

TUURE KINNUNEN

The Role of T Cell Recognition in the Immune Response Against Lipocalin Allergens

Prospects for Immunotherapy

Doctoral dissertation

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ABSTRACT

The increased incidence of allergic diseases is a widely recognized health problem in industrialized countries. Since the role of T cells, particularly T helper type 2 (Th2) cells, in the development of IgE-mediated allergic diseases is well established, the targeted modulation of their function is an attractive therapeutic alternative.

The aim of this study was to analyze the T cell responses of cow- and dog-allergic subjects against the bovine and dog major allergens, Bos d 2 and Can f 1. Moreover, peptide analogs of the immunodominant T cell epitope of Bos d 2 were generated and their potential for allergen immunotherapy was evaluated *in vitro*.

Bos d 2 is known to have a weak stimulatory capacity for human peripheral blood mononuclear cells (PBMCs). In this study, Bos d 2-specific T cell clones were observed to recognize the immunodominant epitope of the allergen suboptimally, as peptide analogs containing single amino acid substitutions were able to stimulate the clones at lower concentrations than the natural ligand. Moreover, the proliferative responses of PBMCs and Can f 1-specific T cell lines of dog-allergic subjects were weak. The weak T cell reactivity observed against Bos d 2 and Can f 1 may be associated with the allergenicity of these lipocalin allergens.

The interaction between the T cell receptor (TCR) and the human leukocyte antigen (HLA) class II molecule-peptide complex plays an important role in initiating the allergen-specific immune response. Therefore, the TCR-HLA associations in responses against Bos d 2 and Can f 1 were investigated. DRB1*0401-restricted T helper cells specific for the immunodominant epitope of Bos d 2 used predominantly the TCR V β 13.1 subtype. Moreover, an increased frequency of TCR V β 5.1+ T helper cells was observed in the Can f 1-specific T cell lines of Can f 1-nonallergic subjects but not in those of Can f 1-allergic subjects. Interestingly, this was preferentially associated with the expression of the DR4-DQ8 HLA haplotype by the nonallergic subjects. These findings suggest that certain TCR-HLA combinations may affect sensitization to lipocalin allergens.

Peptide-based immunotherapy is a promising approach for the treatment of allergic diseases. In this study, the immunomodulatory effect of peptide analogs with enhanced stimulatory capacity on Bos d 2-specific T cell clones was evaluated. These heteroclitic analogs were shown to favor Th1-biased cytokine production and the induction of cell death and hyporesponsiveness of specific T cell clones *in vitro*. Moreover, one of the analogs, pN135D, was observed to induce a Th1/Th0-deviated T cell response cross-reactive with the natural immunodominant epitope in eight different DR4-positive subjects *in vitro*. These findings support the notion that peptide analogs could enhance the efficacy of peptide-based immunotherapy.

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In all things of nature there is something of the marvelous.

Aristotle
Parts of Animals

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Kuopio, January 2007



Tuure Kinnunen

ABBREVIATIONS

7-AAD	7-amino-actinomycin D
A1M	α_1 -microglobulin
AGP	α_1 -acid glycoprotein
AICD	activation-induced cell death
APC	antigen-presenting cell
APL	altered peptide ligand
C8 γ	complement factor C8 γ -subunit
CD	cluster of differentiation
CDR	complementarity-determining region
CFSE	5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
cpm	counts per minute
CTLA	cytotoxic T-lymphocyte-associated antigen
DC	dendritic cell
EBV	Epstein-Barr virus
EC	effective concentration
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
Fc ϵ RI	high-affinity IgE receptor
FITC	fluorescein isothiocyanate
GITR	glucocorticoid-induced tumour necrosis factor receptor family-related protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HA	hemagglutinin
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
IC	inhibitory concentration
ICOS	inducible costimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton

LAR	late asthmatic reaction
mAb	monoclonal antibody
MBP	myelin basic protein
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MS	multiple sclerosis
NGAL	neutrophil gelatinase-associated lipocalin
NK	natural killer
PBMC	peripheral-blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy5	phycoerythrin-cyanine 5
PerCp	peridinin chlorophyll protein
PHA	phytohemagglutinin
PLA ₂	phospholipase A ₂
PSA	prostate-specific antigen
SI	stimulation index
SIT	specific immunotherapy
SPT	skin prick test
SSCP	single-strand conformational polymorphism
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
TCL	T cell line
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
Treg	regulatory T cell
VEGP	human tear lipocalin (von Ebner's gland protein)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by the corresponding Roman numerals I-IV.

- I Kinnunen T, Buhot C, Närvänen A, Rytkönen-Nissinen M, Saarelainen S, Pouvelle-Moratille S, Rautiainen J, Taivainen A, Maillère B, Mäntyjärvi R, Virtanen T. Immunodominant epitope of lipocalin allergen Bos d 2 is suboptimal for human T cells. *Eur J Immunol* 2003;33:1717-26.
- II Kinnunen T, Kwok WW, Närvänen A, Rytkönen-Nissinen M, Immonen A, Saarelainen S, Taivainen A, Virtanen T. Immunomodulatory potential of heteroclitic analogs of the dominant T-cell epitope of lipocalin allergen Bos d 2 on specific T cells. *Int Immunol* 2005;17:1573-81.
- III Kinnunen T, Jutila K, Kwok WW, Rytkönen-Nissinen M, Immonen A, Saarelainen S, Närvänen A, Taivainen A, Virtanen T. Potential of an altered peptide ligand of lipocalin allergen Bos d 2 for peptide immunotherapy. *J Allergy Clin Immunol* 2007 (in press).
- IV Kinnunen T, Taivainen A, Partanen J, Immonen A, Saarelainen S, Rytkönen-Nissinen M, Rautiainen J, Virtanen T. The DR4-DQ8 haplotype and a specific TCR V β T-cell subset are associated with absence of allergy to Can f 1. *Clin Exp Allergy* 2005;35:797-803.

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1. INTRODUCTION

The prevalence of allergic diseases such as allergic rhinitis, asthma and atopic eczema has increased dramatically in the last few decades and these diseases are currently among the commonest causes of chronic health problems (Kay 2001). Allergic diseases are characterized by the inappropriate production of IgE antibodies to common environmental compounds such as pollens, house dust mites and animal dander. At the cellular level, T helper type 2 cells (Th2 cells) are of fundamental importance since they regulate the production of IgE by B cells and the development and activation of mast cells and eosinophils. Therefore, information on allergen-specific T cells is critical for the understanding of the allergenicity of proteins, i.e. their capacity to induce IgE production.

All mammal-derived major respiratory allergens, with the exception of the cat allergen Fel d 1, belong to the lipocalin family of proteins (Virtanen and Mäntyjärvi 2004). Lipocalins are small, extracellular proteins that have been identified in the body fluids and secretions of numerous species including humans (Flower 1996). Despite the fact that lipocalins are common sensitizers, very little is known about the human T cell responses to them.

Currently, specific immunotherapy (SIT), conducted by administering increasing doses of allergen subcutaneously, is the only treatment of allergic diseases which leads to desensitization rather than only controlling the symptoms (Bousquet et al. 1998). Unfortunately, the duration of SIT is long, 3 to 5 years, and there is a considerable risk for systemic IgE-mediated immediate reactions resulting from cross-linking of IgE on the surface of mast cells by allergen molecules. Peptide immunotherapy, in which short linear peptides containing the T cell epitopes of an allergen are used instead of crude allergen extracts, is an interesting alternative for SIT. Recently, several clinical trials have demonstrated its clinical efficiency (Larche and Wraith 2005). Since the therapeutic effect of peptide immunotherapy is thought to be mediated by T cells, one possibility to enhance its therapeutic efficacy is to use altered peptide ligands (APLs). APLs are peptides containing amino acid substitutions as compared with the natural peptide. They are able to induce both quantitatively and qualitatively distinct signals in the responding T cell (Sloan-Lancaster and Allen 1996). The therapeutic potential of APLs has been demonstrated in murine models of autoimmune diseases (Nicholson et al. 1995; Brocke et al. 1996; Alleva et al. 2002) and allergic asthma (Janssen et al. 2000). However, no human studies have evaluated the use of APLs for allergen immunotherapy.

2. REVIEW OF THE LITERATURE

2.1 Allergy

2.1.1 General

The term “allergy” was introduced in 1906 by the pediatrician Clemens von Pirquet for the concept of “altered reactivity” of the immune system (von Pirquet 1906). Allergy was considered a hyperimmune response against a previously encountered antigen (allergen), leading to host damage. Currently, allergy is often equated with type I hypersensitivity (IgE-mediated immediate-type hypersensitivity) as outlined by Coombs and Gell (Coombs and Gell 1975). The related term “atopy” is used for the inherited tendency to produce high levels of IgE against common innocuous environmental allergens (Coca and Cooke 1923).

There has been a marked increase in the prevalence of atopic diseases in industrialized countries during recent decades (Kay 2001). In these countries, the diseases are nowadays among the commonest causes of chronic health problems and have a considerable economic impact on health care costs. Although atopic diseases show complex genetic associations and have a hereditary component (Cookson and Moffatt 2004), the rapidity of this allergy epidemic indicates that environmental factors are important in driving the development of these diseases. The recently formulated “hygiene hypothesis” suggests that an inadequate exposure to environmental microorganisms during infancy leads to a defective development of important immunological regulatory mechanisms that normally protect from allergy (Wills-Karp et al. 2001).

2.1.2 Immune response in allergy

Allergic sensitization is initiated by the generation of allergen-specific Th2 cells (see chapter 2.2.1). Activated Th2 cells produce a variety of cytokines, e.g. IL-4, IL-5 and IL-13. Together with a second signal, i.e. the interaction of the CD40 ligand on T cells and CD40 on B cells, these cytokines promote B cells to undergo isotype switching and produce IgE (Corry and Kheradmand 1999). IgE binds to the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils (Turner and Kinet 1999). Upon subsequent encounter with the allergen, the cross-linking of FcεRI on the surface of these cells leads to degranulation and the release

of preformed mediators such as histamine, proteases, leukotrienes and prostaglandins. These substances are responsible for the immediate early-phase inflammatory response at the allergen contact site (Pearlman 1999). In the skin, vasodilatation, increased vascular permeability and edema lead to the clinical signs of urticaria and the wheal-and-flare reaction. Inhalation of the allergen leads to nasal irritation and increased mucus production, as observed in allergic rhinitis. Contraction of the bronchial smooth muscle and increased mucus production in the lower respiratory tract cause the symptoms of wheezing and shortness of breath in allergic asthma.

The release of *de novo* synthesized mediators, in particular leukotrienes and various Th2 cytokines, from activated mast cells results in the recruitment of other inflammatory cells, such as eosinophils and T helper cells, to the site of allergic inflammation. Their combined effect leads to an allergic late-phase response four to eight hours after the initial allergen exposure (Pearlman 1999). Repeated allergen exposure in the context of an already inflamed tissue leads to chronic allergic inflammation with structural changes (remodeling), such as smooth muscle thickening, tissue fibrosis, and mucous cell hyperplasia. This contributes to the pathogenesis of long-term allergic diseases, for example chronic asthma (Bousquet et al. 2000).

2.1.3 Allergens

Allergens are, by definition, antigens that are bound by specific IgE and trigger allergic symptoms in susceptible individuals. Typical allergen sources include house dust mites, pollens from grass and trees, foods, latex, insect venoms and animal dusts. Usually, allergen sources contain several IgE-binding components, and the pattern of IgE-binding varies between different individuals. If more than 50% of the subjects showing IgE-reactivity to an allergen source have IgE specific for a particular allergenic protein, it is defined as a major allergen (Hoffmann et al. 1994). Other allergens are minor allergens. Allergens are typically proteins or glycoproteins with molecular masses from 3 to 80 kDa. To date, no common structural, functional or chemical features explain their ability to elicit IgE production (Aalberse 2000; Holt and Thomas 2005). Since several allergens are enzymes, proteolytic activity has been proposed as a potential enhancer of allergic sensitization (Comoy et al. 1998; Reed and Kita 2004).

Purified allergens are named according to the guidelines of the The Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS) (Hoffmann et al.

1994). Allergens are designated based on the accepted taxonomic name of their source: the first three letters of the genus, the first letter of the species, and an Arabic number. The numbers are usually assigned to the allergens in the order they have been identified. When necessary, the natural, recombinant and synthetic forms of an allergen are indicated with the prefix n, r or s, respectively.

2.1.4 Lipocalin allergens

Almost all animal-derived major respiratory allergens belong to the lipocalin family of proteins (Virtanen and Mäntyjärvi 2004). The currently identified mammalian lipocalin allergens causing respiratory sensitization are listed in Table I. In addition to mammalian allergens, the pigeon tick allergen Arg r 1 (Hilger et al. 2005), the cockroach allergen Bla g 4 (Arruda et al. 1995), the “kissing bug” (*Triatoma protracta*) allergen Tria p 1 (Paddock et al. 2001), and the cow milk allergen β -lactoglobulin, Bos d 5 (Gjesing et al. 1986), are also lipocalins.

The lipocalin protein family consists of a large group of small extracellular proteins. Lipocalins have been identified in the body fluids and secretions of numerous species including humans (Flower 1996). Although the overall amino acid identity between lipocalins is usually below 20%, their tertiary structure is highly conserved (Flower 1996). Lipocalins contain three short structurally conserved regions. The first contains the signature motif glycine-x-tryptophane (G-x-W) present in all lipocalins. Interestingly, some lipocalins exhibit considerable sequence homology between species. For example, human tear lipocalin (VEGP) has a 57% identity with the dog allergen Can f 1 (Virtanen and Mäntyjärvi 2004).

Lipocalins were originally classified as extracellular transport proteins for diverse molecules, such as vitamins, odorants, steroids and pheromones (Flower 1996). Recently, additional functions have been described for them. Neutrophil gelatinase-associated lipocalin (NGAL), α_1 -acid glycoprotein (AGP), α_1 -microglobulin (A1M), glycodefin (placental protein 14) and the complement factor C8 γ -subunit (C8 γ) are acute-phase proteins associated with anti-inflammatory properties (Logdberg and Wester 2000). In a recent study, glycodefin was shown to exert a direct immunosuppressive effect on T cells by elevating the T cell activation threshold (Rachmilewitz et al. 2001). Although enzymatic activity is not a common property of lipocalins, the lipocalin protein glutathione-independent prostaglandin D₂ synthase is an enzyme (Nagata et al. 1991). In a recent study, two other lipocalins, β -lactoglobulin and

human tear lipocalin were shown to have nonspecific endonuclease activity *in vitro* (Yusifov et al. 2000).

