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Aino Rönkä

Human T cell Response to Dog Lipocalin Allergens

Prospects for Allergen Immunotherapy

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AINO RÖNKÄ

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ABSTRACT

Sensitization to dog lipocalin allergens and other mammalian respiratory allergens is a common cause of allergic disorders. However, surprisingly little is known about the cellular reactivity to these agents. As the activation of T cells is a key event in allergic sensitization, characterization of the human T cell responses to dog lipocalin allergens is an important factor in elucidating the mechanisms by which they promote sensitization. This is also a prerequisite for the development of new modes of allergen-specific immunotherapy.

The aim of this study was to analyze human CD4+ T cell responses to the dog lipocalin allergens Can f 1 and Can f 4 in individuals with and without dog allergy. Moreover, the T cell-stimulating regions (T cell epitopes) of Can f 4 were identified and their potential for the development of peptide immunotherapy was assessed.

The epitope-containing peptides of the major dog lipocalin allergen Can f 1 were found to be weakly antigenic on human T cells *in vitro*. They exhibited only marginally more potent T cell stimulatory capacity than homologous peptides derived from a structurally related human self-protein, tear lipocalin, as assessed by the functional characteristics of the specific T cell lines *in vitro*. One possible mechanism accounting for the low antigenicity of Can f 1 is that its epitopes are recognized suboptimally by human T cells. This was demonstrated by producing several peptide analogues that contained single amino acid substitutions in comparison to the natural epitope. Stimulation of human T cells with these analogues *in vitro* lead to stronger T cell responses. The low antigenicity/immunogenicity of dog lipocalin allergens, potentially associated with suboptimal recognition by human T cells, may be an important factor in explaining their allergenic capacity.

Determining the allergen-specific immune features distinguishing individuals with and without allergy may help to understand the pathogenesis of allergy. Here, the frequency of Can f 4-specific memory CD4+ T cells was found to be substantially higher in allergic subjects than in those without allergy. These T cells exhibited robust immune polarization towards the allergenic T-helper type 2 phenotype. In contrast, the allergen-specific memory T cell responses in nonallergic subjects were observed to be much weaker and of a protective, regulatory T cell phenotype.

The antigenicity of the T cell epitopes identified along the sequence of Can f 4 was found to be associated with their promiscuous capacity to bind to a variety of commonly expressed human leukocyte antigen (HLA) class II molecules. This feature is of special importance in the development of peptide immunotherapy with a population-wide coverage. The 19-mer Can f $4_{46\text{-}64}$ peptide was recognized by 90% of the allergic subjects and it bound strongly to several HLA class II molecules. Therefore, it can be considered as a candidate for the development of peptide immunotherapy of dog allergy.

National Library of Medicine Classification: QW 573, QW 900, QW 940, WF 150, WH 200
Medical Subject Headings: Allergens; Epitopes; HLA Antigens; Hypersensitivity; Immunotherapy; T lymphocytes

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TIIVISTELMÄ

Koiran lipokaliiniallergeenit, kuten muutkin eläinperäiset hengitystieallergeenit, ovat yleisiä allergian aiheuttajia. T-soluilla on keskeinen rooli allergisen immuunivasteen muodostumisessa, mutta syntyvästä T-soluvasteesta tiedetään kuitenkin varsin vähän. Mallilla, jossa tutkitaan ihmisen T-soluvasteita koira-allergeeneja kohtaan, on mahdollista tarkentaa allergisen immuunivasteen syntyyn liittyviä seikkoja ja toisaalta avata uusia lähtökohtia allergian siedätyshoidon kehittämiseksi.

Väitöskirjatutkimuksen tavoitteena oli analysoida koira-allergisten ja terveiden koehenkilöiden T-soluvasteita kahta koiran lipokaliiniallergeenia (Can f 1 ja Can f 4) kohtaan. Lisäksi tutkimuksessa analysoitiin näiden allergeenien sisältämiä T-soluja aktivoivia alueita eli T-soluepitooppeja ja arvioitiin niiden potentiaalia allergian siedätyshoidossa.

Koiran pääallergeenin Can f 1:n havaittiin stimuloivan heikosti ihmisen T-soluja. Havainto perustui siihen, että allergeenille spesifiset T-soluvasteet muistuttivat voimakkuudeltaan ja laadultaan hyvin paljon vasteita, jotka olivat spesifisiä ihmisen endogeeniselle kyynellipokaliinille. Sen aminohappoidentiteetti Can f 1:n kanssa on noin 60 %. Yhdelle Can f 1-allergeenin epitoopeista luotiin sarja peptidianalogeja, eli peptidejä, jotka sisälsivät yhden aminohapon muutoksen epitoopin luonnolliseen rakenteeseen nähden. Näillä peptideillä stimulointi johti T-soluvasteen voimistumiseen *in vitro*, mikä viittaa siihen, että luonnollinen Can f 1-epitooppi tunnistetaan suboptimaalisesti. Tämä löydös voi selittää allergeenin heikkoa kykyä aktivoida ihmisen T-soluja, mikä puolestaan saattaa olla osasyynä niiden allergeenisuuteen.

Kun koira-allergisten ja terveiden kontrollihenkilöiden T-soluvasteita verrattiin, allergisilla havaittiin kahdeksan kertaa enemmän allergeenispesifisiä T-muistisoluja terveisiin nähden. Lisäksi allergisten potilaiden T-soluvaste oli voimakkaampi ja merkitsevästi enemmän polarisoitunut allergiselle immuunivasteelle ominaisen Th2-tyypin suuntaan. Terveiden koehenkilöiden soluvasteissa oli puolestaan havaittavissa piirteitä immuunivastetta vaimentavasta regulatoristen T-solujen aktivaatiosta.

HLA-molekyylit ovat immuunijärjestelmän antigeeneja esitteleviä rakenteita. Can f 4-allergeenin sisältämien epitooppien kyky stimuloida T-soluja havaittiin liittyvän siihen, että ne kykenivät sitoutumaan useaan erilaiseen HLA luokan II-molekyyliin. Tämä ominaisuus on oleellisen tärkeä uudenlaisen allergian siedätyshoitomuodon, peptidi-immunoterapian kehittämisessä. Erityisesti peptidi Can f 4_{46-64} osoittautui tässä suhteessa lupaavaksi. Sille spesifisiä T-soluja havaittiin 90 %:lla Can f 4-allergisista koehenkilöistä.

