cells specific to Bos d 2. The tetramer-based evaluation of allergen-specific T cell frequencies (I, Table 1) largely agreed with those obtained with the split-well method. The frequencies were in the range of 1-10 per 10^6 circulating CD4+ cells in allergic subjects. In nonallergic subjects, Bos d 2-specific memory CD4+ T cells were even rarer.

The frequency of Bos d 2-specific CD4+ T cells in the naïve compartment appeared to be similar to that in the memory compartment of allergic subjects. However, there were no clear differences in the frequencies between the study groups. It has been previously shown that the tetramer staining intensity is proportional to the TCR affinity of the responding T cells (280,288,289). Based on this assumption the MFI of memory and naïve cells of allergic and nonallergic donors were evaluated. Interestingly, a statistically significant difference was detected between the naïve Bos d 2-specific T cells of allergic and nonallergic subjects, with cells of allergic subjects displaying higher functional affinity. Although this finding needs confirmation in future studies, the observed differences in the avidity of the allergen-
specific naïve T cell repertoires could potentially lead to qualitatively different immune responses in allergic and nonallergic individuals (178,237,239,240,326).

Tetramer staining has emerged as a convenient tool for enriching antigen-specific cells by flow-cytometric sorting, and, as a high-resolution detection method, tetramers can even be used to single-cell sort rare antigen-specific cells in order to create antigen-specific T cell clones (see Chapter 4.4.1). Tetramer-based single-cell sorting was employed in study I to generate monoclonal T cell populations specific to Bos d 2. These TCCs proliferated upon stimulation with the whole Bos d 2 allergen, confirming the allergen-specificity of the tetramer-stained cells. Furthermore, the obtained TCCs from allergic subjects proliferated more strongly upon in vitro stimulation and expressed more of the Th2-associated cytokine IL-4 when compared to TCCs from nonallergic subjects.

However, despite the fact that HLA tetramers are efficient tools for detecting and studying antigen-specific T cells, some of their limitations were encountered in study II. Whereas Bos d 2127-142 tetramers in study I reliably detected allergen-specific T cells in all test subjects, the design of functional Equ c 1 tetramers turned out to be considerably more challenging. Several peptides with altering lengths and with various HLA alleles were tested for the generation of tetramers, but with little success (data not shown). In most cases, the Equ c 1 tetramers completely failed to stain TCLs that were screened to be Equ c 1-specific by proliferation tests. This emphasizes the importance of knowing the correct restricting HLA elements and functional peptides (optimal binding to a HLA molecule), as well as the requirement of high enough affinity between the tetramer construct and its specific TCR. It is possible that with many peptide:HLA combinations no stable construct can be achieved, or that additional measures, such as enhanced linker peptides, need be used (253). However, a tetramer composed of HLA-DRB4*0101:Equ c 143-160 was successfully used to examine the HLA restriction of a monoclonal and a polyclonal Equ c 1-specific TCL (II, Figure 6).

In addition to the challenges in the tetramer design itself, there have also been concerns that tetramers could miss relevant antigen-specific T cells due to their low TCR affinity for the HLA-peptide complex and consequent inability to bind in a stable manner. This aspect could be especially important in situations where TCR affinities of the disease-associated T cells are known to be low, such as in autoimmune diseases or cancer (257). In these cases, tetramers could create an artificial cutoff within a cell population, identifying only T cells of sufficient affinity, but failing to identify important cell populations of low-affinity (see Chapter 2.4.3).

6.3.3 CD154-based enrichment method as an alternative approach to detect allergen-specific CD4+ T cells (III)

To circumvent the challenges associated with tetramers, methods relying on activation markers have been recently developed (see Chapter 2.4.3). One of these approaches, the enrichment of antigen-specific T cells based on the expression of the surface molecule CD154, was employed in study III. One of the allergic subjects had an exceptionally high frequency of Can f 5-specific memory CD4+ T cells in their peripheral blood. This allowed us to directly
compare the \textit{ex vivo} frequency of CD154-positive T cells before and after the magnetic enrichment and separation (III, Figure S1). Through this analysis we could verify that the magnetic separation did not alter the frequency or phenotype of the antigen-specific T cells. Next, the CD154 marker in conjunction with CD69 was used to estimate the frequency of Can f 5-specific memory CD4+ T cells in seven allergic and four nonallergic subjects, and these results were compared with those obtained by the conventional split-well method. The comparison demonstrated that the frequencies of allergen-specific cells obtained with these two methods correlated strongly ($r = 0.87$, the Spearman correlation test). However, it should be noted that the split-well method subtly but systematically estimated the frequency to be lower (III, Figure 3C). This could be due to the inability of some of the Can f 5-specific memory CD4+ T cells to undergo long-term expansion \textit{in vitro}, for example, due to their low TCR affinity or terminally differentiated phenotype.