Table I. Mammalian respiratory lipocalin allergens

Allergen	Animal	Reference
Bos d 2	Cow	(Mäntyjärvi et al. 1996)
Can f 1	Dog	(Konieczny et al. 1997)
Can f 2	Dog	(Konieczny et al. 1997)
Cav p 1	Guinea pig	(Fahlbusch et al. 2002)
Cav p 2	Guinea pig	(Fahlbusch et al. 2003)
Equ c 1	Horse	(Gregoire et al. 1996)
Equ c 2	Horse	(Bulone et al. 1998)
Fel d 4	Cat	(Smith W et al. 2004)
Mus m 1	Mouse	(Cavaggioni and Mucignat-Caretta 2000)
Ory c 1 ^a	Rabbit	(Baker et al. 2001)
Ory c 2 ^a	Rabbit	(Baker et al. 2001)
Rat n 1	Rat	(Cavaggioni and Mucignat-Caretta 2000)

^a tentatively named

2.2 T cells

2.2.1 General

T lymphocytes, or T cells, play a key role in adaptive immunity. They derive from hematopoietic stem cells and undergo differentiation in the thymus (Delves and Roitt 2000a). The majority of T cells express T cell receptors (TCRs) that recognize short linear peptides in the context of major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). These T cells can be further divided into two important sublineages: helper T cells expressing CD4 (CD4⁺ T cells) and cytotoxic T cells expressing CD8 (CD8⁺ T cells) (Delves and Roitt 2000b). In addition to helper T cells, distinct subsets of CD4⁺ T cells function as regulatory T cells (see chapter 2.2.6)

CD4⁺ helper T cells are important regulators of adaptive immune responses (Delves and Roitt 2000b). They are activated by professional antigen-presenting cells, such as dendritic cells (DCs), B cells and macrophages. These cells are able to take up extracellular proteins by endocytosis or phagocytosis, degrade them to peptides and present them in the context of MHC class II molecules on their surface (Delves and Roitt 2000a).

CD4⁺ helper T cells can be further divided into two major subtypes based on their cytokine production and function (Mosmann et al. 1986). Th1 cells predominantly secrete interleukin-2

(IL-2) and interferon- γ (IFN- γ). They are effective inducers of cellular immune responses, including the activation of macrophages, natural killer (NK) cells and CD8+ cytotoxic T cells (Delves and Roitt 2000b). Th1 cells are critical for the eradication of intracellular pathogens such as *Mycobacterium tuberculosis*. Th2 cells secrete IL-4, IL-5, IL-6 and IL-13, and are effective in promoting antibody production by B cells (Delves and Roitt 2000b). Th2 cells are essential for the elimination of extracellular pathogens including helminths and nematodes. Both Th1 and Th2 cells also mediate pathologic immune responses. Th1 cells have been associated with the tissue destruction observed in several organ-specific autoimmune diseases, whereas Th2 cells are important effector cells of the allergic immune response (see chapter 2.1.2). T cells capable of producing mixed patterns of Th1 and Th2 cytokines are classified as Th0 cells (Abbas et al. 1996). A new subset of helper T cells, Th17 cells, has recently been described (Weaver et al. 2006). Th17 cells are characterized by the production of a distinct profile of pro-inflammatory cytokines, including IL-17 and IL-6 but not IFN- γ or IL-4. Th17 cells appear to be important in the immune response against extracellular bacteria and fungi, and they contribute markedly to the development of autoimmune disease (Weaver et al. 2006).

CD8+ cytotoxic T cells are able to recognize and eliminate cells infected with viruses and other intracellular microbes (Wong and Pamer 2003). The infected cells display peptides derived from intracellular pathogen-derived proteins in the context of MHC class I molecules on their surface. CD8+ T cells are able to recognize these MHC-peptide complexes and kill the infected cells by at least two different pathways (Barry and Bleackley 2002). They can insert perforins in the target-cell membrane, enabling the passage of apoptosis-mediating enzymes, granzymes. Alternatively, they can bind the Fas molecule on the target cell with their Fas-ligand, which is also able to induce apoptosis of the target cell. In addition, activated CD8+ cytotoxic T cells produce cytokines, such as IFN- γ and tumour necrosis factor α (TNF- α), which contribute to antimicrobial defence (Harty and Bevan 1999).

2.2.2 HLA system

The major histocompatibility complex (MHC) is known in humans as the human leukocyte antigen (HLA) system (Klein and Sato 2000). The HLA complex on chromosome six contains over 200 genes, many of them not related to immune function (Aguado B. et al. 1999). The HLA genes involved in immune recognition fall into two structurally and functionally different classes, I and II. The class I genes are expressed by most somatic cells, and the class

I molecules present peptides derived from endogenous proteins to CD8⁺ T cells. The class II genes, in contrast, are normally only expressed by professional APCs, and the class II molecules present peptides derived from exogenous proteins to CD4⁺ T cells.

Both HLA class I and II molecules are heterodimers formed by α and β chains. There are three genes coding for the α chains of class I molecules HLA-A, -B and -C. The β chain of the class I molecules is formed by a smaller constant chain called β_2 -microglobulin. There are also three pairs of genes coding for the α and β chains of the class II molecules HLA-DR, -DP and -DQ. In many cases the HLA-DR cluster also contains an extra β chain gene. This means that the three sets of genes can give rise to four types of class II molecules.

The HLA class I and II genes are highly polymorphic: that is, there are multiple alleles of each gene within the human population. For example, over 300 different alleles have been identified for HLA-A and over 400 for HLA-DR β (Janeway et al. 2005). The particular combination of HLA alleles found on a single chromosome is called the HLA haplotype. Because of the extensive polymorphism, most individuals are heterozygous at each HLA locus. Thus, humans can express up to six different class I and eight different class II molecules on their cells.

2.2.3 Antigen processing and presentation

The pathways by which antigenic peptides are processed and presented are different for MHC class I and II molecules (Klein and Sato 2000). In the MHC class I pathway, endogenous proteins are degraded into short peptides in the cytosol by a multicatalytic proteinase complex, the proteasome. These peptides are translocated into the endoplasmic reticulum (ER) lumen by molecules called TAPs (transporters associated with antigen processing). In the ER, chaperone-assisted assembly generates a stable complex from a newly synthesized MHC class I α chain, β_2 -microglobulin and a peptide. Finally, the assembled MHC-peptide complexes are transported to the cell surface (Gromme and Neefjes 2002).

MHC class II molecules are also synthesized separately and brought together in the ER. Unlike class I molecules, class II molecules associate with a protein produced in the ER, the invariant chain, which blocks the loading of endogenous peptides to the peptide-binding groove of the molecules. Vesicles containing class II molecules complexed with invariant chains fuse with endosomes containing extracellular proteins taken up by endocytosis to form the MHC class II compartment. In this compartment, proteolytic enzymes degrade the

exogenous proteins and the invariant chains. Only a small peptide fragment of the invariant chain called CLIP is left in the peptide-binding groove of the class II molecules. Finally, the CLIP peptide is released with the help of a special protein, HLA-DM, allowing peptides derived from exogenous proteins to bind. The peptide-loaded MHC class II molecules are then transported to the cell surface (Villadangos 2001).

In both MHC class I and II molecules, the peptides are bound between two α -helical segments. In class I molecules, the α helixes converge to close the peptide-binding groove, whereas in the class II molecules the groove ends are open (Klein and Sato 2000). Consequently, class I molecules accommodate shorter peptides (usually 8-10 amino acid residues) than class II molecules (13 to 17 residues) (Janeway et al. 2005). The side chains of the amino acid residues can either protrude out of the peptide-binding groove to make contact with the TCR or point into small pockets within the groove (Fig. 1A). The residues that bind to the pockets of the MHC are called anchor amino acid residues. The pattern of amino acid residues required for the binding of a peptide to a particular MHC molecule is known as a peptide-binding motif (Rammensee et al. 1995). The first residue of this motif is designated P1, the next is P2, and so on (Fig. 1A).

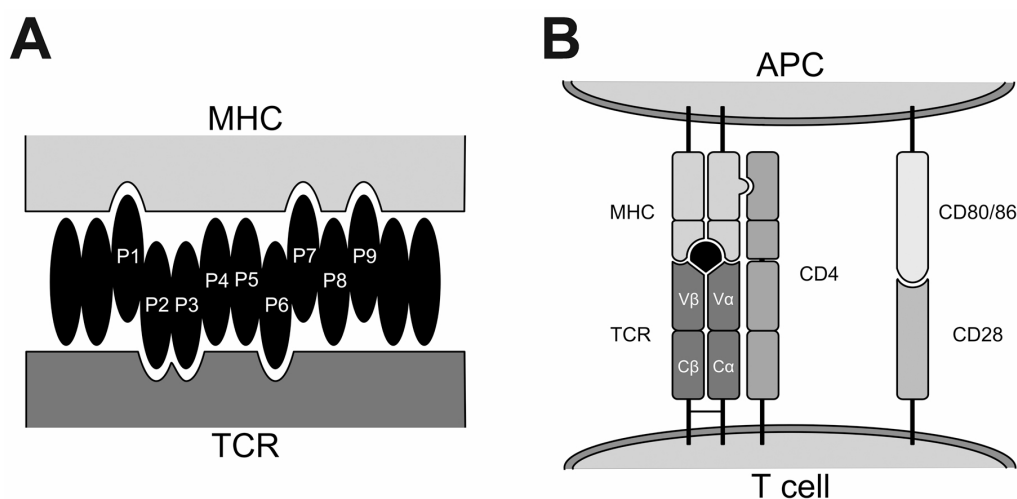


Figure 1. A, Peptides bind to MHC molecules through anchor amino acid residues (P1, P7 and P9 in the figure). Amino acid residues protruding out of the peptide-binding groove make contact with the TCR (P2, P3 and P6 in the figure). B, CD4⁺ T cells recognize peptides bound by MHC class II molecules. The TCR makes contact with both the MHC molecule and the peptide. The coreceptor CD4 binds to an invariant part of the MHC molecule and stabilizes the TCR-MHC complex. Interaction of CD80 or CD86 with CD28 provides a costimulatory signal enhancing the activation of the T cell.

2.2.4 T cell receptor structure and T cell activation

T cell receptors (TCRs) are plasma membrane-bound heterodimers, usually composed of α and β chains (Delves and Roitt 2000a). Both the α and β chains of the TCR have an amino-terminal variable (V) region and a carboxy-terminal constant (C) region (Fig. 1B). Each chain spans the lipid bilayer by a hydrophobic transmembrane domain, and ends in a short cytoplasmic tail. The TCR α and β chain genes are composed of discrete segments, variable (V), diversity (D), joining (J) and constant (C), which are joined by somatic recombination during the development of the T cell. There are about 70 V_α , 61 J_α and 1 C_α , and 52 V_β , 2 D_β , 13 J_β and 2 C_β gene segments (Janeway et al. 2005). Each T cell uses a different combination of these gene segments to form its TCR, creating a huge spectrum of possible TCR structures.

The peptide-MHC binding site of the TCR is formed mainly from three complementarity-determining regions (CDR1-3). The hypervariable CDR3 loops of α and β chains lie in the center of the TCR-MHC interface and make direct contact with the antigenic peptide, while the less variable CDR1 and CDR2 loops contact mainly the MHC component (Davis et al. 1998). Since the TCR makes contact with both the peptide and the MHC component, the specificity of the interaction is determined by both of them (Fig. 1B). This phenomenon is called MHC restriction. A small fraction of T cells bears an alternative form of TCR, consisting of γ and δ chains instead of α and β chains. The $\gamma\delta$ T cells do not generally recognize their target antigens as peptides presented by MHC molecules in the way that $\alpha\beta$ T cells do, and their precise biological function is unclear (Carding and Egan 2002).

T cell receptors are associated on the surface of T cells with the CD3 complex of molecules that transmit signals into the cell when the TCR binds to the MHC-peptide complex. TCR-MHC interaction leads to phosphorylation of tyrosine residues in the cytoplasmic tails of the CD3 complex, and the following transduction of down-stream signals initiates the transcriptional activation of various genes involved in T cell differentiation, proliferation and effector functions (Delves and Roitt 2000b). The coreceptors CD8 and CD4 bind to the invariant parts of MHC class I or II molecules, respectively, and enhance TCR recognition by stabilizing the TCR-MHC complex (Fig. 1B) (van der Merwe and Davis 2003).

In addition to TCR-MHC interaction, efficient T cell activation also requires a second, costimulatory signal. The best-characterized costimulatory molecules are two structurally related glycoproteins, B7-1 (CD80) and B7-2 (CD86) expressed on the surface of APCs. These molecules are recognized by the CD28 protein expressed by T cells (Fig. 1B) (van der Merwe and Davis 2003). Ligation of CD28 provides a second signal that is necessary for the

activation of naïve T cells. In contrast, the requirement for costimulation is less stringent for the activation of memory T cells (Croft et al. 1994). Interestingly, activation through CD28 costimulation results in gradual up-regulation of a related molecule the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) by the T cell. CTLA-4 binds to the same B7 molecules as CD28, but with more avidity, and delivers an inhibitory signal to the activated T cell. This provides a self-limiting mechanism to prevent excessive T cell activation and proliferation (Bour-Jordan and Bluestone 2002). The inducible costimulator (ICOS), another member of the CD28 family, is also upregulated upon T cell activation, and appears to be an important regulator of Th2 immune responses (Greenwald et al. 2005).