Yleinen Suomalainen asiasanasto: allergia; allergeenit; lymfosyytit; siedätyshoito

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Kuopio, December 2014
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List of the original publications

This dissertation is based on the following original publications:

I Juntunen R, Liukko A, Taivainen A, Närväinen A, Kauppinen A, Nieminen A, Rytkönen-Nissinen M, Saarelainen S, Maillère B, Virtanen T and Kinnunen T. Suboptimal recognition of a T cell epitope of the major dog allergen Can f 1. *Mol Immunol 46:3320-7, 2009.*

II Liukko A, Kinnunen T, Rytkönen-Nissinen M, Kailaanmäki A, Randell J, Maillère B and Virtanen T. Human CD4+ T cell responses to the dog major allergen Can f 1 and its human homologue tear lipocalin resemble each other. *PLoS One 29: 9(5):e98461, 2014.*

III Rönkä A, Kinnunen T, Goudet A, Rytkönen-Nissinen M, Sairanen J, Kailaanmäki A, Randell J, Maillère B and Virtanen T. Characterization of human memory T cell responses to the dog allergen Can f 4. *Submitted.*

Contents

Abbreviations

AICD	activation-induced cell death
APC	antigen-presenting cell
APL	altered peptide ligand
ASIT	allergen-specific immunotherapy
CCR	C-C chemokine receptor
CD	cluster of differentiation
CDR	complementary-determining region
CPM	counts per minute
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
CTLA	cytotoxic T-lymphocyte-associated antigen
DC	dendritic cell
EAACI	European Academy of Allergy and Clinical Immunology
EBV	Ebstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EC	effective concentration
FACS	flurescence-activated cell sorting
FCεRI	high-affinity IgE receptor
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3
GITR	glucocorticoid-induced THFR family related gene
HA	hemagglutinin
HEL	hen egg lysozyme
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
IFN	interferon
IEDB	Immune Epitope Database
Ig	immunoglobulin
IL	interleukin
ICAM	intracellular adhesion molecule
ICOS	inducible T cell costimulator
IC	inhibitory concentration
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
kDa	kilodalton
LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MAIT	mucosal associated invariant T cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
NK	natural killer
PBMC	peripheral blood mononuclear cell

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy5	phycoerythrin-cyanine 5
PHA	phytohemagglutinin
PLA_2	phospholipase A_2
PMC	perimedullary cortex
PPD	purified protein derivative
ROR	RAR-related orphan receptor
RPMI	Roswell Park Memorial Institute
SCIT	subcutaneous immunotherapy
SI	stimulation index
SLIT	sublingual immunotherapy
SMAC	supramolecular activation cluster
SPT	skin prick test
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
T-bet	T-box expressed in T cells
TCC	T cell clone
TCL	T cell line
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TL	tear lipocalin
Treg	T regulatory

1 Introduction

Allergy is a hypersensitivity disease of the immune system against innocuous environmental substances, such as food, pollens and animal dust. At the cellular level, it is characterized by the activation of T-helper type 2 cells (Th2), which leads to the production of allergen-specific IgE antibodies by B cells and the release of inflammatory mediators that eventually cause the classical allergic symptoms in the target organ (1). Common clinical manifestations of allergy include allergic asthma, rhinitis, conjunctivitis, eczema and certain gastrointestinal disorders.

The prevalence of allergic diseases has increased rapidly all around the world during recent decades. Currently, about 10-20% of the world population has been estimated to suffer from an allergic disorder (2). Allergies are chronic, often life-long diseases that affect the quality of life and represent considerable economic burden on society (3). Although the interactions of several genetic and environmental factors are known to contribute to the development of allergy, the exact cellular mechanisms involved in the allergic immune response are unclear. Moreover, the basis of allergenicity, i.e. the capacity of allergens to induce the development of Th2 immunity, remains largely elusive.

Sensitization to dog dander is a common cause of respiratory allergy (4). Dog allergens disperse efficiently and they can be detected in the indoor air in domestic houses and public buildings, such as schools (5). To date, six dog allergens, Can f 1-6, have been identified from dog dander and urine. Four of them belong to the family of lipocalin proteins that represents the largest group of mammalian inhalant allergens. The molecular structure and IgE reactivity of these allergens have been characterized in detail (6), however, little is known about the human T cell reactivity to these agents.

The treatment of allergy relies largely on palliative medication, such as antihistamines and corticosteroids. The only disease modifying therapy currently available for the treatment of allergy is allergen-specific immunotherapy (ASIT) during which progressive doses of allergen extract are administered subcutaneously (7). The main drawbacks of the treatment are its long duration (3-5 years) and the risk of severe IgE antibody-mediated side effects. A promising way to improve the efficacy and safety of allergen-specific immunotherapy is to administer small allergen-derived peptide fragments that are capable of activating specific T cells but too short to induce IgE-mediated reactions. Mapping of these T cell activating regions (T cell epitopes) of an allergen is a prerequisite for the development of new peptide immunotherapy vaccines.

Previously, the T cell epitopes of only one dog allergen, Can f 1, have been characterized (8). An interesting feature of Can f 1 is its weak capacity to stimulate the proliferation of peripheral blood mononuclear cells (PBMCs) from sensitized subjects *in vitro* (8,9). Importantly, this characteristic is also shared by other lipocalin allergens, such as cow Bos d 2 (10,11), horse Equ c 1 (12) or rat Rat n 1 (13). Therefore, the weak T cell stimulatory capacity of lipocalins has been postulated to be a possible determinant of their allergenicity (14).

The primary focus of this thesis was to assess the allergenic properties of the dog lipocalin allergens Can f 1 and Can f 4 by characterizing the human T cell responses to these agents. In addition, allergen-specific T cell responses between allergic and nonallergic individuals were compared in order to clarify the immunological background of allergic sensitization and tolerance. Finally, to extend the knowledge of dog allergen-specific human T cell responses, the T cell epitopes of Can f 4 were mapped and their potential for the development of peptide-based allergen immunotherapy was analyzed.

2 Review of the Literature

2.1 ALLERGY

2.1.1 Nomenclature

First coined by Austrian pediatrician Clemens von Pirquet in 1906, the term allergy (from Greek *allos ergos*; altered reaction) initially referred to a general concept of changed immune reactivity upon exposure to a foreign substance, an allergen (15). It was utilized to distinguish "supersensitivity" reactions from protective immunity against infectious agents. The term atopy was later introduced by Coca and Cooke to describe more specifically the inherited tendency of developing immediate allergic symptoms (16). The reaction was suggested to be mediated by "reagins" that were later recognized to belong to the IgE-class of antibodies (17,18). In 1975, Coombs and Gell classified the different types of hypersensitivities into four subgroups with the immediate, IgE-mediated allergy being categorized as a type I hypersensitivity reaction (19). Today, the definition of allergy has been broadened to cover also antibody-independent hypersensitivity reactions to an allergen stimulus dominated by cellular interactions (20).

The clinical manifestations of the classical IgE-mediated allergy include allergic asthma, rhinitis, conjunctivitis, certain gastrointestinal symptoms and eczema. Typically, atopic individuals develop eczema and food allergies with gastrointestinal symptoms in childhood, whereas respiratory symptoms, induced mostly by inhalant allergens, dominate those appearing later in life (the so-called atopic march) (21). An example of the less frequent, non-IgE-mediated allergy is allergic contact dermatitis, where the skin reaction is directly caused by the activation of T lymphocytes (22). Allergic anaphylaxis, an acutely developing multiple organ reaction, which causes characteristic symptoms of reduced blood pressure and respiratory distress, is regarded as the most severe and potentially fatal response to an allergen stimulus (23). The term was first proposed by Charles Richet already in 1902 (24). The nomenclature for common allergy-related terms, revised by the World Allergy Organization in 2004 (20), is summarized in Table 1.