The CD154 enrichment method can also be used as an alternative for HLA class II tetramers in T cell phenotyping (243). In many regards, these two methods can be utilized in a similar manner, as tetramers labeled with, for example, PE can also be used to magnetically enrich for antigen-specific cells in a similar way, as described above for the CD154 marker. Therefore, if high-affinity tetramer reagents are available for the antigens of interest, this method offers a viable alternative with a capability to detect antigen-specific cells with great specificity \textit{ex vivo}. However, the issue of the affinity threshold still remains, potentially causing the failure to detect antigen-specific T cells of lower avidity (see Chapter 2.4.3). Also, each HLA allele may have a different affinity to the antigenic peptide, and the peptide:HLA complex itself may exhibit dissimilar affinities depending on its components. Similarly, examining larger numbers of non-HLA-matched test subjects is often impractical with the tetramer approach, since typically only a limited selection of functional peptide:HLA tetramer combinations are available. In the case of Can f 5 there were no pre-existing data regarding the T cells epitopes of the allergen. Therefore, the CD154-based detection was considered an ideal approach for direct \textit{ex vivo} T cell analysis. In line with the cytokine profile of the TCLs obtained with the split-well approach (III, Figure 2), the Th2-deviated phenotype of Can f 5-specific CD4+ memory T cells, shown by the expression of chemokine receptors (CCR6\textsubscript{low} / CXCR3\textsubscript{low} / CRTh2\textsubscript{high}), was clearly evident when compared to those of T cells specific to the microbial antigen PPD or unstimulated total memory CD4+ T cells (III, Figures 4 and S3). However, a subtle shift in the expression levels of certain chemokine receptors, notably the reduction in CXCR3, was already observed after 16 h of \textit{in vitro} stimulation (data not shown). While this did not impair the analysis of the data, it clearly indicates that in order to determine the unaltered \textit{ex vivo} phenotype of specific CD4+ T cells the incubation times should be minimized. In a very recent study, it was shown that stimulation for only 3 hours could be sufficient for CD154 to be upregulated (273). Therefore, to preserve the exact \textit{ex vivo} phenotype of antigen-specific T cells, it is conceivable that shorter \textit{in vitro} stimulation times should be preferred in future experiments. However, it is possible that three hours
may not be a sufficient enough time for complete protein antigens to be internalized and processed by APCs.

Collectively, the above data indicate that the CD154-based detection of allergen-specific CD4+ T cells yields results comparable with both the conventional long-term in vitro expansion and the more recent tetramer-based detection methods. In terms of cell frequencies in allergy, the estimates agree well with each other. Based on the results obtained by these three methods, it can be estimated that the frequency of mammalian allergen-specific memory CD4+ T cells is low, generally in the range of 1-30 per 10^6 memory T cells. The highest detected frequency in an allergic subject was as high as 469 per 10^6 T cells. Similarly, the Th2-polarization of the allergen-specific memory CD4+ T cells from allergic, but not from nonallergic subjects, was evident with both methods. Since each of the aforementioned detection methods for antigen-specific CD4+ T cells has been successfully put to use in multiple studies, choosing the right approach depends on the careful evaluation of the strengths and weaknesses of each method.

6.4 POTENTIAL OF BOS D 2, EQU C 1 AND CAN F 5- DERIVED PEPTIDES FOR PEPTIDE-BASED ALLERGEN IMMUNOTHERAPY (I, II, III)

In ideal cases, the candidate peptides for immunotherapeutic purposes should elicit robust T cell responses in allergic individuals without binding to IgE. Also, the peptides should be presented by a wide range of HLA class II molecules to ensure sufficient coverage within the target population. The cow Bos d 2 (study I) and horse Equ c 1 (II) allergens have been previously shown to contain immunodominant epitopes (75,76), and their capacity to induce in vitro allergen-specific T cell responses in allergic individuals was further affirmed in this work. In study II, the Equ c 1143-160 peptide was identified as a promising candidate for PIT due to its distinctly immunodominant nature and the fact that it was able to be presented by multiple common HLA class II molecules. Furthermore, as both Bos d 2 and Equ c 1 are major allergens (4,83,90), they cover a substantial portion of cow and horse-sensitized subjects, 90% and 76% respectively. That possibly reduces the need for including peptides from additional allergens in the prospective immunotherapeutic products.