2.2.5 T cell development and survival

T cell precursors arise from hematopoietic stem cells and migrate to the thymus from the bone marrow (Starr et al. 2003). Upon entry in the thymus, T cell precursors lack the expression of CD4 and CD8, and their T cell receptor genes are unrearranged. At this stage the precursors are called double-negative (DN). The developing double-negative T cells rearrange first the TCR β -chain, which is then complexed with CD3 and an invariant pre-TCR α -chain (Starr et al. 2003). Proper assembly of this complex induces the cells to express the CD4 and CD8 coreceptors and to progress to the double-positive (DP) stage. At the DP stage the T cells start to rearrange the TCR α -chain and try to recognize self peptide-MHC complexes on the surface of cortical epithelial cells with their newly formed receptors (Starr et al. 2003). Most of the developing T cells fail to produce TCRs able to interact with peptide-MHC complexes with sufficient avidity and they die of neglect. Only a minority of T cells (less than 5%) capable of weakly binding peptide-MHC complexes with their TCRs receive a signal that blocks the pathway to apoptosis, i.e. they undergo positive selection (Goldrath and Bevan 1999). The recognition of peptide-MHC complexes not only promotes T cell survival but also determines the commitment of the DP T cells to either the CD4 or CD8 lineage. Recognition of peptides in the context of MHC class I results in upregulation of CD8 expression and a complete loss of CD4 expression, whereas MHC class II-restricted recognition results in upregulation of CD4 expression and a complete loss of CD8 expression (Goldrath and Bevan 1999).

T cells at the single-positive stage (SP) migrate to the medulla of the thymus, where they recognize self peptide-MHC complexes on the surface of bone marrow-derived DCs and macrophages (Klein and Sato 2000). T cells with high-affinity TCRs for self peptides receive

a signal from the antigen-presenting cells to undergo apoptosis. In this way, the developing T cell population is purged of most self-reactive T cells that might otherwise initiate an autoimmune response, i.e. the T cells undergo negative selection (Goldrath and Bevan 1999). The TCR affinity required for negative selection is higher than that required for positive selection, otherwise all the T cells that survive positive selection would be deleted during negative selection (Hogquist et al. 2005). Of all the precursor T cells less than 1 percent mature into naïve T cells and leave the thymus (Klein and Sato 2000).

In the periphery, naïve T cells circulate throughout the secondary lymphoid organs (Goldrath and Bevan 1999). When a naïve T cell encounters an antigen-presenting cell bearing a peptide-MHC complex for which its TCR has a high affinity, it becomes activated only if it also receives a sufficient costimulatory signal from the APC. Upon activation, the naïve cell starts to proliferate and differentiates to an effector T cell. When exposure to the antigen ceases, most of the effector T cells die by apoptosis (Goldrath and Bevan 1999). However, some of the effector cells return to a resting state and survive as memory T cells, maintaining a heightened ability to mount a response to the same antigen.

Peripheral T cell homeostasis is tightly regulated by programmed cell death and responses to survival factors, so that the numbers of naïve and memory T cells remain roughly constant (Goldrath and Bevan 1999). Naïve T cells are sustained by repeated contact with the low-affinity self peptide-MHC complexes that originally positively selected them (Marrack et al. 2000). Memory T cells, in contrast, appear not to require constant stimulation through the TCR for survival. Both naïve and memory T cells are highly dependent on the presence of cytokines, in particular IL-7, for their persistence (Bradley et al. 2005).

The deletion of self-reactive T cells in the thymus, i.e. central tolerance, constitutes the primary form of tolerance. Additional mechanisms, which are collectively referred to as peripheral tolerance, supplement central tolerance by preventing the activation of self-reactive T cells in the periphery (Walker and Abbas 2002). The simplest form of peripheral tolerance is T cell ignorance: some self-antigens are physically separated from T cells, e.g. by the blood-brain barrier, or their level of expression is too low to activate specific T cells (Kamradt and Mitchison 2001). Alternatively, recognition of self-antigens in the absence of costimulation can lead to incomplete activation of the T cells, causing a state of specific unresponsiveness termed anergy (Schwartz 2003). Repeated stimulation can lead to apoptosis of the T cells by a process called activation-induced cell death (AICD), providing another mechanism to control T cell activation (Green et al. 2003). In addition to intrinsic

mechanisms, peripheral tolerance is also maintained by distinct subsets of T cells with a regulatory function (see chapter 2.2.6).

2.2.6 Regulatory T cells

Regulatory T cells (Treg cells) are subsets of CD4⁺ T cells involved in the maintenance of peripheral tolerance by actively suppressing the activation and expansion of both Th1 and Th2 type CD4⁺ T cells as well as CD8⁺ T cells and B cells. There are at least two major types of regulatory T cells: CD4⁺CD25⁺ natural Tregs and adaptive Tregs (Bluestone and Abbas 2003).

Natural CD4⁺CD25⁺ Treg cells develop in the thymus and constitutively express the high-affinity α chain of the IL-2 receptor, CD25. Depletion of these cells leads to autoimmunity against multiple tissues (Sakaguchi et al. 1995; Asano et al. 1996). Natural CD4⁺CD25⁺ Treg cells express high levels of CTLA-4 and the glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR) (Takahashi et al. 2000; Shimizu et al. 2002) but low levels of the IL-7 receptor, CD127 (Liu et al. 2006; Seddiki et al. 2006). Recently, a transcription factor, Foxp3, has been identified as a critical regulator of the development and function of natural CD4⁺CD25⁺ Treg cells (Hori et al. 2003). Mutations in the *Foxp3* gene result in a lymphoproliferative disorder with a predilection to multi-organ autoimmunity and severe allergic inflammation in both mice and humans (Ochs et al. 2005).

Natural CD4⁺CD25⁺ Treg cells utilize a wide diversity of TCR V β genes, comparable to CD4⁺CD25⁻ effector T cells, suggesting a similar capacity to recognize diverse antigens (Bluestone and Abbas 2003; Kasow et al. 2004). The generation of natural CD4⁺CD25⁺ Treg cells in the thymus appears to require a relatively high TCR affinity for self-antigens, near the threshold for negative selection (Jordan et al. 2001; Hsieh et al. 2004). Natural CD4⁺CD25⁺ Treg cells are presumably activated in the periphery by self-ligands and function by suppressing the effector T cell response against these ligands (Bluestone and Abbas 2003). Natural CD4⁺CD25⁺ Tregs exert their immunosuppressive functions by several distinct mechanisms. *In vitro*, the dominant immunosuppressive mechanism appears to be cell contact- and CTLA-4-dependent, while *in vivo* the suppression is also mediated by the immunosuppressive cytokines IL-10 and TGF- β (von Boehmer 2005).

Recently, it has been shown that naïve peripheral CD4⁺ T cells can also differentiate to CD4⁺CD25⁺ Tregs. These adaptive CD4⁺CD25⁺ Tregs have properties similar to those of their natural counterparts (Apostolou and von Boehmer 2004; Kretschmer et al. 2005). In

addition to CD4⁺CD25⁺ Tregs, there are other types of adaptive regulatory T cells that are Foxp3⁻ and CD25⁻negative (Bluestone and Abbas 2003). These include Tr1 and Th3 cells, that secrete large amounts of IL-10 and TGF- β , respectively (Roncarolo et al. 2001; Weiner 2001). Adaptive Tregs can be induced both *in vitro* and *in vivo* under particular conditions of repeated sub-optimal antigen exposure and/or costimulation (Bluestone and Abbas 2003; Apostolou and von Boehmer 2004; O'Garra et al. 2004; Kretschmer et al. 2005). Natural CD4⁺CD25⁺ and adaptive Tregs appear to have separate roles in the maintenance of peripheral tolerance: the first population has a central homeostatic function, suppressing autoreactive T cells, whereas the latter prevents pathological overactivation of the immune system leading to self-damage (Bluestone and Abbas 2003; Chatila 2005).

2.2.7 T helper cell differentiation

Naïve CD4⁺ T cells can differentiate into Th1, Th2 or Th17 cells, which differ in cytokine production and function (see chapter 2.2.1). The fate of the naïve CD4⁺ T cell is decided during the clonal expansion phase that follows activation through the TCR (Murphy and Reiner 2002). Many factors influence the differentiation of naïve cells into Th1 or Th2 lineages but it appears that the cytokines IFN- γ , IL-12 and IL-4 are key determinants of the outcome (Abbas et al. 1996). IFN- γ induces the expression of the transcription factor T-bet through the actions of signal transducer and activator of transcription 1 (STAT1) in naïve CD4⁺ T cells (Szabo et al. 2000). Analogously, IL-4 induces the expression of GATA-3 through STAT6 in naïve CD4⁺ T cells (Zheng and Flavell 1997). The transcription factors T-bet and GATA-3 mediate chromatin remodeling events at the IFN- γ and IL-4 genes, respectively, leading to the commitment of the naïve CD4⁺ T cells to the Th1 or Th2 lineages (Lee et al. 2001; Mullen et al. 2001). In addition, IL-12 is essential for Th1-differentiation, as it amplifies the production of IFN- γ by activated T cells and NK cells through the action of STAT4. The cytokines IL-6, TGF- β and IL-23 appear to be important for the development of Th17 cells (Weaver et al. 2006).

Cytokine signals from the innate immune system are crucial for Th1-development (Murphy and Reiner 2002). IL-12 is produced mainly by pathogen-activated macrophages and DCs (Hsieh et al. 1993). Additionally, pathogen-activated NK cells produce IFN- γ , which increases the expression of T-bet and also induces the up-regulation of the IL-12 receptor by the T cells (Afkarian et al. 2002), further enhancing Th1 commitment. For Th2 development the requirement for innate immune signals appears to be less stringent. In naïve cells, the

basal expression of GATA-3 is low, but might be sufficient for low-level production of IL-4 upon TCR activation (Ouyang et al. 2000). The absence of IFN- γ and IL-12 potentially allows Th2 development to be driven by a positive feedback loop through IL-4 and GATA-3 as a default pathway (Murphy and Reiner 2002).

Th1 and Th2 cells also cross-regulate each other's development by the action of their specific cytokines. The IFN- γ and IL-12 signaling pathways acting through T-bet have been implicated in the suppression of GATA-3 expression and in repressing the production of Th2 cytokines (Ouyang et al. 2000; Szabo et al. 2000). On the other hand, IL-4-induced expression of GATA-3 has been shown to inhibit the production of IFN- γ (Ouyang et al. 1998). It seems that the transcription factors T-bet and GATA-3 are able to silence the opposing Th lineage by directly regulating each other's expression (Szabo et al. 2003). Th17 development appears to be inhibited by both Th1 and Th2 cytokines, IFN- γ and IL-4 (Weaver et al. 2006).

In addition to cytokine signals, the strength of the TCR-MHC-peptide interaction and the presence of costimulatory signals have an impact on the lineage commitment of naïve T cells into Th1 and Th2 phenotypes. Several studies have shown that Th2 development is favored when antigenic peptides are presented at low concentrations (Constant et al. 1995; Hosken et al. 1995; Rogers and Croft 1999). In addition, studies with altered peptide ligands (APLs, see chapter 2.3.4) have demonstrated that low affinity binding of the TCR to the MHC-peptide complex also favors Th2 development (Pfeiffer et al. 1995; Tao et al. 1997; Rogers and Croft 1999; Brogdon et al. 2002). These studies collectively suggest that weak signaling through the TCR promotes Th2 development, whereas strong signaling promotes Th1 development. Interestingly, the TCRs of Th1 and Th2 cells appear to be structurally different, with longer CDR3 α loops on Th2 cells than on Th1 cells (Boyton et al. 2002), further suggesting the importance of the TCR-MHC-peptide interaction in lineage commitment. The CD28/B7 costimulatory pathway also plays a major role in the regulation of Th1/Th2 differentiation (Bour-Jordan and Bluestone 2002). Several studies have demonstrated that CD28 costimulation is a critical requirement for Th2 development but not for Th1 development (King et al. 1995; Webb and Feldmann 1995; Tao et al. 1997; Schweitzer and Sharpe 1998).

2.3 Allergen immunotherapy

2.3.1 General

The current treatment of allergic diseases consists of allergen avoidance, patient education, antiallergic medication and allergen immunotherapy (Kay 2001). Unfortunately, allergen avoidance is often difficult to achieve and all the current drugs used to treat allergic diseases (antihistamines, anticholinergic agents and corticosteroids) tend to control the symptoms rather than cure the disease.

Today, allergen immunotherapy is the only routinely administered antigen-specific treatment for any immunologic disease (Till et al. 2004). In allergen immunotherapy (specific immunotherapy, SIT), increasing quantities of an allergen extract are gradually administered subcutaneously to an allergic subject to ameliorate the symptoms associated with the exposure to the allergen (Bousquet et al. 1998). The approach was first described by Leonard Noon in 1911 in a short report on immunization treatment of hay fever with a distilled water extract of the pollen of timothy grass (Noon 1911). Allergen immunotherapy has been shown to be effective for the treatment of insect venom anaphylaxis and rhinoconjunctivitis caused by inhalant allergens, especially seasonal pollen allergens (Bousquet et al. 1998; Norman 1998; Norman 2004). It appears to confer long-term benefit for several years after discontinuation (Golden et al. 1996; Durham et al. 1999). The effectiveness of allergen immunotherapy in the treatment of asthma, however, is controversial (Adkinson 2001; Bousquet 2001). In children, allergen immunotherapy has been shown to prevent the onset of new sensitizations (Pajno et al. 2001) and to reduce the progression of rhinoconjunctivitis to physician-diagnosed asthma (Moller et al. 2002). Recently, several studies have confirmed that sublingual immunotherapy (SLIT) may be a safe and effective alternative to the traditional subcutaneous immunotherapy (Bousquet and Demoly 2006).

At present, allergen immunotherapy is associated with several problems. Firstly, allergen immunotherapy takes a long time: weekly to monthly allergen injections for 3 to 5 years are required for a long-lasting clinical effect (Bousquet et al. 1998). Secondly, the allergen extracts are usually crude preparations containing several allergenic components, which can lead to the development of new IgE sensitizations (Moverare et al. 2002). Thirdly and importantly, allergen immunotherapy can induce adverse systemic reactions, such as asthma attacks and anaphylaxis, resulting from IgE cross-linking by the allergen (Bousquet et al. 1998).