Table 1. Allergy nomenclature. Modified from *(20)* by permission of Macmillan Publishers Ltd: *Nature Reviews Immunology*, copyright 2014.

Term	Definition
hypersensitivity	A state that causes objectively reproducible symptoms or signs. Initiated by exposure to a defined stimulus that is tolerated by normal subjects.
allergy	A hypersensitivity reaction initiated by immunological mechanisms. Can be antibody- (usually the IgE isotype) or cell-mediated.
allergen	An antigen causing allergic disease
atopy	A personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposure to allergens, and to develop typical symptoms such as asthma, rhinoconjunctivitis or eczema.
anaphylaxis	A severe, life-threatening generalized or systemic hypersensitivity reaction.
IgE	Immunoglobulin E, a subtype of immunoglobulins in mammals. Exists as a monomer consisting of two heavy chains (ε chain) and two light chains. Binding of IgE to FcεRI on mast cells triggers the degranulation of inflammatory mediators.

2.1.2 Prevalence of allergy

The global prevalence of allergic disorders has been reported to be around 10-20% (2). In western countries, the steepest increase in the prevalence took place towards the end of the 20th century (25-27), but the upward trend has been reported to be levelling off during recent years in some European countries (28,29). The overall prevalence, however, continues to rise, mainly due to the increasing occurrence of allergy in developing countries (2). In Finnish young men, the prevalence of asthma remained stable from 1926 to 1961 (30) but has increased steadily since the 1960s (12-fold increase between the years 1966-2003) as has the prevalence of allergic rhinitis (31). Recently, the prevalences estimated for common allergic disorders in Finland were as follows: rhinitis 30%, asthma 8-10%, eczema 10-12% and conjunctivitis 15% (32). Pollen and animal dust are the most common sensitizers, causing allergic symptoms in up to 20% of the population (32). Due to their common occurrence and chronic nature, allergic diseases represent a considerably economic burden on society, not only via direct health care costs but also through the loss of productivity (3).

2.1.3 Risk factors of allergy

Genetic factors contribute significantly to the development of allergic diseases. The atopic phenotype has been associated with >100 susceptibility genes that regulate the inflammatory pathways of both innate and adaptive immunities (33). Many of these genes belong to the human leucocyte antigen (HLA) system or code for inflammatory signaling molecules, such as interleukins, thus they are directly involved in the pathogenesis of allergy. Other genes may contribute to the disease susceptibility indirectly, for example the gene *ADAM33*, which is involved in the development of bronchial hyperresponsiveness in asthmatic conditions (34).

The increasing incidence of allergic disorders, however, is clear evidence that also environmental factors must play a role in the disease pathogenesis. This hypothesis is further supported by the observation that the occurrence of allergic symptoms varies largely in populations with similar genetic backgrounds but differing lifestyle (2). For example, in the comparison of socio-economically distinct but genetically related populations of East and West Germany, marked differences in the prevalences of allergic disorders were demonstrated after the fall of the communist system (35). Along with the changes towards western lifestyle in the former East Germany, these differences were reported to even out (36). The observation is in line with the hygiene hypothesis, according to which the modern lifestyle is favorable for allergic development. Mechanistically, it has been postulated to result from a lack of microbial exposure in the urban environment, which may disrupt the normal tolerance development and the regulation of hypersensitivity of the immune system (37-40). The risk factors of allergy supporting the hygiene hypothesis from the epidemiological point of view are listed in Table 2. In addition, there is evidence that two life-style factors, tobacco smoke (41) and obesity (42) are associated with an increased risk of suffering allergic disorders.

Table 2. Environmental risk factors associated with the risk of allergy/atopy.

Environmental factor	Risk of allergy ↑	↓	Reference
habitation	urban	rural/farm	*(29)*
mode of delivery	c-section	vaginal	*(43)*
postnatal diet		breastfeeding	*(44)*
family size & birth order		older siblings	*(45)*
daycare	home	daycare center	*(46)*
medication	childhood antibiotics		*(47)*

2.2 ALLERGENS

2.2.1 Nomenclature

Most allergens are soluble proteins or glycoproteins. Allergens are defined as antigens that are capable of binding specific IgE and inducing allergic symptoms in susceptible individuals (20). To date, 796 allergens from mites, plants, animal dander, pollens, foods and insects are listed in the official site for the systematic allergen nomenclature approved by the World Health Organization and International Union of Immunological Societies (IUIS; www.allergen.org, accessed 8/13/2014). In order to become officially listed as an allergen, a set of biochemical criteria and demonstration of allergenic activity must be fulfilled, including clearly defined molecular and structural properties and a demonstration of IgE-binding capacity (48).

The systematic allergen nomenclature was originally developed in 1986 and later revised in 1994 by the IUIS Subcommittee for Allergen Nomenclature (49,50). Purified allergens are named using the first three letters of the taxonomic genus and the first letter of the species followed by an Arabic number that indicates the chronological order of the allergen purification (50,51). For example, Can f 1, the first dog allergen identified, is abbreviated from the Linnean *Canis familiaris*. The polymorphism of allergens, i.e. isoallergens and isoforms (or variants) of allergens, is denoted with additional suffixes of a period followed by four digits in the nomenclature, the first two distinguishing between isoallergens and the last two between isoforms. Isoallergens, by definition, are multiple molecular forms of an allergen in a single species that share a similar biological function, molecular size and an amino acid identity of ≥67%, whereas the identity of isoforms of allergens is typically ≥90% with only a limited number of amino acid substitutions (51). Moreover, a prefix letter in the name indicates the natural (n), synthetic (s) or recombinant (r) origin of an allergen, for example rCan f 1 (50). Allergens are generally called major or minor depending on the percentage (greater or less than 50%, respectively) of clinically allergic subjects tested showing specific IgE reactivity (50).

2.2.2 Lipocalin allergens

Lipocalins are a large family of small extracellular proteins that are capable of binding small hydrophobic molecules such as odorants and pheromones (52). In addition, other biochemical functions for lipocalins have been reported, for example, the non-specific

endonuclease activity of human tear lipocalin and cow milk β-lactoglobulin or the immunomodulatory activity of placental protein 14 (53,54).

Most of the known mammalian inhalant allergens belong to the lipocalin family of proteins (6,55-57). In addition, β-lactoglobulin and four arthropodan allergens are lipocalins (58). Mammalian lipocalin allergens can be found mainly in saliva, urine and dander (58). The sequential length of these allergens is around 150-180 amino acids (aa), and they have a molecular mass of 15-20 kDa and an acidic isoelectric point (6).