In dog allergy, several major allergens (Can f 1, Can f 4 and Can f 5) have been identified (4). Can f 1 shows IgE reactivity in 50-75%, Can f 4 in 35-60% and Can f 5 in up to 70% of subjects sensitized to dog (as reviewed in (4)). Therefore, choosing peptides for the immunotherapy of dog allergy can be more challenging. Furthermore, Can f 5 had not been previously characterized in detail. In study III, overlapping peptides spanning the whole Can f 5 amino acid sequence were used to map the allergen for CD4+ T cell epitopes. In Can f 5, six prominent epitope regions were discovered and they covered all the allergic subjects in the study (III, Figure 5). The most prominent region was recognized by 7/12 (58%) of the study subjects. Since many Can f 5-sensitized individuals are not sensitized to Can f 1 (327)
and only a minority is sensitized exclusively to Can f 4 (328), the inclusion of Can f 5 peptides in a potential PIT preparation against dog allergy could markedly enhance the outcome. For this purpose, the core peptides of the six immunodominant regions offer a feasible alternative, especially if combined with peptides containing the major epitopes of Can f 1 and Can f 4. Together, these peptides would help to ensure a maximal coverage among dog-allergic individuals.
7 Conclusions

The purpose of this work was to analyze the immunological mechanisms underlying animal allergy. CD4+ T cell responses against major airborne animal allergens, cow Bos d 2, horse Equ c 1 and dog Can f 5, were studied in allergic and nonallergic individuals in vitro and/or ex vivo. Furthermore, different methods for detecting allergen-specific CD4+ T cells were compared while analyzing these responses.

The frequency of allergen-specific memory CD4+ T cells was found to be consistently higher in allergic than nonallergic subjects with all three allergens studied. The T cells of allergic donors were also distinctly Th2-deviated, whereas those of nonallergic donors were mostly Th1 or Th0-deviated. This was evident by both the cytokine production and the chemokine receptor analyses of the T cells. Furthermore, results from the tetramer staining and proliferation assays suggested that the allergen-specific T cells of allergic subjects were of higher affinity than those of nonallergic subjects. Collectively, these findings indicate that there are marked differences in the frequency and phenotype of allergen-specific CD4+ memory T cells between allergic and nonallergic subjects. Understanding these differences and their underlying reasons are prerequisites for understanding the allergenic immune response.

In this work, CD4+ T cell responses to Can f 5 were investigated for the first time. Whereas Bos d 2 and Equ c 1 contain single distinct immunodominant T cell epitope regions, six immunologically important epitope regions, dispersed evenly along the sequence, were found in Can f 5. The identified T cell epitopes of Can f 5 can be utilized for developing peptide-based immunotherapy for dog allergy.

Conventional methods for analyzing allergen-specific T cells, such as the long-term in vitro cultivation after specific stimulation, including the split-well method, have provided substantial knowledge on the T cell responses in healthy and allergic individuals. However, these methods contain caveats that have limited their usefulness in several settings. Therefore, they are being superseded by novel approaches. In this work, both the HLA class II tetramer and the activation marker CD154-based assays were employed to successfully gather detailed information about allergen-specific CD4+ T cells. These methods avoid artefacts possibly produced by prolonged in vitro cultivation, which are typical for the conventional methods. The approaches adopted helped to confirm that the frequency and phenotype of allergen-specific CD4+ T cells ex vivo largely corresponds to the findings obtained earlier with the long-term in vitro culture assays.
8 References


(21) Barrett EG, Rudolph K, Bowen LE, Bice DE. Parental allergic status influences the risk of developing allergic sensitization and an asthmatic-like phenotype in canine offspring. Immunology 2003;110(4):493-500.

(22) Hicks WB, Nageotte CG, Wegienka G, Havstad S, Johnson CC, Ownby DR, et al. The association of maternal prenatal IgE and eczema in offspring is restricted to non-atopic mothers. Pediatric Allergy and Immunology 2011;22(7):684-687.


(35) Schaub B, von Mutius E. Ovid: Obesity and asthma, what are the links?. Current Opinion in Allergy and Clinical Immunology 2005;5(2):185-193.


(81) Yamane H, Paul WE. Cytokines of the gamma(c) family control CD4+ T cell differentiation and function. Nat Immunol 2012 Nov;13(11):1037-1044.


(106) Munir AK, Einarsson R, Schou C, Dreborg SK. Allergens in school dust. I. The amount of the major cat (Fel d 1) and dog (Can f I) allergens in dust from Swedish schools is high enough to probably cause perennial symptoms in most children with asthma who are sensitized to cat and dog. J Allergy Clin Immunol 1993 May;91(5):1067-1074.


(131) Briner TJ, Kuo MC, Keating KM, Rogers BL, Greenstein JL. Peripheral T-cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen Fel d I. Proc Natl Acad Sci U S A 1993 Aug 15;90(16):7608-7612.


The aim of this study was to analyze human CD4+ T cell responses to mammalian allergens in subjects with and without allergy using in vitro assays and novel ex vivo detection methods.

Functional and phenotypic differences were detected in allergen-specific CD4+ T cells between the study groups. Furthermore, the CD4+ T cell responses to the major dog allergen Can f 5 were analyzed for the first time in detail in this work. These results could aid the development of immunotherapy against allergy.