Recombinant allergen technology offers a way to diminish the problems associated with crude allergen extracts and allows the creation of hypoallergenic allergen variants that show reduced IgE binding while still containing the relevant T cell epitopes (Valenta 2002). An interesting alternative of this approach is to use synthetic peptides containing only the T cell epitopes of allergens (see chapter 2.3.3). Further strategies for immunotherapy include the use of novel Th1-deviating adjuvants, such as immunostimulatory DNA sequences containing CpG motifs (Tighe et al. 2000; Marshall et al. 2001), or the use of a humanized monoclonal anti-IgE antibody (omalizumab) in combination with conventional allergen immunotherapy (Casale et al. 2006).

2.3.2 Mechanisms of immunotherapy

Allergen immunotherapy inhibits both the early-phase and late-phase reactions to allergen exposure (Till et al. 2004). Early studies of patients treated with allergen immunotherapy reported a reduction of T cell proliferative responses and a shift away from Th2 to Th1 response *in vitro* (Secrist et al. 1993; Jutel et al. 1995; Bellinghausen et al. 1997; Ebner et al. 1997). The induction of T cell anergy, AICD or immune deviation were considered to be the mechanisms behind the observed phenomena (Rolland and O'Hehir 1998). Subsequently, several studies have demonstrated that the decrease in the allergen-specific Th2 response is accompanied by the induction of T cells producing the immunosuppressive cytokine IL-10 sometimes together with TGF- β (Bellinghausen et al. 1997; Akdis et al. 1998; Jutel et al. 2003). The IL-10 producing allergen-specific T cells appear to be CD4+CD25+ (Francis et al. 2003; Jutel et al. 2003), suggesting a role for CD4+CD25+ Treg cells in the induction of tolerance in allergen immunotherapy (Chatila 2005). Interestingly, increased levels of both IFN- γ and IL-10 have also been reported in skin or nasal mucosa biopsy specimens from patients undergoing allergen immunotherapy (Varney et al. 1993; Nasser et al. 2001; Wachholz et al. 2002; Nouri-Aria et al. 2004).

In allergen immunotherapy, serum IgE antibody concentrations do not seem to decrease, but the levels of allergen-specific IgA, IgG1 and in particular IgG4 increase considerably (Gehlhar et al. 1999; Jutel et al. 2003). It has been proposed that allergen-specific IgG4 act as blocking antibodies by competing with IgE for allergen binding, and in this way inhibit the IgE-dependent activation of mast cells and basophils (Till et al. 2004). IgG4 may also reduce IgE-facilitated allergen presentation by preventing the binding of allergen-IgE complexes to APCs (van Neerven et al. 1999; Wachholz et al. 2003). Intriguingly, IL-10 has been shown to

modulate the IgE-production of B cells in favor of IgG4 (Jeannin et al. 1998), further supporting the central role of this immunosuppressive cytokine in allergen immunotherapy.

2.3.3 Peptide immunotherapy

Short synthetic peptides corresponding to the T cell epitopes of allergens generally lack the capacity to bind allergen-specific IgE while retaining T cell reactivity. Therefore, they have been envisaged to be a safer alternative for conventional SIT. Early murine studies demonstrated that T cell tolerance to the whole allergen molecule could be established by administering peptides containing its T cell epitopes (Briner et al. 1993; Hoyne et al. 1993). As a first approach in humans, cat-allergic patients were treated subcutaneously with two long peptides (27 amino acids each) containing multiple T cell epitopes of the dominant cat allergen Fel d 1 (Norman et al. 1996; Simons et al. 1996; Pene et al. 1998; Maguire et al. 1999). In some of these studies, treatment with high peptide concentrations resulted in a modest improvement in pulmonary function and symptom scores, but this was associated with a high frequency of both immediate and late allergic reactions. Early adverse reactions may have resulted from cross-linking of IgE by the peptides, as the generation of peptide-specific IgE was frequently observed (Norman et al. 1996; Maguire et al. 1999). Moreover, in a recent study, 4 of the 9 subjects treated with long (> 40 amino acids) peptides from the bee venom allergen phospholipase A₂ (PLA₂) became skin test positive to the peptides and two of them developed mild generalized reactions a few hours following peptide injection (Fellrath et al. 2003). The late allergic reactions observed in the early Fel d 1 peptide studies were probably T cell-dependent isolated late asthmatic reactions (LARs), as described by Haselden et al. (Haselden et al. 1999).

More recently, several studies have demonstrated the clinical efficacy of using mixtures of shorter T cell epitope-containing peptides of both Fel d 1 and PLA₂, with reduced cutaneous reactions to allergen injection (Muller et al. 1998; Oldfield et al. 2001; Oldfield et al. 2002; Alexander et al. 2005a; Tarzi et al. 2006), reduced airway hyperreactivity (Alexander et al. 2005a) and improvements in symptom scores after nasal allergen challenge (Alexander et al. 2005b). None of these studies reported early adverse reactions. In contrast, LARs were observed frequently in some of the studies (Oldfield et al. 2001; Oldfield et al. 2002; Alexander et al. 2005b). After subsequent peptide administrations, the LARs were markedly attenuated, demonstrating that in peptide immunotherapy transient T cell activation is followed by the induction of tolerance.

Peptide immunotherapy appears to reduce allergen-specific proliferation of PBMCs together with reductions in both Th2 (IL-4 and IL-13) and Th1 (IFN- γ) cytokine production, and increases in the production of the immunosuppressive cytokine IL-10 (Oldfield et al. 2001; Oldfield et al. 2002; Tarzi et al. 2006). One study reported the induction of IFN- γ production together with IL-10 (Fellrath et al. 2003). As in whole allergen immunotherapy, IL-10 appears to be essential for the induction of tolerance in peptide immunotherapy. Elevated IL-10 levels have also been reported in peptide immunotherapy trials of autoimmune diseases, such as type 1 diabetes (Raz et al. 2001) and rheumatoid arthritis (Prakken et al. 2004). In contrast to SIT, peptide immunotherapy appears not to be associated with the induction of CD4+CD25+ regulatory T cells, as CD4+CD25+ T cells obtained before and after treatment did not differ in their ability to suppress allergen-specific T cell responses (Smith TR et al. 2004). Interestingly, in another study CD4+ T cells isolated after peptide immunotherapy were shown to be suppressive in coculture experiments, but no induction of CD25 was observed (Verhoef et al. 2005). Finally, in a recent study, the expression of Foxp3 in PBMCs was not affected by peptide immunotherapy (Tarzi et al. 2006). Collectively, these studies suggest that adaptive regulatory T cells other than CD4+CD25+ Tregs mediate the induction of tolerance in peptide immunotherapy. It has been speculated that in peptide immunotherapy T cells are activated in the absence of inflammatory signals, possibly via presentation by immature DCs, resulting in the generation of anergic, IL-10 secreting regulatory T cells (Larche and Wraith 2005).

2.3.4 Altered peptide ligands

Altered peptide ligands (APLs), or peptide analogs, are peptides containing amino acid substitutions as compared with a natural antigenic peptide (Sloan-Lancaster and Allen 1996). Alterations in the TCR contact residues of a peptide ligand lead to changes in the stability of the TCR-MHC complex and consequently to an altered activation of the responding T cell. Based on the functional outcome, peptide ligands can be classified as agonists, partial agonists, antagonists and null ligands (Bielekova and Martin 2001). In principle, agonists are able to induce all the functions of a given T cell (for example, proliferation, cytokine production, upregulation of surface receptors/ligands) while partial agonists can only induce some of the functions. Antagonists usually do not induce any of the functions, but are engaged by the TCR and can inhibit the activation of the T cell by an agonist if both ligands are present at the same time. Engagement of null ligands has no effect on the T cell because

their binding affinity is below the threshold of TCR signaling. APLs capable of activating a T cell at lower peptide concentrations than the natural peptide ligand are called superagonists or heteroclitic ligands.

The potential of APL-based immunotherapy has been demonstrated in animal models of multiple sclerosis (MS) (Karin et al. 1994; Nicholson et al. 1995; Brocke et al. 1996; Gaur et al. 1997; Santambrogio et al. 1998), type 1 diabetes (Alleva et al. 2002; Hartemann-Heurtier et al. 2004) and allergic asthma (Janssen et al. 2000). These studies have demonstrated that APLs may block T cell responses by acting as TCR antagonists or partial agonists, or by inducing AICD in the responding T cells. Some of these studies have also reported the induction of novel APL-specific T cell populations with immunoregulatory properties that were able to cross-recognize the native antigen, and upon its encounter modulate the response of neighboring pathogenic T cells (Nicholson et al. 1995; Santambrogio et al. 1998; Alleva et al. 2002). This regulatory mechanism is referred to as bystander suppression (Bielekova and Martin 2001).

Based on the promising findings in animal models, two phase II clinical trials were initiated to evaluate the effect of APL immunotherapy in human autoimmune disease (Bielekova et al. 2000; Kappos et al. 2000). In these trials an APL of the immunodominant epitope of the autoantigen myelin basic protein (MBP) was administered to MS patients. The response to treatment was heterogeneous with no clear improvement in clinical parameters, and both studies were suspended as a result of adverse effects. In one study, a proportion of the treated patients developed hypersensitivity reactions associated with Th2-skewing of the T cells during the treatment (Kappos et al. 2000). In the other study, a substantial expansion of T cells specific for the APL and cross-reactive with the native auto-antigen was observed in every patient (Bielekova et al. 2000). Unfortunately, a majority of these T cells were Th1-skewed, which presumably led to disease exacerbations in two of the eight patients in the study. In both studies, subjects receiving lower doses of APL seemed to tolerate the treatment better, and in the larger study the volume and number of enhancing lesions in the brain were reduced in patients receiving the lowest APL dosage (5mg weekly) (Kappos et al. 2000). Recently, the results of a phase I trial of APL immunotherapy for type 1 diabetes strongly suggested that APL treatment induced a Th1 to Th2 shift in the treated patients (Alleva et al. 2006). A phase II study evaluating the clinical significance of the finding is currently in progress.

3. AIMS OF THE STUDY

The aims of the study were

- To analyze the response of Bos d 2-specific human T cell clones to single amino acid analogs of the peptide p127-142 containing the immunodominant epitope of the allergen, and to identify cross-reacting peptides from endogenous proteins (I)
- To characterize the immunomodulatory potential of heteroclitic analogs of the peptide p127-142 on Bos d 2-specific T cell clones (II)
- To analyze the T cell response of DR4-positive subjects to peptide p127-142 and to one of its heteroclitic analogs (pN135D), and to evaluate the potential of the analog for peptide immunotherapy (III)
- To investigate the significance of particular TCR V β subtypes and HLA class II genotypes in the T cell response against Can f 1 (IV)

4. MATERIALS AND METHODS

4.1 Subjects (III, IV)

Clinically diagnosed cow- and dog-allergic subjects were recruited from the patients of the Pulmonary Clinic of Kuopio University Hospital (Virtanen et al. 1996; Saarelainen et al. 2004). Nonatopic dog owners were recruited as control subjects. Allergic subjects had positive skin prick tests (SPTs; ≥ 3 mm) to either commercial cow-allergen (III) or dog-allergen preparations (IV) (ALK-Abellø, Hørsholm, Denmark). All the cow-allergic subjects and a proportion of the dog-allergic subjects also had positive SPTs to recombinant (r) Bos d 2 and Can f 1, respectively. SPTs of nonallergic control subjects were negative. Heparinized peripheral blood samples were collected from the patients and peripheral blood mononuclear cells (PBMCs) were separated by Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. The subjects were HLA-genotyped for DRB1, DQB1 and DPB1 loci using the standard Inno-LIPA reversed dot blot assays (Innogenetics, Ghent, Belgium). The study was approved by the Ethics Committee of Kuopio University Hospital, and written informed consent was given by all the subjects.

4.2 Antigens

4.2.1 Recombinant allergens (IV)

rBos d 2 and rCan f 1 were produced in *Pichia Pastoris* as previously described (Rautiainen et al. 1998; Saarelainen et al. 2004).

4.2.2 Synthetic peptides (I-III)

The Bos d 2 peptide (p)127-142 (ELEKYQQQLNSERGVNP) containing the immunodominant epitope of the allergen, its 16-mer single amino acid analogs and truncated derivatives were synthesized using a PerSeptive 9050 Plus automated peptide synthesizer (Millipore, MA, USA) with the Fmoc strategy. The peptides were purified by HPLC and verified by mass spectrometry.

4.3 Cells

4.3.1 Antigen-presenting cells (I-III)

Autologous EBV-transformed B cell lines were produced from cow-asthmatic patients by infecting PBMCs with the supernatant from the EBV producer line B57-8. The EBV-transformed homozygous cell lines PITOUT (DRB1*0701, DRB4*0101), VEU (DRB1*0403) and SCHU (DRB1*1501, DRB5*0101) were from the European Collection for Biomedical Research, Essen, Germany, and BOLETH (DRB1*0401, DRB4*0103) was kindly provided by Dr. J. Choppin (Hôpital Cochin, Paris, France). The T2.DR4 cell line expressing the DRB1*0401 allele (Turvy and Blum 1998) was kindly provided by Dr. H. Zarour (Pittsburgh, USA).

Mature dendritic cells were prepared according to the procedure of Ponsaerts et al. (Ponsaerts et al. 2002). Briefly, PBMCs were incubated for 2 h in serum-free AIM-V medium (Life Technologies, Paisley, UK) in six-well plates (Corning Inc., Corning, NY, USA). Non-adherent cells were gently removed by repeated washing and the adherent cells were cultured for 2 days in AIM-V supplemented with 100 ng/ml GM-CSF (Strathmann Biotech, Hannover, Germany). PolyI:C (Sigma, St. Louis, MO, USA) was added at a concentration of 25 µg/ml 24 h after starting the culture. The typical purity of the DC culture was 60-70% according to staining with FITC-labeled anti-CD14, anti-CD86 and anti-CD83 mAbs (BD Biosciences, San Jose, CA, USA).