Lipocalins share a highly conserved three-dimensional structure with a β-barrel core that constitutes their ligand-binding site (58). They are also characterized by having one to three structurally conserved regions (SCRs 1-3) in their primary amino acid sequences. The N-terminal SCR1, which contains the motif glycine-x-tryptophan (GxW), is considered as the hallmark of the protein family since it is present in >90% of the known lipocalins. The SCR2 and 3 are characterized by the motifs threonine-aspartic acid-tyrosine-x-x-tyrosine (TDYxxY) and arginine/lysine (R/K), respectively (14). Based on the number of SCRs, lipocalins are subdivided into two groups: kernel lipocalins contain all three motifs whereas outlier lipocalins contain 1-2 motifs (58). In spite of the structurally conserved regions and the conserved three-dimensional structure, the amino acid sequence identity between the lipocalin family members is generally low, around 20-30% (the SIB BLAST network service, web.expasy.org/blast/; (6,14,59)). However, there are a few exceptions e.g. the homology of dog Can f 1 with cat Fel d 7 (63%) and human tear lipocalin TL (57%) and the homology of dog Can f 6 with cat Fel d 4 (67%). These pairs have also exhibited IgE cross-reactivity (60,61).

2.2.3 Dog lipocalin allergens

Up to 30% of individuals suffering from atopic conditions are sensitized to dog dander (4,62). Four of the six dog allergens identified to date (Can f 1-6) belong to the lipocalin protein family. The major dog allergen Can f 1 (45-70% of dog-allergic patients sensitized) was the first to be identified, along with the minor dog allergen Can f 2 (around 25% sensitized) (63,64). The two more recently identified dog lipocalin allergens are Can f 4 (around 35-60%) (65,66) and Can f 6 (38%) (67). Dog lipocalin allergens are found in dog dander or body secretions, such as saliva. The dog serum albumin Can f 3 (around 20-40%) is IgE cross-reactive with several other mammalian albumins (68), and the dog prostatic kallikrein Can f 5 (70%) exhibits IgE cross-reactivity with human prostate-specific antigen (69). The characteristics of dog lipocalin allergens are summarized in Table 3.

Table 3. Dog lipocalin allergens.

Allergen	Source	Amino acids	Molecular weight (kDa)	Sensitization (%)	UniProtKB* accession No	Reference
Can f 1	Saliva, Dander	156	22-25	45-70	O18873	*(63,64)*
Can f 2	Saliva, Dander	162	22-27	25	O18874	*(63,64)*
Can f 4	Saliva, Dander	158	16-18	35-60	D7PBH4	*(65,66)*
Can f 6	Dander	175	20	38	E2QYS2	*(67)*

* UniProt Knowledgebase (www.uniprot.org; accessed 8/13/2014)

2.2.4 Allergenicity

Although allergenic proteins exhibit a diversity of molecular structures and biological functions (51), they can be found in only about 5% of all structural protein families (PFam database; (70)) suggesting that allergens possess special physicochemical or functional features that contribute to their allergenicity, i.e. the capacity of inducing a Th2-biased immune response in the host.

The specific physical properties of allergens may facilitate their presentation to the immune system. The allergenicity of food allergens is potentiated by their heat and acid stability as well as by their resistance to proteolytic degradation (71). Instead, the optimal molecular weight and aerodynamics are critical for the efficient dispersion of respiratory allergens (72). For some allergens, the natural enzymatic activity has been proved to be an important factor in determining their allergenicity. For example, the major house dust mite allergen Der p 1 exhibits cysteine protease activity, which has been shown to facilitate its entry into mucous tissue by cleaving the intracellular junctions of epithelium cells (73) and to disrupt the immune cell function by acting on lymphocyte surface receptors (74,75). For others, the capacity to activate the innate immune system appears central. For instance, the binding capacity of the peanut allergen Ara h 1 to the dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN), a receptor on antigen-presenting dendritic cells, has been shown to enhance allergen uptake and to stimulate the allergen-specific T cell response (76).

Not only the allergen proteins but also other components in the allergen source can possess properties promoting allergic sensitization. This type of an adjuvant effect has been observed e.g. with diesel exhaust particles (77), phytoprostanes derived from pollen grain (78) and bacterial lipopolysaccharide (LPS) at low concentrations (79). Interestingly, higher concentrations of LPS have been associated with conferring protection from allergic sensitization (80).

Furthermore, the allergen concentration itself also appears to play a role in allergenicity. For example, a prospective study analyzing the mouse Mus m 1 allergen concentrations in the air demonstrated that development of specific antibodies peaked at the exposure level of approximately 1.2 ng/m^3 whereas the risk of becoming IgE-positive to the allergen decreased at lower and higher concentrations of exposure (81). In line with this study, high-dose exposure to the cat allergen Fel d 1 has been shown to favor the induction of tolerance (82).

T lymphocytes are central players in allergy (see 2.3) and the T cell recognition of allergens is an important determinant of the subsequent response. Interestingly, in previous studies investigating cellular immune response towards the allergens of the lipocalin protein family, both human and murine responses have constantly been reported to be weak (8-13). For example, the spleen cell response in BALB/c mice towards the cow allergen Bos d 2 has been found to be significantly weaker than that mounted to a control antigen hen egg lysozyme (HEL) (11). In addition, the activation of human peripheral blood T lymphocytes in response to dog Can f 1 has been observed to be significantly weaker than that to the microbial streptokinase and a tubercle bacillus-derived preparation, purified protein derivative (PPD) (83). Given that the weak T cell recognition of an antigen is a factor favoring the deviation of T cells towards the allergenic T-helper type 2 phenotype

(see 2.5.3; (84)), the low capacity of lipocalin allergens to stimulate specific T lymphocytes may be one important determinant of their allergenicity.

2.3 INTRODUCTION TO ALLERGIC IMMUNE RESPONSE

The allergic sensitization process is characterized by the activation of allergen-specific T lymphocytes (Figure 1), which eventually leads to the immediate and late-phase inflammatory responses, i.e., allergic inflammation.

Allergen-derived peptides are presented to T cells in lymph nodes by antigen-presenting cells (APCs), such as dendritic cells (DCs). T cells recognize the peptides with their T cell receptors (TCR), and become classically polarized into type 2 helper T cells (Th2 cells, see 2.5.3). These cells mediate activation signals to B lymphocytes and other cells involved in the allergic immune response via cell surface molecule interactions and the release of proinflammatory cytokines, such as interleukin (IL)-4, IL-5, IL-9 and IL-13 (85). The production of IL-5 and IL-9 by Th2 cells activates the proliferation of eosinophils and mast cells, respectively (85).

Importantly, B cells are activated through the release of the cytokines IL-4 and IL-13 and the ligation of specific cell surface molecules (CD40L on T cells – CD40 on B cells). These signals are the main inductors of the synthesis of the immunoglobulin (Ig)E antibody by B cells. IgE is considered to be one of the major mediators of type I hypersensitivity reactions underlying atopic conditions. Naïve B cells express only the IgM (or IgD) antibody isotypes on their cell surface. In order to produce IgE, activated B cells must undergo isotype class switching. In this process, the gene coding the variable region of an IgM antibody with specificity to the encountered allergen is recombined with the gene coding the constant region of IgE (86). Once formed, the IgE molecules are secreted by the activated B cells and bound by high-affinity Fcε receptors (FcεRI) on the surface of mast cells and basophils (87). Upon subsequent allergen exposure, the allergen molecules attach directly to the specific FcεRI-bound IgE-molecules, which leads to the cross-linking of the FcεRI receptors. The process triggers mast cell degranulation, a rapid release of preformed mediators, such as prostaglandins, proteases and histamines, from the cytoplasmic vesicles of the cells (88). These powerful mediators cause the classical allergic symptoms such as bronchoconstriction, vasodilatation and plasma exudation within minutes.