4.3.2 T cell clones (I, II)

Bos d 2-specific T cell clones F1-9, T144, T215, K3-2 and K9-10 from three cow-asthmatic farmers with positive SPTs to Bos d 2 were isolated by the limiting dilution method, as reported previously (Zeiler et al. 1999). The T cell clones were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 20 µM 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (BioWhittaker, Verviers, Belgium), 5% inactivated human AB serum (Finnish Red Cross, Helsinki, Finland) and recombinant interleukin-2 (rIL-2; 25 IU/ml; Strathmann Biotech). The clones were restimulated every two to three weeks with γ -irradiated (6000 rad) allogeneic PBMCs and 1 µg/ml phytohemagglutinin (PHA; Murex Biotech, Dartford, UK).

The clonality of the T cell clones was determined by identifying the TCR genes expressed. In brief, total RNA was isolated from the clones by the RNAgents® total RNA isolation

system (Promega, Madison, WI, USA) following the manufacturer's instructions. The first-strand cDNA was synthesized using TCR C α or TCR C β -specific primers following amplification with a set of primers specific for the TCR V α and TCR V β sequences as described previously (Genevee et al. 1992). The amplification products were fractionated on a 2.0% agarose gel. The amplified products were purified from agarose gel using an Ultrafree-DA Centrifugal Filter device (Millipore, Bedford, MA, USA) and sequenced using a Thermo Sequenace CY5 Dye Terminator Kit and an A.L.F. Express DNA Sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). The nomenclature used for TCR V α and TCR V β sequences was according to Arden et al. (Arden et al. 1995) and for TCR J α and J β sequences according to Rowen et al. (Rowen et al. 1996).

4.3.3 Generation of T cell lines (III, IV)

For the generation of long-term peptide-specific CD4⁺ T cell lines (III), autologous CD4⁺ T cells were positively selected from PBMCs with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cells was routinely > 98%. Peptide-pulsed DCs (1×10^4) and 5×10^4 CD4⁺ T cells were seeded in 96-well U-bottomed plates (30 wells per peptide) in complete RPMI 1640. After four days, rIL-2 (10 IU/ml) and rIL-7 (Strathmann; 5 ng/ml) were added to the cultures. The cultures were restimulated with fresh peptide-pulsed DCs every 10 to 11 days. After the second and third stimulation, 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 (1×10^4 per well) pulsed for 4 h with or without the peptides (10 μ M). After 3 days, the antigen-specific proliferation was determined as described in 4.4.1. The stimulation index (SI; cpm in the presence of a peptide divided by cpm in the absence of a peptide) was determined. Positive cultures (SI > 2 and a Δ cpm of at least 5,000) were transferred to a 24-well plate and expanded with 2×10^6 autologous PBMCs, 10 μ M of the peptide, 10 IU/ml of rIL-2 and 5 ng/ml of rIL-7.

For the generation of Can f 1-specific T cell lines (IV), PBMCs were stimulated with rCan f 1 at a concentration of 100 μ g/ml in 12-well plates (Corning) at a density of 6×10^6 cells per well in complete RPMI 1640. PHA at a concentration of 100 ng/ml was used to establish control T cell lines. On day 6, rIL-2 was added to the cultures at a final concentration of 5 IU/ml. On day 9, the rIL-2 concentration was raised to 20 IU/ml. On day 14, the cells were harvested, washed and counted. Then the cells were restimulated on 12-well plates at a density of 3×10^6 cells per well, together with 6×10^6 γ -irradiated (3000 rad) autologous

PBMCs as feeder cells and with rCan f 1 or PHA as previously. The restimulation was completed as described above.

4.4 *In vitro* T cell analyses

4.4.1 Lymphocyte proliferation assay (I-IV)

The antigen-specific proliferation was measured by incubating PBMCs (2×10^5), or T cells (2.5×10^4 to 5×10^4) together with γ -irradiated (3000 to 6000 rad) APCs (1×10^4 to 1×10^5), in 200 μ l volumes in round-bottomed 96-well plates with or without antigen. Three to five replicate wells were used. After an incubation of three (T cell lines and clones) to five (PBMCs) days, [3 H]thymidine was added (1 μ Ci per well; Amersham Pharmacia Biotech, Little Chalfont, UK), and after an additional 16 h, the cells were harvested on to glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac MicroBeta 1450), and the results were expressed as counts per minute (cpm).

4.4.2 Measurement of cytokine production (I-III)

For cytokine analyses, 5×10^5 T cells and 5×10^5 γ -irradiated APCs were incubated at different concentrations of peptides in a total volume of 0.5 to 1 ml (I, II). After 24 hours, culture supernatants were collected and stored at -70°C until analyzed. Alternatively, supernatants from the 96-well microcultures (see 4.4.1) were collected after 72h and samples from replicate wells were pooled (III). The concentrations of IFN- γ , IL-4 and IL-10 were measured in duplicate using commercial ELISA kits (DuoSet, R&D Systems, Minneapolis, MN, USA) with AMDEX amplification (Amersham Pharmacia Biotech), according to the manufacturers' instructions. For the measurement of IL-5 levels in the supernatants, the LyH7.B13 cell line was used as described previously (Zeiler et al. 1999).

4.4.3 Flow cytometry (I-IV)

For analyzing the TCR and CD25 expression by T cell clones (II), the cells were stained with FITC-labeled anti-TCR $\alpha\beta$, PE-labeled anti-CD25 and PerCP-labeled anti-CD4 (BD Biosciences) for 20 minutes at $+4^\circ\text{C}$, washed twice with PBS and analyzed with a FACScan flow cytometer (BD, Mountain View, CA, USA).

The number of live T cells in a sample (II) was analyzed by staining the cells with PE-labeled anti-CD4 and FITC-labeled anti-TCR $\alpha\beta$ for 20 minutes at +4°C. After washing twice, the cells were incubated for an additional 10 minutes at room temperature with the vital dye 7-amino-actinomycin D (7-AAD; BD Biosciences). After this period, the cell samples were suspended in a constant volume of 200 μ l of PBS and events were counted for 60 seconds with the flow cytometer to allow comparative analysis between samples. The number of CD4-positive, 7-AAD-negative cells was calculated, and the TCR expression was analyzed in this population.

TCR V β subtypes expressed on CD4⁺ T cells (I, III, IV) were assessed using the IOTest Beta Mark kit (Immunotech, Marseille, France). The kit contains PE, FITC or PE-FITC double-labeled antibodies against the following V β subtypes: V β 1, V β 2, V β 3, V β 4, V β 5.1, V β 5.2, V β 5.3, V β 7.1, V β 7.2, V β 8, V β 9, V β 10, V β 11, V β 12, V β 13.1, V β 13.2, V β 14, V β 16, V β 17, V β 18, V β 20, V β 21.3, V β 22 and V β 23. For the gating of CD4⁺ cells, anti-CD4-PE-Cy5 (Immunotech) was used. Cells were incubated with the anti-V β and anti-CD4 antibodies for 30 min at room temperature in the dark. After washing twice with PBS, the cells were analyzed with the flow cytometer. V β distribution was calculated and expressed as a percentage of CD4⁺ cells. The expansions of TCR V β subtypes in the T cell lines were regarded as significant when the level of expression was more than 5% (IV) or 10% (III) and at least twice greater than the expression in PBMCs.

4.4.4 TCR RT-PCR/SSCP analysis (III)

Total RNA was isolated from the T cell lines and the first strand cDNA was synthesized as described in 4.3.2. The amplification product was subjected to single-strand conformational polymorphism (SSCP) analysis (Yoshida et al. 2002). Briefly, the sample was diluted in a denaturing solution containing 95% formamide and 20 mM EDTA and boiled for 5 minutes. The sample was loaded onto 6% polyacrylamide TBE gel containing 10% of glycerol. The gel was run at 8 Vcm⁻¹ constant voltage for 2.5 hours in 0.5 x TBE buffer at room temperature. After electrophoresis, the gel was silver-stained and the individual DNA bands of interest were reamplified with V β 13 and C β primers using the "bandstab" technique (Wilton et al. 1997). The V β 13-C β fragments were cloned into pCR2.1-TOPO vectors and the constructs were transformed into *E.coli* using the TOPO TA Cloning kit (Invitrogen, CA, USA). The nucleotide sequences of the V β 13-C β clones were confirmed by DNA sequencing. In some cases, identical sequences were obtained from two independent SSCP-derived clones of a specific T cell line.

4.5 MHC class II tetramer staining of T cells (II, III)

The generation of soluble DRA1*0101/DRB1*0401 molecules and the procedure used for peptide loading have been described earlier (Novak et al. 1999). The peptides described in 4.2.2 were used to load DRB1*0401 molecules to generate DRB1*0401/peptide tetramers. The prostate-specific antigen (PSA) peptide 64-78 (QVFQVSHSFPHPHYD) or the hemagglutinin (HA) 306-318 peptide (PRYVKQNTLKLAT) were used to generate DRB1*0401 control tetramers for staining. Streptavidin-PE (BioSource International, Camarillo, CA, USA) was used for cross-linking the MHC molecules. All the tetramers were filtered through a Sephadex G-50 size exclusion column before use. The staining was performed by incubating T cells with different concentrations of the PE-labeled tetramers in 50 μ l of culture medium for 2h at 37°C. Anti-CD4-FITC or anti-CD4-PE-Cy5 was added and the cells were incubated for an additional 20 minutes at +4°C. The cells were washed twice and analyzed with the flow cytometer. Staining with FITC-labeled anti-TCR $\alpha\beta$ (BD Biosciences) was performed separately and the dose-response curve of median fluorescence intensity (MFI) of tetramer staining was normalized to that of the TCR staining (II), as described previously (Holzer et al. 2003).

For the CFSE/tetramer analysis (III), purified CD4⁺ T cells were stained with 1 μ M of 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, Netherlands) in PBS for 10 min at 37°C. The staining was stopped by adding 100% human AB serum and subsequent washing. T cells (1×10^6) were stimulated with 2×10^5 autologous peptide-pulsed DCs in 48-well plates (Corning) in 1 ml of complete RPMI 1640 supplemented with 5 ng/ml of rIL-7. On day 8, fresh medium supplemented with 10 IU/ml of rIL-2 and 5 ng/ml of rIL-7 was added to the cultures. The T cells were stained with tetramers and anti-CD4-PE-Cy5 and analyzed by flow cytometry on days 8 and 15.

4.6 Measurement of peptide binding to HLA-DRB1*0401 (I)

HLA-DRB1*0401 molecule was purified from the EBV-transformed homozygous cell line BOLETH by affinity chromatography using mAb L243 (Texier et al. 2000). Competitive binding assays were performed as previously described (Texier et al. 2000). Briefly, an appropriate dilution of HLA-DRB1*0401 molecule was incubated with a biotinylated HA 306-318 peptide (30 nM) and serial dilutions of competitor peptides. After a 24 h incubation,

complexes were incubated on plates coated with 10 µg/ml L243 mAb and revealed using streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech) and 4-methylumbelliferyl phosphate substrate (Sigma, France). Results were expressed as the peptide concentration that prevented the binding of 50% of the labeled peptide (IC₅₀). They were based on at least two independent experiments.

4.7 Data analysis

4.7.1 Database search (I)

The sequences of the homologous peptides SP1-SP9 were obtained from the SWISS-PROT protein sequence database using the program FindPatterns with a search motif (I, Table III). The sequences for the peptides SP10-SP20 corresponding to the p127-142 of Bos d 2 were obtained by the sequence homology alignments of lipocalin protein sequences from the SWISS-PROT protein sequence database using the program BestFit. Both programs are a part of the Wisconsin Package of the Genetics Computer Group, Inc.

4.7.2 Statistical analyses (III, IV)

Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, CA, USA). The Mann-Whitney U test was used to analyze the differences between the specific proliferative and cytokine responses of p127-142- and pN135D-induced long-term T cell lines (data in III, Fig. 2). The χ^2 test for trend was used to analyze the differences between the T cell lines in the distribution of cytokine phenotypes and the distribution of avidities for the homologous peptide (data in III, Tables III and IV). The Kruskal-Wallis test, and post hoc comparisons with Dunn's test (data in IV, Figs. 1 and 2) or the Mann-Whitney U-test (data in IV, Fig. 3) were used to analyze the differences in proliferative responses or expression of TCR V β subtypes in PBMCs and Can f 1-induced T cell lines. Fisher's exact test was used to analyze the association between the occurrence of DR4-DQ8 haplotype and the expansion of V β 5.1+ T cells in the Can f 1-specific T cell lines (data in IV, Table I). P values of 0.05 or less were considered significant.

5. RESULTS

5.1 *Bos d 2*-specific T cell clones (I, II)

5.1.1 Clonality and MHC restriction (I, II)

The T cell clones F1-9, T144, K3-2 and K9-10 recognizing the immunodominant epitope (p127-142, ELEKYQQLNSERGPVN) of *Bos d 2* were previously isolated from three cow-asthmatic subjects (Zeiler et al. 1999). The concentration of p127-142 inducing the half-maximal response (EC_{50}) was between 1 μ g/ml and 10 μ g/ml (0.5 to 5 μ M) for all the clones (I, Fig. 1). The clonality of the T cell clones was demonstrated by flow cytometric analysis with a panel of 24 TCR V β -specific mAb. All the clones stained uniformly with a single V β -specific mAb. The finding was confirmed by PCR using a panel of TCR-subfamily-specific primers. The TCR usage of the clones was V α 2/V β 7.2 for F1-9, V α 14.1/V β 13.1 for T144, V β 13.1 for K3-2 and V β 14.1 for K9-10. The V α usage of the clones K3-2 and K9-10 could not be identified with the primer panel used.

The MHC restriction of the T cell clones was determined using a panel of EBV-transformed B-cell lines expressing different MHC-alleles as APCs. The clones F1-9, K3-2 and K9-10 were shown to be restricted by DR4 (DRB1*0401) and clone T144 by DR2 (DRB1*1501 or DRB5*0101).

5.1.2 Recognition of peptide analogs by T cell clones (I, II)

The TCR recognition motif of the T cell clones F1-9 and K3-2 was assessed by alanine-substituted peptide analogs and truncated variants of p127-142. The critical residues for TCR recognition were mapped to be between the residues 131 and 138 of the epitope for F1-9 (I, Figs. 3A and 4A) and between the residues 127 and 138 for K3-2 (I, Figs. 3B and 4B). Additional peptide analogs with single amino acid substitutions were synthesized for the positions between Y131 to R138. The binding of the peptide analogs to DRB1*0401 was measured (I, Table II). A strong decrease was observed in binding with the analogs pY131A and pS136Y, indicating that Y131 and S136 are the MHC-binding amino acids in the positions P1 and P6, respectively (Sette et al. 1993).

Most of the substitutions resulted in an abrogated T cell response by the T cell clones (I, Fig. 5). For clone F1-9, one of the analogs, pN135D, however, was discovered to be a strong superagonist. This peptide, with a substitution of aspartic acid for asparagine at position 135, was able to stimulate the clone at a 100-fold lower peptide concentration than the natural ligand p127-142 (I, Fig. 6A and II, Fig. 1A). Similarly, two peptide analogs, pQ133K and pL134I, were observed to activate clone K3-2 at 10- to 30-fold lower concentrations than p127-142 (I, Fig. 6B and II, Fig. 1B). For the T cell clones T144 and K9-10 no heteroclitic peptide analogs were found within the panel of peptide analogs synthesized (data not shown). In MHC binding assays the heteroclitic peptide ligands were observed to bind to DRB1*0401 with similar affinities as p127-142 (I, Table II), indicating that the superagonist activity is attributable to increased TCR affinity of the T cell clones for the analogs.

5.1.3 Database search for cross-reacting peptide ligands (I).

A database search motif for stimulatory peptide ligands was formulated for clone F1-9 according to the tolerated/non-tolerated amino acid substitutions observed with the peptide analogs (I, Table III). Based on the search in the Swiss-Prot protein sequence database, nine peptides (SP1-9) were synthesized. Five of these peptides, four of human and one of bacterial origin, were able to induce the proliferation of clone F1-9, although more weakly than p127-142 (I, Table IV). One of the self peptides (SP4) was also able to stimulate clone K3-2. Eleven peptides corresponding to p127-142 in other lipocalin allergens and endogenous human lipocalin proteins (SP10-20) were also synthesized. None of these peptides was observed to stimulate the T cell clones.

5.1.4 MHC class II tetramer staining (II)

Fluorescent-labeled DRB1*0401-tetramers loaded with p127-142, pN135D or pQ133K were used to directly assess the TCR avidity of the T cell clones (Holzer et al. 2003) for the different peptide ligands. An example of staining with DRB1*0401/p127-142 tetramer is shown in II, Fig. 2A. To allow comparison between the clones, the binding of the tetramers was measured over a range of concentrations and the results were normalized to the TCR $\alpha\beta$ expression of the clones (II, Fig. 2B). Clones K3-2 and K9-10 showed approximately similar avidities for DRB1*0401/p127-142. Clone F1-9, however, failed to bind DRB1*0401/p127-142 at any of the concentrations used, suggesting a much weaker TCR avidity for the

tetramer. When the tetramers loaded with the heteroclitic analogs were used for staining, clone F1-9 was positive with DRB1*0401/pN135D while clone K3-2 bound the DRB1*0401/pQ133K tetramer at a higher avidity than DRB1*0401/p127-142. These findings confirm the increased TCR avidity of F1-9 and K3-2 for the heteroclitic analogs pN135D and pQ133K, respectively. None of the three DRB1*0401-restricted T cell clones was stained with the control tetramer DRB1*0401/PSA64-78. The DR2-restricted clone T144 was also tested, but it was not stained with the DRB1*0401-tetramers.

5.1.5 Immunomodulatory properties of peptide analogs (II)

All the T cell clones produced IFN- γ together with IL-4 and IL-5 upon stimulation with p127-142 (II, Fig. 3). According to their cytokine production, F1-9 and K9-10 can be regarded as Th0-like and K3-2 and T144 as Th2-like T cell clones. For all the clones, the production of IL-4 and IL-5 tended to reach a plateau at lower peptide concentrations than did the production of IFN- γ . This was reflected in the ratios of IFN- γ /IL-4 and IFN- γ /IL-5, which increased with increasing concentrations of p127-142 (II, Fig. 4). Moreover, when the clones F1-9 and K3-2 were stimulated with the heteroclitic analogs pN135D and pQ133K, the ratios were higher than those observed with equimolar concentrations of p127-142.

To evaluate the effect of p127-142 or the heteroclitic peptide analogs on the growth and function of the T cell clones F1-9 and K3-2, the TCR expression and the number of viable T cells were monitored for 10 days after stimulation with the peptides (II, Fig. 5). Stimulation of the clones with the heteroclitic ligands resulted in a stronger and longer-lasting TCR down-regulation than did the stimulation with p127-142. Upon stimulation with p127-142, both F1-9 and K3-2 expanded steadily after an initial decrease on day 1. In contrast, when clone F1-9 was stimulated with the heteroclitic analog pN135D the cells rapidly started to die. The extent of cell death on day 10 varied between 88% and 98% in independent experiments. The heteroclitic analog pQ133K was not able to induce the death of clone K3-2. The clone expanded throughout the culture period in numbers comparable with those observed with p127-142.

To analyze whether the long-lasting TCR down-regulation of K3-2 observed upon stimulation with pQ133K had an impact on the capacity of the clone to respond to stimulation with the natural peptide ligand, live K3-2 cells were collected 10 days after stimulation with either p127-142 or pQ133K by density gradient centrifugation and restimulated with p127-142 and fresh APCs (II, Fig. 6). The T cells initially stimulated with p127-142 proliferated as

vigorously as the unstimulated T cells. In contrast, the T cells initially stimulated with pQ133K showed a diminished proliferative response upon restimulation with p127-142 on day 10, indicating that a hyporesponsive state was induced upon stimulation with the heteroclitic peptide analog.

5.2 T cell response of DR4-positive subjects to peptide p127-142 containing the immunodominant epitope of Bos d 2 and to its heteroclitic analog pN135D (III)

5.2.1 Expansion of specific CD4⁺ T cells upon stimulation with p127-142 and pN135D

To evaluate the potential of peptide analogs for allergen immunotherapy, the *in vitro* CD4⁺ T cell responses of eight DR4-positive subjects, six cow-asthmatic patients (A-1 to A-6) and 2 nonatopic controls (NA-1 and NA-2), to p127-142 or the heteroclitic analog pN135D were analyzed. For the analysis, CD4⁺ T cells isolated by immunomagnetic sorting were stained with CFSE and stimulated with DCs pulsed with the peptides. The cells were stained with DRB1*0401 tetramers on days 8 and 15. On day 8, the percentage of divided tetramer-positive CD4⁺ T cells was very low. Distinct populations of DR0401/p127-142- and DR0401/pN135D-positive T cells could be observed in the peptide-induced cultures of the subject A-3 only (0.12% and 0.16%, respectively, III, Fig. 1A). However, after culturing the cells for an additional 7 days together with IL-2 and IL-7, DR0401/p127-142- and DR0401/pN135D-positive CD4⁺ T cells were observed in the p127-142- and pN135D-induced cultures from six and five subjects, respectively. A representative example is shown for subject A-3 in III, Fig. 1B. The percentage of DRB1*0401/p127-142-positive T cells ranged from 0.36 to 6.69% in the p127-142-induced cultures, and that of DRB1*0401/pN135D-positive T cells ranged from 0.22 to 5.11% in the pN135D-induced cultures (III, Table I).

Several of the tetramer-positive cultures exhibited cross-reactivity for the homologous peptide, as shown for subject A-3 (III, Fig. 1A and B). However, only two out of the six p127-142-induced cultures that were positively stained with DR0401/p127-142 were stained with DR0401/pN135D. In contrast, positive staining with DR0401/p127-142 was observed in four out of the five pN135D-induced T cell cultures that were positively stained with DR0401/pN135D (III, Table I).

5.2.2 Characteristics of long-term T cell lines induced with p127-142 and pN135D

As pN135D was observed to induce T cells cross-reactive with p127-142, the phenomenon was further analyzed by generating long-term CD4⁺ T cell lines (TCLs) from all subjects with p127-142 and pN135D. Eighteen DRB1*0401-restricted TCLs were induced with each of the peptides (III, Table II). Interestingly, at least one pN135D-specific TCL was obtained from all eight subjects, whereas p127-142-specific T cell lines were obtained from only five subjects. The proliferative responses of the p127-142- and pN135D-induced T cell lines were of the same magnitude (III, Fig. 2A). Moreover, the functional TCR avidity of the T cell lines, estimated by EC₅₀ values, was similar (median EC₅₀ of p127-142-induced TCLs: 1.0 μ M, pN135D-induced TCLs: 1.9 μ M, $p=0.38$, Mann-Whitney U test).

Most of the long-term TCLs were stained with the specific MHC tetramers: only one p127-142-induced and five pN135D-induced TCLs failed to stain with DRB1*0401/p127-142 and DRB1*0401/pN135D, respectively (less than 1% tetramer positive).

Interestingly, there was a difference in cytokine production by the T cell lines, with the pN135D-induced T cell lines producing significantly higher levels of IFN- γ than the p127-142-induced T cell lines (III, Fig. 2B, $p=0.02$). In contrast, there was no statistically significant difference in the production of IL-4 or IL-10 (III, Figs. 2C and D). Consequently, the p127-142-induced TCLs were predominantly of the Th2/Th0-phenotype, whereas the pN135D-induced TCLs were of the Th1/Th0-phenotype (III, Table III, $p=0.004$).

5.2.3 Cross-reactivity of the p127-142- and pN135D-induced T cell lines

Both the p127-142- and pN135D-induced T cell lines frequently showed reactivity to the homologous peptide. Nine of the eighteen p127-142-induced TCLs exhibited a cross-reactive response to pN135D. Importantly, 14 of the 18 pN135D-induced T cell lines recognized p127-142. At least one cross-reactive pN135D-induced TCL was obtained from every subject in the study. Both p127-142- and pN135D-induced T cell lines displayed a spectrum of functional avidities for the homologous peptide (III, Table IV). The different types of TCL cross-reactivity are demonstrated in III, Fig. 3. Although most of the cross-reactive T cell responses were of lower or similar avidity than the responses to the peptide used for inducing the TCLs, five pN135D-induced TCLs (from 4 different subjects) recognized p127-142 with a

higher avidity than pN135D (III, Table IV). In contrast, only one p127-142-induced TCL recognized pN135D with a higher avidity. In general, the avidity of the pN135D-induced TCLs for p127-142 tended to be higher than that of the p127-142-induced TCLs for pN135D (III, Table IV, $p=0.06$). Importantly, upon stimulation with the homologous peptide, the cytokine phenotype of the T cell lines remained unaltered, i.e. it was the same as upon stimulation with the peptide that was used for inducing the cell lines (data not shown).

5.2.4 TCR V β subtypes used by the p127-142- and pN135D-induced T cell lines

The expression of TCR V β subtypes in the p127-142- and pN135D-induced long-term T cell lines was analyzed by flow cytometry and compared with the expression in PBMCs of the same subjects (III, Fig. 4A). Interestingly, an expansion of V β 13.1+ T cells was observed in 14 of the 18 p127-142-induced TCLs but only in 4 of the 18 pN135D-induced TCLs. All the pN135D-induced TCLs with an expansion of V β 13.1+ T cells were cross-reactive with p127-142. Expansion of a specific V β subtype other than V β 13.1 was observed in only 1 to 3 of the p127-142- or pN135D-induced TCLs. The specificity of the expanded V β 13.1+ T cell populations for p127-142 was confirmed by staining the TCLs with both the V β 13.1-specific antibody and the DRB1*0401/p127-142 (a representative example is shown in III, Fig. 4B).

The T cell lines showing an expansion of peptide-specific V β 13.1+ T cells were subjected to SSCP analysis. In 16 of the 18 T cell lines, a monoclonal SSCP pattern (Yamamoto et al. 1992) was detected, while in 2 TCLs the SSCP pattern was oligoclonal. The SSCP bands were re-amplified directly from the gels, and their nucleotide sequences were determined after subcloning. The deduced TCR β -chain CDR3 amino acid sequences are summarized in III, Table V. Interestingly, a common amino acid motif RTGG, or minor variations of it, was observed in 11 TCLs from six different subjects. Moreover, a CDR3 sequence was shared by p127-142-induced TCLs #4 and #5 from subject A-5, whereas the TCLs #7 and #8 from the same subject shared a different sequence. This finding suggests that at least two different p127-142-specific T cell clones had expanded in the subject A-5 *in vivo*.

5.3 T cell response of dog-allergic and nonatopic subjects against Can f 1 (IV)

5.3.1 Proliferative responses of PBMCs and T cell lines to rCan f 1

Twenty-six dog-allergic subjects with positive SPTs to a commercial dog dander preparation participated in the study. Thirteen of these subjects had a positive SPT to rCan f 1. Twelve non-atopic dog-owners were recruited as control subjects. The proliferative responses of PBMCs were generally very low (IV, Fig. 1A). Only four subjects (three with positive SPTs to rCan f 1) exhibited a SI of > 2 in the proliferation assay. T cell lines were established from the PBMCs of all the subjects by two cycles of *in vitro* stimulation with rCan f 1. The proliferative response of most of the TCLs to rCan f 1 remained weak (IV, Fig. 1B). Although both the PBMCs and TCLs of dog-allergic subjects with positive SPTs to rCan f 1 exhibited a tendency for slightly stronger proliferative responses in comparison with those of subjects from other groups, the differences were not statistically significant. T cell lines were considered to be antigen-specific when the SI was greater than 1.5 and the difference of the proliferative response between the stimulated and unstimulated cultures was greater than 1000 cpm. According to these criteria, eight of the 13 TCLs from dog-allergic subjects with positive SPTs to rCan f 1 were specific for the allergen. Similarly, four of the 13 TCLs from dog-allergic subjects with negative SPTs to rCan f 1 and six of the 12 TCLs from nonatopic control subjects were antigen-specific. None of the T cell lines had a positive proliferative response to the irrelevant control allergen rBos d 2.