The late-phase allergic response occurs hours after the exposure to the allergen. It is mediated by *de novo* synthesized mediators, including leukotrienes, prostaglandins and interleukins, in mast cells. The mediators stimulate the growth and differentiation of eosinophils and other inflammatory cells and alter vascular permeability in order to facilitate inflammatory cell migration into the target tissues (85). A recurrent release of proinflammatory cytokines and invasion of inflammatory cells may lead to a state of chronic inflammation, which can eventually result in tissue remodeling such as thickening of the smooth muscular wall, mucous cell hyperplasia and tissue fibrosis. If this occurs in the asthmatic lung, the process may permanently reduce airway caliber (89).

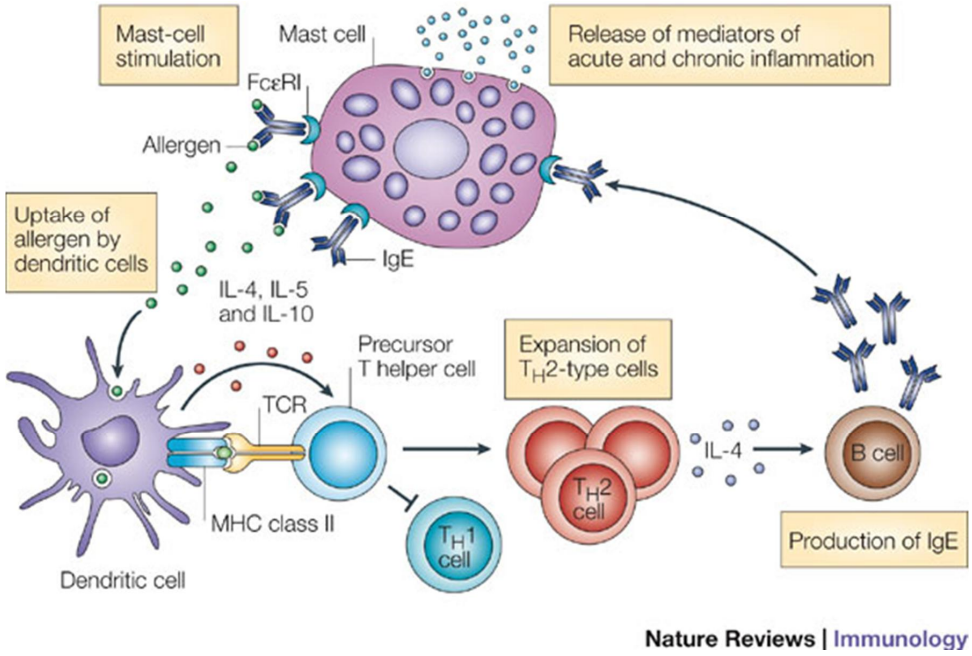

Figure 1. Allergic immune response. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (90), copyright 2014.

2.4 ANTIGEN PROCESSING AND PRESENTATION

Antigen-derived peptide fragments are presented to T lymphocytes on antigen-presenting cells in the context of cell surface major histocompatibility complex (MHC) molecules. MHC molecules are transmembrane glycoproteins with short cytoplasmic domains which are normally divided into two subtypes based on their structure and function. The MHC class I molecules consist of a larger (43 kDa), membrane-spanning α-chain and a smaller (12 kDa) non-covalently bound β_2-microglobulin (91). MHC class I molecules are expressed by virtually all cells in the body with the exception of erythrocytes. They present peptides derived from cytosolic proteins to a subtype of T cells called CD8+ T cells. The MHC class II molecules are formed by a noncovalent complex of α- (34 kDa) and β- (29 kDa) chains which both span the membrane, and are expressed on the surface of specialized antigen-presenting cells, such as dendritic cells, B cells, monocytes and thymic epithelial cells (Figure 2). These cells present extracellular peptides, taken up by endocytosis, to CD4+ T cells (91).

Before they can be presented on the cell surface, peptides must first be degraded from proteins and then become associated with MHC molecules in the lumen of the endoplasmic reticulum (ER). Peptides forming complexes with MHC class I molecules are processed from cytosolic proteins by proteasomes and transported subsequently to the ER lumen by a transmembrane transporter associated with antigen processing (TAP). In the ER lumen, the binding of a peptide to a partially folded MHC class I molecule attached to TAP completes

the MHC folding after which the complex is translocated to the cell surface (92). In the formation of a peptide:MHC class II complex, an MHC class II molecule transported to the ER is associated with a membrane protein known as the MHC class II-associated invariant chain (Ii). A part of the chain, called CLIP, lies within the peptide-binding groove of MHC class II and prevents the molecule from binding to the peptide prematurely. The peptides bound by MHC class II molecules are degraded from exogenous proteins taken up by endocytosis. The endosomes fuse with vesicles containing MHC II molecules to form an MHC class II compartment, which becomes increasingly acidic and this in turn activates proteases that degrade the endocytic proteins and the invariant chain. The CLIP fragment is released from the peptide-binding groove in a reaction catalyzed by an MHC class II-like molecule called HLA-DM, which also helps with the binding of the antigenic peptide in the groove. The complex is then ready to be presented on the cell surface (92).

The peptide-binding groove on both MHC class I and II molecules consists of an eight-stranded β-sheet floor and two antiparallel α-helical walls. In an MHC class I molecule, the ends of the cleft converge and the space can accommodate short peptides of 8-10 amino acid residues. The cleft of an MHC class II molecule is open-ended and can hold longer peptides, typically of 13-17 amino acid residues (93). The MHC molecule makes contact with the amino acid residues of a peptide via anchoring cavities in the peptide binding groove. The residues that point into the cavities are called anchor residues and the binding pattern is referred to as the peptide-binding motif (94). In the peptide:MHC I complexes, the anchor residues at the ends of the peptide are the major stabilizing contacts. In the context of MHC class II molecule, the anchor residues are typically scattered along the peptide and the cleft usually binds three to five anchor residues. Most commonly, the binding pockets of MHC class II molecules are accommodated by four anchor residues at positions 1, 4, 6 and 9 (starting from the first N-terminal residue of the motif), with the residues 1 and 9 often being hydrophobic (91,95).

In humans, MHC molecules are known as human leukocyte antigens (HLA). The HLA region is located on the chromosome six and contains more than 200 genes, many of which are involved in antigen presentation or other related immune functions (94). The HLA molecules expressed by an individual must be diverse to enable the presentation of the vast diversity of antigens encountered. Therefore, the HLA system is both polygenic, meaning that several different genes encode for the HLA molecules and polymorphic, meaning that there are multiple allelic variants of a specific HLA gene at the population level. Three HLA class I genes, HLA-A, -B and –C, encode for the HLA-I α-chains (the β_2-microglobulin is coded by a gene outside the HLA locus), and three HLA-II genes, HLA-DR, -DP and –DQ, encode for the HLA II α- and β-chains. In addition, the HLA-DR cluster often contains an extra β-chain gene. Thus, due to polygeny, up to eight different HLA-II molecules can be expressed on an antigen-presenting cell (94). The allelic polymorphism of the HLA system is largely concentrated on the parts of the HLA gene sequences that encode the peptide binding pockets of the binding groove. For example, to date, more than 800 allelic variants of the most polymorphic HLA II locus, DRB, have been identified (91).