5.3.2 TCR V β subtype distribution of peripheral blood CD4 $^{+}$ T cells

The TCR V β distribution of peripheral blood CD4 $^{+}$ T cells was analyzed for the 38 subjects (IV, Fig. 2). Significantly higher frequencies of V β 21.3 $^{+}$ and V β 22 $^{+}$ T cells were observed in the peripheral blood samples of dog-allergic subjects with positive SPTs to rCan f 1 compared with non-atopic control subjects ($p < 0.05$ and $p < 0.01$, respectively). Dog-allergic subjects with negative SPTs to rCan f 1 also had a greater frequency of these V β subtypes in their peripheral blood than the non-atopic controls, although this did not reach statistical significance. Other V β subtypes did not show statistically significant differences between the three groups.

5.3.3 Expansion of TCR V β subtypes in Can f 1-specific T cell lines and its association with HLA class II genotypes

The expression of different TCR V β subtypes in the Can f 1-specific T cell lines was analyzed by flow cytometry and compared with the expression in PBMCs of the same subjects (IV, Table I). rCan f 1 was able to induce the expansion of 0-4 V β subtypes in each of the 18 Can f 1-specific TCLs studied. Out of the 24 V β subtypes examined, seventeen were significantly expanded in at least one of the TCLs. When the subjects in the study were arranged into two groups according to their SPT reaction to rCan f 1, an expansion of V β 5.1+ T cells was observed in the Can f 1-specific TCLs of five of the ten subjects with negative SPTs to rCan f 1, but in none of the TCLs of the eight subjects with positive SPTs to rCan f 1 and in none of the 18 PHA-induced control T cell lines (IV, Fig. 3). In contrast, three TCLs of the subjects with positive SPTs to rCan f 1 showed a strong expansion of V β 7+ T cells. This TCR V β subtype was not expanded in any of the T cell lines of the subjects with negative SPTs to rCan f 1 or in any of the PHA-induced control T cell lines.

The subjects with positive SPTs to rCan f 1 did not show accumulation of a specific HLA class II haplotype. The subjects with negative SPTs to rCan f 1 had, however, a higher frequency of the haplotype HLA-DRB1*04-DQB1*0302 (DR4-DQ8). This was found in six out of the ten subjects in this group, whereas only one of the eight subjects with positive SPTs to rCan f 1 had the same haplotype. Four of the subjects with the haplotype had the DRB1*0401 allele and three the DRB1*0404 allele. Interestingly, the occurrence of the DR4-DQ8 haplotype was associated with the expansion of V β 5.1+ T cells in the cell lines. Four of the five subjects with negative SPTs to rCan f 1 and a significant expansion of V β 5.1+ T cells had the DR4-DQ8 haplotype ($p < 0.05$).

6. DISCUSSION

6.1 *T cell recognition of lipocalin allergens*

This study demonstrated that human T cell clones recognize the immunodominant epitope of lipocalin allergen Bos d 2 in a suboptimal way (I). The EC₅₀ values of Bos d 2-specific T cell clones were between 0.5 and 5.0 μ M, resembling in this respect human MBP-specific T cell clones, which are generally of low avidity (Hemmer et al. 1997; Vergelli et al. 1997; Hemmer et al. 2000). In contrast to the self antigen, the EC₅₀ values for influenza virus hemagglutinin-specific T cell clones appear to be at the level of 0.1 μ M or below (Korb et al. 1999; Zhao et al. 2001). Importantly, it was observed that a single amino acid analog of the immunodominant epitope of Bos d 2, pN135D, could stimulate the T cell clone F1-9 at a 100-fold lower concentration than the natural peptide. Similarly, two analogs, pQ133K and pL134I were discovered to exhibit an increased stimulatory capacity for another T cell clone K3-2. The heteroclitic activity of these peptides is to be attributed to improved recognition of these peptides by the TCR, since they bound to MHC with equal affinities as compared with the natural peptide ligand. Staining with MHC class II tetramers confirmed the increased TCR avidity of the clones for the heteroclitic ligands (II).

Heteroclitic peptide ligands have been described for T cell clones specific for self antigens (Hemmer et al. 1997; Hemmer et al. 1998; Nicholson et al. 1998). In contrast, little improvement over the natural ligand has been observed with APLs with clones specific for true foreign antigens, such as determinants in influenza virus (Germain and Stefanova 1999). Accordingly, it has been suggested that for autoreactive T cell clones the natural ligand is often suboptimal, whereas for T cell clones specific for foreign antigens the natural ligand is much closer to optimal (Zhao et al. 2001).

In this study, it was observed that the proliferative responses of PBMCs of dog-allergic patients and nonatopic control subjects upon stimulation with the major dog allergen Can f 1 were weak (IV). Moreover, Can f 1-specific T cell lines could be established in only 62% of the Can f 1-allergic subjects and the proliferative capacity of these T cell lines upon stimulation with the allergen was mainly weak. Interestingly, the capacity of other lipocalin allergens, such as Bos d 2 (Zeiler et al. 1999), Rat n 1 (Jeal et al. 2004) and Equ c 1 (Immonen A et al., submitted), to induce the proliferation of PBMCs from allergic subjects has also been observed to be weak.

The poor stimulatory capacity of lipocalin allergens may be a characteristic associated with their allergenicity. It has been previously suggested that through central or peripheral tolerance mechanisms the presence of cross-reacting endogenous lipocalin proteins limits the T cell responses to exogenous lipocalin allergens (Virtanen et al. 1999). The presence of endogenous lipocalins during thymic maturation may lead to the elimination of T cells that could potentially react strongly against exogenous lipocalin allergens. Peripheral regulatory mechanisms, such as induction of anergy or AICD, or the presence of natural CD4+CD25+ Tregs, may additionally modulate the T-cell response against exogenous 'self-mimicking' lipocalin allergens. Collectively, central and peripheral tolerance mechanisms could lead to a T cell repertoire containing only low-affinity allergen-specific T cells that are suboptimally activated by their ligands, as observed with the Bos d 2-specific T cell clones. Since weak stimulation through the TCR of naïve T cells has been shown to favor Th2 deviation (Pfeiffer et al. 1995; Tao et al. 1997; Rogers and Croft 1999; Brogdon et al. 2002), a T cell repertoire lacking high-affinity T cells for lipocalin allergens could predispose the immune response against these allergens to be Th2-dominated.

It was demonstrated that Bos d 2-specific T cell clones were able to weakly recognize several self-protein-derived peptides, though none of these was derived from endogenous lipocalins (I). Clone F1-9, for which the search motif was designed, recognized four self peptides, and clone K3-2 one of these. Interestingly, the most stimulatory self peptide for F1-9, SP9, contains the superagonist substitution of aspartic acid for asparagine at the same position as the analog pN135D. This peptide is derived from the DNA-repair protein RAD51 homolog 2 (R51H2) expressed in various human tissues, including the thymus (Rice et al. 1997). Partial agonists such as the R51H2-derived peptide can be hypothesized to be involved in the positive selection of Bos d 2-specific T cells in the thymus and their survival in the periphery. As TCR recognition has been shown to be degenerate up to a point that no sequential homology with the original ligand is needed to activate a T cell clone (Hemmer et al. 1998), it is conceivable that all T cells are capable of weakly recognizing multiple self ligands. In fact, cross-reactivity between allergen-derived and self peptides has been previously demonstrated in murine models (Kristensen et al. 1996; King and Lu 1997).

6.2 T cell receptor V β subset and HLA associations with the T cell response to lipocalin allergens

The Bos d 2-specific clones F1-9, K3-2 and K9-10 isolated from two cow-asthmatic subjects were shown to be restricted with HLA-DRB1*0401 (I, II). Additional DRB1*0401-restricted T cell lines were generated from these subjects (A-1 and A-2) as well as from 6 other subjects expressing the allele with both the Bos d 2 peptide p127-142 and its heteroclitic analog pN135D (III). An expansion of TCR V β 13.1+ T cells was observed in 14 of the 18 p127-142-induced and in 4 of the 18 pN135D-induced TCLs, all of which also recognized p127-142. A common amino acid motif, RTGG, or minor variations of it, was observed in the TCR β -chain CDR3 sequences of 11 of these 18 T cell lines. Interestingly, one of the previously isolated DRB1*0401-restricted T cell clones, K3-2, also used the V β 13.1 subtype, although its TCR β -chain CDR3 sequence did not contain the RTGG motif (I, Fig. 2). Collectively, these data suggest a common structural requirement for TCRs recognizing the Bos d 2 peptide p127-142 in the context of DRB1*0401.

The significance of the highly homogenous TCR V β repertoire for the allergenicity of Bos d 2 in DR4-positive subjects needs to be clarified in further studies. The identification of common TCR structures in the pathogenic T cell population could potentially allow the development of TCR-based therapies, such as TCR-based vaccination (Ria et al. 2001). Although several Bos d 2-specific T cells from allergic subjects were shown to be restricted with DRB1*0401, it is of interest to note that the allele is not present in a higher frequency among Bos d 2-sensitized subjects in comparison with a normal population (Immonen A, unpublished observations). Therefore, it appears that DRB1*0401 does not predispose to allergy to Bos d 2.

TCR V β -MHC associations were also analyzed in Can f 1-specific T cell lines from allergic and non-allergic subjects (IV). Three of the eight TCLs from subjects with positive SPTs to rCan f 1 exhibited a strong expansion of V β 7+ T cells. Two of the three subjects had the same HLA-DRB1*0101-DQB1*0501(DR1-DQ5) haplotype. Importantly, a significant expansion of V β 5.1+ T cells was observed in five of the ten TCLs from subjects with negative SPTs to rCan f 1. Four of these subjects had the same HLA-DR4-DQ8 haplotype. This haplotype was also found in two additional subjects with negative SPTs to rCan f 1, but only in one subject with a positive SPT to the allergen.

TCR V β -MHC associations have not been previously reported with respect to lipocalin allergens, but have been observed in responses against other antigens, such as the ragweed

allergen Amb a 5 (Huang et al. 1995), and the self-antigen MBP (Oksenberg et al. 1993; Musette et al. 1996). Moreover, HLA associations with immune responsiveness to lipocalin allergens have remained largely unexplored. In one study, the HLA-DRB1*0405 allele was reported to be important in the T cell response against cow milk allergen β -lactoglobulin (Bos d 5) (Sakaguchi et al. 2002). Also, in another study, both positive (DR7) and negative (DR3) HLA associations were observed with sensitization to rat urine (Jeal et al. 2003), the main allergen of which is Rat n 1. In agreement with previous reports (Young et al. 1994; Howell et al. 1999), no association between a specific HLA class II allele and allergy to Can f 1 was found in this study. In contrast, it was observed that protection against sensitization to Can f 1 was associated with the HLA haplotype DR4-DQ8. DR4 is an interesting allele because it has been shown to predispose to autoimmune diseases, such as type I diabetes and rheumatoid arthritis (Tuokko et al. 2001; Hermann et al. 2003), but appears to confer protection against sensitization to various allergens (Lympny et al. 1990; Reid et al. 1992; Cho et al. 2000; Kim et al. 2001). Although not examined in detail, it has been suggested that the protective effect of an HLA allele might be mediated by antigen-specific regulatory T cells (Lympny et al. 1990; Reid et al. 1992). In support of this view, a recent study demonstrated that a protective response (modified Th2 response) to cat allergen Fel d 1 was associated with DR7, and that the allergen-specific T cells from DR7-positive subjects produced high levels of IL-10 in response to Fel d 1 peptides (Reefer et al. 2004). Whether the protective TCR V β -MHC association observed in this study is attributable to a regulatory T cell response against Can f 1 remains to be verified in future studies.

In this study, a significantly higher frequency of V β 21.3+ and V β 22+ CD4+ T cells was observed in the peripheral blood of dog-allergic subjects with positive SPTs to rCan f 1 compared with nonatopic controls (IV). As the frequency of the V β subtypes was not further increased in the Can f 1-specific T-cell lines of these subjects, the greater frequency of the V β subtypes in the peripheral blood was probably not associated with allergy to Can f 1. It is possible that other factors, such as *in vivo* expansion of T cells reactive against other dog allergens or against other allergen sources, or the atopic status of the subjects, explain the finding. This view is supported by the observation that dog-allergic subjects with negative SPTs to rCan f 1 also had a greater frequency of these V β subtypes in their peripheral blood than the non-atopic control subjects. Some studies have suggested that allergic subjects can have increased frequencies of allergen-specific T cells with particular V β subtypes in the peripheral blood (Beyer et al. 1999; Kircher et al. 2002). In one of these studies, birch pollen-

sensitized individuals had higher frequencies of T cells with specific V β subtypes in their peripheral blood only during the pollen season (Beyer et al. 1999). This finding suggests that an *in vivo* expansion of allergen-specific T cells may only be detectable upon strong and constant allergen exposure.

6.3 Detection of allergen-specific T cells by MHC class II tetramer staining

MHC tetramers are multimeric complexes of soluble recombinant MHC molecules and antigenic peptides (Mallone and Nepom 2004). They allow the direct identification of both MHC class I and II specific T cells within diverse polyclonal populations by flow cytometry. MHC class II tetramers have been successfully used in detecting CD4⁺ T cells specific for viral (Novak et al. 1999; Kwok et al. 2000), bacterial (Meyer et al. 2000) and autoantigens (Kotzin et al. 2000; Quarsten et al. 2001; Reijonen et al. 2002).

The potential of MHC class II tetramers in detecting Bos d 2-specific CD4⁺ T cells was demonstrated in this study. As a first approach, DRB1*0401 tetramers loaded with the peptide containing the immunodominant epitope of Bos d 2 were used to stain Bos d 2-specific T cell clones (II). This was the first time that MHC class II tetramers were shown to specifically stain allergen-specific T cells, although recently the detection of rye grass allergen Lol p 1-specific T cells by MHC class II tetramers has also been reported (Macaubas et al. 2006). Two of the three DRB1*0401-restricted T cell clones, K3-2 and K9-10, bound the DRB1*0401/p127-142 tetramer in a dose-dependent manner. Clone F1-9, however, failed to bind the tetramer despite the fact that p127-142 efficiently induced the proliferation and cytokine production of the clone in the context of DRB1*0401 (II, Figs. 1 and 3). F1-9 appears to have a low TCR avidity for p127-142, since the TCR down-regulation observed with the clone upon stimulation with p127-142 was weaker than with the other two clones (II, Fig. 1). Interestingly, one of the eighteen p127-142-induced and five of the eighteen pN135D-induced long-term T cell lines also failed to bind the DRB1*0401/p127-142 or DRB1*0401/pN135D tetramers, respectively (III). These findings suggest that the low TCR avidity of some Bos d 2-specific T cells may preclude their detection by MHC class II tetramers. It is of interest to note that Bos d 2-specific T cells resemble in this manner autoantigen-reactive T cells, as they also appear to have a reduced capacity to bind MHC class II tetramers (Buckner et al. 2002; Reijonen et al. 2002).

CD4⁺ T cells that are directed toward a specific antigen occur at a very low frequency in the periphery. Therefore, an *in vitro* expansion of the cells with the antigen prior to tetramer staining is usually necessary (Mallone and Nepom 2004). In this study, tetramer staining in conjunction with CFSE labeling (Novak et al. 1999) was used to evaluate the potential of p127-142 and pN135D to induce a T cell response *in vitro* (III). On day 8 after stimulation of peripheral blood CD4⁺ T cells with the peptides, the frequency of divided, tetramer-positive T cells was very low and below the detection limit (0.1%) in the T cell cultures of all subjects except one. In this subject, A-3, both p127-142-specific (0.12%) and pN135D-specific (0.16%) T cells could be observed. Precursor frequency of the T cells can be estimated by dividing the percentage of tetramer-positive cells by 2^x , where x is the average number of cell divisions (Novak et al. 1999). As the T cells appeared to have undergone 5 to 6 cell divisions (III, Fig. 1A), the estimated T cell precursor frequency of both p127-142- and pN135D-specific T cells in the peripheral blood appears to be 1 in 20,000 or less, which is in line with previous estimates of the frequency of antigen-specific CD4⁺ T cells in the periphery (1 in 6,000 to 1 in 100,000) (Mallone and Nepom 2004). When IL-2 was added to the T cell cultures and the cells were reanalyzed one week later, tetramer-positive T cell populations were observed in the p127-142- and pN135D-induced cultures of six and five of the eight subjects, respectively. This finding demonstrates that both p127-142 and pN135D were stimulatory for T cells *in vitro* but exogenous IL-2 was necessary for the expansion of the cells.

6.4 Potential of altered peptide ligands for peptide-based allergen immunotherapy

The identification of heteroclitic APLs of the immunodominant epitope of Bos d 2 (I) prompted their evaluation for peptide-based allergen immunotherapy. Downregulation of pathogenic T cell responses in peptide immunotherapy may arise as a result of numerous mechanisms, such as the induction of anergy or AICD, immune deviation from Th2- to Th1-type cytokine profile, or the induction of regulatory T cells (Ali and Larche 2005).

In this study, the immunomodulatory potential of the heteroclitic analogs pN135D and pQ133K on the Bos d 2-specific T cell clones F1-9 and K3-2 was examined first (II). When the kinetics of cytokine production by Bos d-specific T cell clones upon stimulation with the natural peptide p127-142 were analyzed, it was observed that the ratios of IFN- γ /IL-4 and

IFN- γ /IL-5 increased with increasing concentrations of the peptide. This can be a common feature of allergen-specific T cell clones, as similar findings have been reported previously with clones specific for the bee venom allergen PLA₂ (Carballido et al. 1992; Carballido et al. 1997). Interestingly, the clones F1-9 and K3-2 elicited greater Th1/Th2 cytokine ratios upon stimulation with the heteroclitic analogs than with p127-142 at equimolar concentrations. Increased Th1 to Th2 cytokine ratios have also been observed in conventional SIT (Secrist et al. 1993; Jutel et al. 1995; Bellinghausen et al. 1997; Ebner et al. 1997), in which considerably higher allergen doses are administered than encountered naturally (Bousquet et al. 1998). As both a high antigen dose and high affinity of the TCR-MHC interaction promote Th1 type responses (see chapter 2.2.7), it can be expected that in an immunotherapeutic setting immune deviation to Th1 could be induced more efficiently with heteroclitic peptides than with a natural allergen.

Supraoptimal stimulation of T cells has also been observed to lead to hyporesponsiveness and cell death, probably through the induction of anergy and AICD (Suzuki et al. 1988; Critchfield et al. 1994; Liblau et al. 1996; Gardner et al. 2004). Both phenomena were observed upon stimulation of the T cell clones F1-9 and K3-2 with the heteroclitic analogs. Clone F1-9 died rapidly after stimulation with pN135D, whereas after stimulation with pQ133K clone K3-2 became hyporesponsive to restimulation with p127-142. In contrast, stimulation with similar concentrations of the natural peptide appeared to favor the expansion of the clones. It appears that stimulation with the heteroclitic analogs provided the critical TCR occupancy for the induction of anergy or AICD (Viola and Lanzavecchia 1996; Falk et al. 2000), which could not be achieved with an equimolar concentration of the natural peptide.

Although APLs have been shown to be efficient in directly modulating T cell responses *in vitro*, and *in vivo* in animal models, their therapeutic application in human diseases appears to be much more complicated (Bielekova and Martin 2001). The extensive polymorphism of the HLA class II genes and the considerable flexibility of TCR recognition make it highly unlikely that a single APL could have a desired effect on all the pathogenic T cell clonotypes inter- or even intraindividually. For example, in a murine study APLs of MBP shown to be antagonists for clonal T cells *in vitro* were actually encephalitogenic as they acted as agonists for some of the pathogenic T cells in the polyclonal population *in vivo* (Anderton et al. 1998). Moreover, in one of the clinical trials of APL immunotherapy the precursor frequencies of T cells specific for MBP decreased in only two of eight patients, although previous *in vitro* experiments had demonstrated the capability of the APL to induce anergy or antagonism

(Bielekova et al. 2000). Therefore, it appears that direct modulation of the pathogenic T cells with APLs may not be a viable alternative in immunotherapy.

Some murine studies have described an alternative mechanism for the therapeutic effect of APLs in immunotherapy (Nicholson et al. 1995; Santambrogio et al. 1998; Alleva et al. 2002). In these studies APLs were shown to prime and expand APL-specific T cells with immunoregulatory properties. Notably, these cross-reactive T cells were able to recognize the natural peptide and to down-regulate the ongoing inflammatory response through bystander suppression. This phenomenon was also demonstrated in one of the clinical trials of APL immunotherapy (Bielekova et al. 2000). In that study, an *in vivo* expansion of APL-specific T cells, a large fraction of which were cross-reactive with the natural MBP peptide, was observed in every patient treated with the APL. Unfortunately, some of these T cells were not of the desired Th2-phenotype but of the Th1-phenotype, which presumably led to disease exacerbations in two of the eight subjects in the study.

The data in this study support the view that APL-induced bystander suppression can potentially be beneficial in allergen immunotherapy (III). Almost all the pN135D-induced T cell lines cross-recognized the natural peptide p127-142. Importantly, the Th1/Th0 cytokine phenotype of these TCLs remained unaltered upon stimulation with p127-142. Finally, the difference in the TCR V β usage by the p127-142- and pN135D-induced T cell lines supports the idea that most of the TCLs induced with the APL were derived from a different pool of T cell precursors.

Hypothetically, the induction of bystander suppression with an APL in allergen immunotherapy requires two steps *in vivo*. Firstly, immunization with the APL should create a novel Th1-biased T cell population cross-reactive with the natural allergen epitope. Subsequently, upon allergen exposure, these T cells would become activated with the natural peptide and be able to modulate the Th2-deviated immune response. Another possibility is that immunization with the APL could result in the induction of regulatory T cells, a phenomenon previously observed in both conventional allergen immunotherapy (Bellinghausen et al. 1997; Akdis et al. 1998; Francis et al. 2003; Jutel et al. 2003) and immunotherapy with natural allergen peptides (Verhoef et al. 2005). In the future, immunomodulatory treatments that, for example, enhance the production of IL-10 (Hawrylowicz and O'Garra 2005) might be combined with APL immunotherapy to promote the induction of regulatory T cells.

Most of the previous research on APL immunotherapy has been directed against autoimmune diseases, in particular MS, in which unsuccessful therapy can have detrimental

consequences. It is possible that allergic disorders may be a safer alternative for the clinical trials of APL immunotherapy (Larche 2001). The efficacy and safety of immunotherapy with natural peptides has been evaluated in several clinical trials of allergic diseases (Norman et al. 1996; Muller et al. 1998; Pene et al. 1998; Oldfield et al. 2002; Fellrath et al. 2003), while experience with autoimmune diseases is more limited (Raz et al. 2001; Prakken et al. 2004). It is also conceivable that potential disease exacerbations associated with APL immunotherapy (Bielekova et al. 2000) are more easily controllable in allergic subjects than in patients with autoimmune diseases (Oldfield et al. 2002).

7. CONCLUSIONS

The purpose of this study was to investigate the T cell response of allergic and nonatopic subjects against lipocalin allergens. The potential of altered peptide ligands for peptide-based allergen immunotherapy was also investigated.

In this study, the lipocalin allergen Bos d 2 was shown to be suboptimally recognized by human T cell clones, as heteroclitic analogs of the immunodominant epitope could readily be found. Moreover, the proliferative responses of PBMCs and T cell lines of both dog-allergic patients and nonatopic dog owners against the major dog allergen Can f 1 were observed to be weak. Collectively these data support the hypothesis that the presence of endogenous lipocalins limits the T cell responses against exogenous lipocalin allergens through central or peripheral tolerance mechanisms.

Two interesting TCR V β -MHC associations were observed in the study. First, it was demonstrated that DRB1*0401-restricted CD4⁺ T cells specific for the immunodominant epitope of Bos d 2 used predominantly the TCR V β 13.1 subtype with a common CDR3 motif. Second, subjects with negative SPTs to rCan f 1 exhibited an increased frequency of TCR V β 5.1⁺ CD4⁺ T cells in their Can f 1-specific T cell lines, and this was associated with the expression of the DR4-DQ8 haplotype by these subjects. These observations point to the possibility that certain TCR-MHC combinations may contribute to sensitization to lipocalin allergens. Moreover, the identification of common CDR3 motifs in the allergen-specific T cell population could allow the development of TCR-based therapies.

The potential of MHC class II tetramers in detecting allergen-specific CD4⁺ T cells was analyzed in this study. First, the specificity of MHC tetramers was demonstrated by dose-dependent staining of Bos d 2-specific T cell clones. Subsequently, by using tetramer staining in conjunction with CFSE labeling it was possible to detect specific T cells after a single *in vitro* stimulation of peripheral blood T cells. The use of MHC class II tetramers opens new prospects for the analysis of T cell responses in allergy research.

Finally, this study demonstrated that stimulation of Bos d 2-specific T cell clones with heteroclitic APLs favored Th1-biased cytokine production and the induction of cell death and hyporesponsiveness *in vitro*. Moreover, the APL pN135D was shown *in vitro* to induce a Th1/Th0-deviated T cell response cross-reactive with the natural peptide in eight different DR4-positive subjects studied. *In vivo*, these Th1-biased T cells might be able to modulate the

Th2-biased immune response against the allergen through bystander suppression. Collectively, these results support the notion that, with a careful selection of patients, APLs may offer a way to enhance the efficacy of peptide-based allergen immunotherapy.

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APPENDIX: ORIGINAL PUBLICATIONS

Kuopio University Publications D. Medical Sciences

D 385. Perola, Outi. Hospital water supply as a source of nosocomial infections. 2006. 99 p. Acad. Diss.

D 386. Kuittinen, Taru. Autologous stem cell transplantation in patients with non-Hodgkin's lymphoma: progenitor cell mobilisation, toxicity of high-dose therapy, and progressive disease after transplantation. 2006. 115 p. Acad. Diss.

D 387. Siloaho, Maritta. Utilization of quality management systems in Finnish medical laboratories. 2006. 105 p. Acad. Diss.

D 388. Haara, Mikko. Osteoarthritis and osteoporosis assessed from hand radiographs: prevalence, determinants, and associations with morbidity and mortality. 2006. 87 p. Acad. Diss.

D 389. Tenhola, Sirpa. Cardiovascular risk factors and adrenal function in 12-year-old children born small for gestational age or after a preeclamptic pregnancy. 2006. 85 p. Acad. Diss.

D 390. Zacharova, Jelena. Insulin-sensitizing genes and risk of type 2 diabetes: studies on the PGC-1 α , PPAR γ , adiponectin, leptin receptor and hepatic lipase genes. 2006. 103 p. Acad. Diss.

D 391. Rilla, Kirsi. Hyaluronan synthase: intracellular traffic, activity at plasma membrane, and impact on keratinocyte migration, proliferation and formation of microvilli. 2006. 119 p. Acad. Diss.

D 392. Pesonen, Tuula. Trends in Suicidality in Eastern Finland, 1988–1997. 2006. 119 p. Acad. Diss.

D 393. Tuhkanen, Hanna. DNA copy number changes in the stromal and epithelial cells of ovarian and breast tumours. 2006. 112 p. Acad. Diss.

D 394. Koskelo, Reijo. Säädettyjen kalusteiden vaikutukset tuke- ja liikuntaelämänterveysten lukiolaisilla. 2006. 96 p. Acad. Diss.

D 395. Elo, Mika. Stress-Related Protein Synthesis in Mammalian Cells Exposed to Hydrostatic Pressure. 2006. 74 p. Acad. Diss.

D 396. Remes-Pakarinen, Terhi. Influences of genetic factors and regular exercise on bone in middle-aged men. 2006. 95 p. Acad. Diss.

D 397. Saarela, Tanja. Susceptibility genes of diabetes and endothelial dysfunction in preeclampsia. 2006. 103 p. Acad. Diss.

D 398. Piippo-Savolainen, Eija. Wheezy babies - wheezy adults? Adulthood asthma, bronchial reactivity and lung function after hospitalization for bronchiolitis in early life. 2006. 91 p. Acad. Diss.

D 399. Kauppinen, Anu. Lipocalin Allergen-Induced T Cell Response: Prospects for Peptide-Based Immunotherapy. 2006. 81 p. Acad. Diss.