2.5 T CELLS

T cells are key players in the adaptive immune response. They are characterized by the expression of T cell receptors (TCR) on their cell surface (Figure 2). TCRs are heterodimer cell-surface molecules that recognize antigens in the context of MHC molecules. They are typically composed of α- and β-chains that are disulfide-linked. Both of the chains consist of two segments: an amino-terminal, membrane-distal V (variable) region and a carboxy-terminal C (constant) region that passes through the cell surface membrane domain ending in a short cytoplasmic segment (96).

T cells are classically divided into two main subclasses based on their phenotypic and functional characteristics. C*ytotoxic T cells* are distinguished by the expression of the CD8 molecule (CD8+ T cells) and *helper T cells* by the expression of the CD4 molecule (CD4+ T cells) (97). The T cell receptors of CD8+ T cells recognize host cells that have been infected with intracellular pathogens, such as viruses, since they now display the microbial peptide in the context of their MHC class I molecules. CD8+ T cells act directly against the infected host cells mainly by inducing apoptosis (98). In contrast, the T cell receptors of CD4+ T cells recognize antigenic peptides of extracellular origin in the context of MHC class II molecules on professional antigen-presenting cells (see 2.4). Stimulus via TCR induces CD4+ T cells to provide activation and/or inhibitory signals to other immune cells, such as B cells, eosinophils or macrophages.

In addition to CD4+ and CD8+ T cells, other T cell subtypes with unique functions have been characterized. These include γδ T cells designated by their unique T cell receptor composed of γ- and δ-chains, natural killer T cells (NKT cells) and mucosal associated invariant T cells (MAIT). These cells are abundant in gut mucosa and possess characteristics of both innate and adaptive immune system (99-101).

2.5.1 Development of CD4+ and CD8+ T cells

T lymphocytes originate from bone marrow stem cells, but they mature in thymus where they are called thymocytes. Thymus is composed of two anatomically and functionally distinct areas, medulla and cortex. Bone marrow-derived progenitors first occupy the perimedullary cortex of thymus. During their development, thymocytes move on to the subcapsular regions of the thymic cortex. Finally, thymocytes enter the medulla to be ultimately released into the circulation (102).

Three major developmental stages, named the double-negative (DN), double-positive (DP) and single-positive (SP) stages, dictate the T cell maturation (103). Each developmental step is controlled by the unique regional microenvironment provided by specific thymic stromal cells (102). The progenitors entering the thymus are CD4/CD8 double negative (CD4-CD8-). During their migration towards the outer parts of the cortex, the formation of the $\alpha\beta$ T cell receptor starts with the rearrangement of the TCRβ gene. Concurrently, thymocytes undergo extensive proliferative expansion.

A successful TCRβ gene rearrangement leads to the expression of the preTCR molecule, an immature form of T cell receptor that consists of a TCRβ chain and an invariant pre-α chain. The preTCR expression, in turn, induces the DP stage in the cells, i.e. the expression of both CD4 and CD8 molecules on their surface. At this point, the rearrangement of the

TCRα gene takes place, and the cells start expressing mature TCRs (102). While migrating back towards medulla, the DP thymocytes undergo positive selection, i.e. they try to engage a self peptide:MHC complex expressed by the thymic stromal cells. The vast majority, 90%, of the thymocytes fail and die by neglect. If the TCR of a thymocyte recognizes a peptide bound to an MHC class I molecule, the cell produces a survival signal and downregulates the expression of CD4 becoming single positive (SP) for the CD8 expression. Respectively, a thymocyte expressing TCR that recognizes a peptide bound to an MHC class II molecule becomes single positive for the CD4 expression. In addition to the MHC ligation, the commitment of thymocytes to either the CD4 or the CD8 lineage seems to require an optimal cytokine milieu (104).

Finally, the SP thymocytes undergo negative selection in the medullary region of thymus rich with professional antigen-presenting cells that express MHC molecules with a variety of peptides from self antigens. Those thymocytes that carry TCRs that bind too strongly to self peptides, undergo apoptosis. As a result of positive and negative selections, cells with low but significant avidity for self peptide:MHC ligands survive and ultimately exit the thymus to join the circulating T cell repertoire (102,105).

2.5.2 T cell activation

Signaling through the T cell receptor is critical for the development, activation and polarization of T cells. In order to achieve correct T cell activation, the TCR needs to recognize the structures of both the antigenic peptide and the MHC molecule presenting it (96,106). To ensure the recognition of the diversity of antigens encountered, an individual must harbor a vast array of different TCR structures. The variability is achieved by the somatic recombination of three types of gene segments of the TCR α and β chains, termed V (variable), J (joining) and D (diversity). There are numerous copies of each segment. During somatic recombination, the segments assemble randomly, producing a unique coding sequence for the TCR of each lymphocyte (97). In the mouse, the process has been estimated to result in a theoretical repertoire of $>10^{15}$ unique $\alpha\beta$ TCRs (107). Considering that both TCR and MHC molecules also exhibit conformational flexibility at the peptide-binding site (108), virtually almost any peptide structure can be recognized by the TCRs of an individual.

The cytoplasmic tail of the TCR molecule is too short to transmit an activation signal into the cell. Instead, the intracellular downstream signaling is initiated by the nonpolymorphic CD3 molecule complex associated constitutively with the T cell receptor. In the process, the cytosolic domains of CD3 containing the immunoreceptor tyrosine-based activation motifs, ITAMs, are phosphorylated by the lymphocyte-specific protein tyrosine kinase, LCK (109). The following phosphorylation cascade initiated by LCK culminates in the activation of several intracellular signaling routes that activate transcription factors (such as NF-κB, NFAT and AP-1) and eventually the genes related to the proliferation and differentiation of T cells (110). In addition, the coreceptor molecules CD8 and CD4 on T cells bind to the nonpolymorphic regions of the membrane-proximal domains of MHC class I and II molecules, respectively. They are thought to enhance TCR triggering at least by stabilizing the T cell receptor:peptide MHC (TCR:pMHC) interaction (111).

Stimulation through the TCR:pMHC complex is not alone sufficient for the proper activation of the T cell. In fact, it can result in T cell anergy, a hyporesponsive state where the T cell proliferation and effector functions are inhibited (112,113). Therefore, T cells require a secondary signal transmitted through a set of costimulatory receptors on the T cell surface. The T cell activation also requires a third signal from certain cytokines, such as interleukin (IL)-12 or interferon (IFN)-I, in order to achieve efficient expansion and good effector functions (114) (Figure 2).

The most important costimulatory receptor on the T cell surface is CD28, which not only enhances the TCR-mediated activation of the cell but also induces the expression of other costimulatory receptors (115). CD28 is ligated to the B7-1 (CD80) and B7-2 (CD86) glycoproteins on APCs. B7-2, which is constitutively expressed on APCs at low levels, is upregulated before B7-1 and plays a more significant role in the priming of T cell activation. Instead, B7-1 is virtually absent in non-activated APCs, but becomes highly expressed once upregulated. B7-1 has been speculated to play more of an inhibitory role in the T cell activation (116). In addition to a number of other effects, CD28 signaling increases substantially the production of the cytokine IL-2, which enhances T cell growth, proliferation and differentiation (115). The binding of CD28 to its ligands leads to the up-regulation of another molecule, cytotoxic T-lymphocyte-associated protein (CTLA)-4, on T cells. CTLA-4 is also ligated to B7 molecules, but releases an inhibitory signal by blocking the TCR downstream pathways and controlling T-cell adhesion and motility (117). Numerous other recently identified molecules have also been shown to play costimulatory roles in T cell activation. These include the molecules OX40, inducible T-cell costimulator (ICOS), 4-1BB and CD30 that are upregulated only upon TCR-mediated signaling (118).

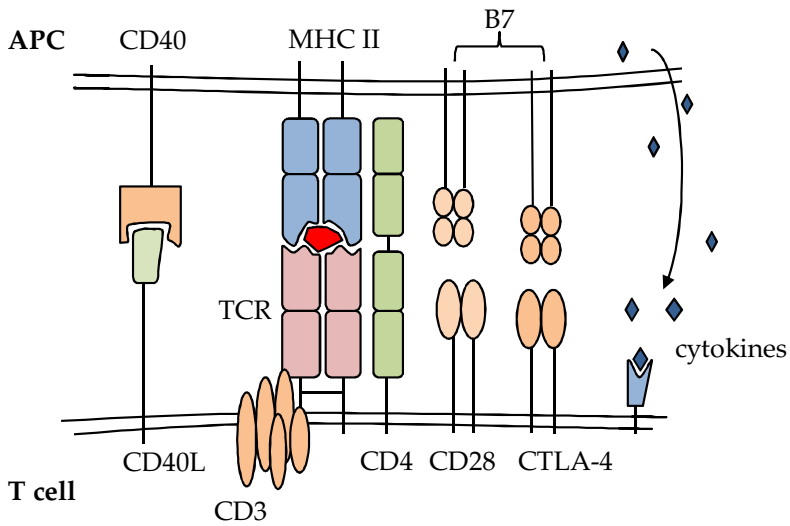

Figure 2. TCR-mediated signaling and costimulation.

During TCR signaling, the pMHC:TCR-complexes cluster in the contact site between the T cell and APC. The contact area is called the immunological synapse (IS). It is formed by an outer ring (peripheral supramolecular activation cluster, pSMAC) that mostly consists of adhesion molecules and a central area (cSMAC) enriched in TCRs and costimulatory molecules. The immunological synapse is a dynamic structure that has been hypothesized to sustain TCR signaling and focus the signal transmission and cytokine secretion between the interacting cells (119).

In addition to receiving costimulatory signals, T cells also provide costimulatory help to the other cells of adaptive immunity. For example, an important contact point between activated T and B cells is the ligation of CD40L (CD154), a transiently expressed ligand on T cells upon their activation, to CD40, which is required for the isotype-switching and the production of antibodies by B cells (120).

2.5.3 CD4+ T cell differentiation

T cells that have not encountered an antigen are called naïve T cells. Upon encountering an antigen, CD4+ T cells become activated, undergo clonal expansion and differentiate into effector T cells. Different effector T cell subtypes promote differential inflammatory responses that are mediated mainly by the secretion of cytokines. The three major CD4+ T cell subsets include the well-characterized Th1 and Th2 cells discovered almost 30 years ago (121) and the more recently identified Th17 (122) cells. Th1-type CD4+ T cells are the main inducers of the macrophages and the CD8+ T cells that are responsible for the cytolytic immune response. They also provide support for the IgG isotype class switching of B cells. IgG antibodies are effective in opsonizing pathogens, allowing their ingestion by phagocytes. Therefore, the Th1-type CD4+ T cells play a major role in the protection against intracellular pathogens and they participate in the elimination of cancerous cells (123). Th2-type cells support the immune response against helminth infections and other parasites by stimulating eosinophils, mast cells and the IgE-producing B cells (123). Th17 cells are involved in conferring resistance against extracellular bacteria and fungi (122). The three CD4+ T cell subtypes are also involved in many harmful immune responses, such as the tissue destruction seen in type 1 diabetes, multiple sclerosis or other autoimmune diseases (Th1, Th17) as well as the pathogenesis of allergy (Th2, see 2.3) (124).

Several other CD4+ T cells populations, such as Th9 (125), Th22 (126) and follicular T helper (T_{FH}) cells (127), have also been recognized during recent years, although it is still a matter of debate whether they represent unique CD4+ T cell lineages or simply reflect the heterogeneity of the existing subtypes. The Th9 cells have been shown to exert a proinflammatory effect in allergies and autoimmune diseases (125) whereas the Th22 cells are thought to play a role in the regulation of inflammatory response, especially in the skin (126). The follicular helper T cells are specialized in providing help in B cell activation in lymphoid follicles (127). In addition, several T cell populations with regulatory functions (see 2.5.5), are also subsets of CD4+ T cells. The growing number of CD4+ T cell subtypes, along with the finding that some T cell populations exhibit the capacity to convert from one type into another (128), highlights the plasticity and complexity of the helper T cell network.

The CD4+ T cell differentiation is regulated by all the elements of TCR signaling (see 2.5.2), i.e. the quality of the pMHC:TCR contact, costimulatory interactions and the surrounding cytokine microenvironment. The strength of TCR signaling is mainly affected by the antigen dose, its affinity and the duration of the stimulation. In general, weak signaling appears to favor Th2 differentiation whereas stronger signaling promotes Th1 differentiation (84,129-131). The polarizing effect of the stimulation through costimulatory receptors is unclear, however, at least the activation of intercellular adhesion molecules (ICAM-1 and 2) has been postulated to contribute to Th1 polarization whereas the inducible T cell costimulator (ICOS) activation seems to promote the Th2 phenotype (132-134).

The cytokines produced by antigen-presenting cells (mainly dendritic cells, DCs) are a major determinant of CD4+ T cell differentiation. Their commitment to express particular cytokines is largely determined by the recognition of pathogens via their pattern recognition receptors (PRRs) and by the signals they receive from other inflammatory cells. Binding of the T cell surface molecule CD40L to CD40 on APCs induces the secretion of the cytokines at the optimal time point (135).

With respect to the induction of Th1-type CD4+ T cells, the most important DC-derived cytokine is IL-12. This cytokine activates the transcription factor T-bet, a key regulator of the Th1 commitment via the STAT1 pathway and subsequently induces the production of IFN-γ by Th1 cells. IL-12 also inhibits Th2 deviation by blocking the transcription factor GATA-3, a hallmark of the Th2 phenotype (124). The cytokine IL-4, instead, is the main activator of GATA-3 through STAT6 in Th2 cells. The initial source of IL-4 is not clear, but it has been thought to be produced by basophils, macrophages, group 2 innate lymphoid cells or by the T cells themselves (131,136). DCs appear to be incapable of producing IL-4 (123). GATA-3 expression upregulates the production of the Th2 cytokines IL-4, IL-5 and IL-13 and inhibits Th1 differentiation (124). The respective commitment pathways of the Th17 and iTreg cells (see also 2.5.5) are shown in Table 4.

Table 4. Hallmarks of CD4+ T cell subtypes (summarized from (124,135,137)).

	Th1	**Th2**	**Th17**	**iTreg**
inductive cytokine	IL-12	IL-4	TGF-β (+ IL-6)	TGF-β (- IL-6)
STAT	STAT1	STAT6	STAT3	STAT5
transcription factor	T-bet	GATA-3	RORγ	FOXP3
surface marker	CXCR3	CCR4, CRTH2	CCR6, CD161	(CD25)
cytokines produced	IFNγ, LT-α	IL-4, IL-5, IL-13	IL-17, IL-17F	IL-10, TGF-β

2.5.4 CD4+ T cell homeostasis

Continuous low-level contact with MHC molecule-bound self peptides in combination with the exposure to IL-7 is needed for the survival of resting naïve T cells (114). In the quiescent state, they mainly reside in secondary lymphoid organs and are characterized by the expression of the respective homing receptors CCR7 and L-selectin (CD62L) (138). The recognition of an antigenic peptide by naïve T cells induces their rapid proliferation and differentiation into effector T cells (139). Effector T cells lose the expression of CCR7 and L-selectin and start expressing receptors for inflammatory chemokines and homing molecules

that allow their extravasation and entrance into the infected parenchymal tissue (138). After an antigen challenge, the vast majority (>90%) of effector cells are destroyed (139). Nonetheless, a fraction of them do survive to become long-lived effector memory T cells that reside in peripheral tissues and are capable of rapidly responding and exerting effector functions ($CCR7^-$ T_{em}). In addition to effector memory T cells, an antigen challenge leads to the development of another type of memory T cell population, central memory T cells. These cells differ from T_{em} in that they lack any effector function and reside in secondary lymphoid organs ($CCR7^+$ T_{cm}) (140). Their role is regarded as being important in retaining the T cell memory, as they have a self-renewal capacity and are capable of evolving into T_{em} cells. Both central and effector memory T cells are distinguished from naïve cells in terms of their expression of the surface receptor CD45R. Memory T cells typically downregulate the expression of CD45RA, the high molecular weight isotype of the protein that is largely expressed on naïve T cells, and start expressing its low molecular weight isotype, CD45RO, that facilitates T cell activation (141). The cytokine IL-7 also plays a key role in the long-term survival and homeostasis of memory T cells. However, these cells appear to maintain their numbers independently of contact with self peptide:MHC complexes (114).

2.5.5 Regulation of T cell response

The deletion of self-reactive T cells in the thymus is the primary way of establishing tolerance to self structures (central tolerance; see 2.5.1). In the periphery, it is complemented by a number of mechanisms that suppress harmful immune responses and regulate inflammation, collectively referred to as peripheral tolerance (142). Ignorance is the state where the triggering point of T cell activation is not reached, for example, due to low expression levels of a self antigen. Anergy refers to nonresponsiveness to an antigen e.g. it can result from the lack of essential TCR costimulation. T cells may also become apoptotic by repetitive TCR signaling, a phenomenon known as activation-induced cell death *(AICD)* (142).

Regulatory T cells (Tregs) are the key mediators of peripheral tolerance. The two best characterized Treg subgroups are the forkhead box P3 transcription factor-positive Tregs (FOXP3+ Tregs) and the T regulatory type 1 (Tr1) cells. Both subgroups share common properties, such as the expression of the inhibitory receptors cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced TNFR family related gene (GITR) and the ability to suppress effector T cell proliferation and cytokine production (137,143).

FOXP3+ Tregs are characterized by the constant expression of the CD25 molecule, the α-chain of the IL-2 receptor. They can originate from thymus (natural Tregs) as fully functional suppressor T cells or develop in the periphery from naïve CD4+ T cells (inducible or peripheral FOXP3+ Tregs). The natural and inducible FOXP3+ Tregs cannot be clearly differentiated functionally from each other. *In vitro,* both subtypes exert their suppressive effects primarily by contact-dependent inhibition (e.g. via costimulatory ligands), but *in vivo*, they have been reported also to induce suppression through the production of the cytokines TGF-β or IL-10 (144).

The second major subset of CD4+ Treg cells, the Tr1 cells, arises in the periphery from naïve CD4+ T cells activated by tolerogenic antigen-presenting cells independently of

FOXP3 and in the presence of IL-10. Despite the eager attempts, a specific marker to distinguish the human Tr1 cells from other CD4+ T cell subsets remains undefined (145). Importantly, once activated by the TCR ligation to a peptide:MHC complex present on an antigen-presenting cell, Tr1 cells can mediate their suppressive function against cells that recognize other antigens via bystander activity, secreting large amounts of IL-10 as well as transforming growth factor (TGF)-β, but low levels of IL-2 (137,146,147).

Compromised Treg function has been demonstrated to have severe autoimmune-related consequences. For example in mice, neonatal thymectomy that leads to the development of multiple-tissue autoimmunity has been linked to CD4+CD25+ Treg cell deficiency (148). In humans, a fatal autoimmune lymphoproliferative disorder termed the immune dysregulation polyendocrinopathy enteropathy-X-linked (IPEX) syndrome is caused by loss-of-function mutations in the FOXP3 gene (149). In addition to autoimmune diseases, the regulatory T cells appear to play a role also in the pathogenesis and the treatment of allergic diseases (see 2.6.2).

2.6 TREATMENT OF ALLERGY

2.6.1 Symptom control

The symptoms of allergy are mainly controlled either pharmaceutically or by avoiding exposure to the allergen. The symptomatic drugs include antihistamines, corticosteroids and leucotriene receptor antagonists that provide immunosuppression relieving the symptoms effectively. However, the use of symptomatic drugs in allergy is often long-standing (even life-long), which can be a factor hampering patient adherence to the treatment, e.g. due to the side effects or the cost of medication (150). Allergen avoidance is considered as a necessity in the case of severe allergic reactions. However, complete allergen avoidance is often problematic as allergens are present not only in domestic houses but also in public places, such as schools and day care centers (5,151).

2.6.2 Allergen-specific immunotherapy

Allergen-specific immunotherapy (ASIT) refers to the practice of administering gradually increasing quantities of an allergen product to an individual with an IgE-mediated allergic disease in an attempt to ameliorate the symptoms associated with the exposure to the causative allergen (7). ASIT is the only disease-modifying approach available for the treatment of allergy. The first immunotherapeutic trials were conducted on hay fever patients with grass pollen extract by Leonard Noon and John Freeman already a century ago, establishing the therapy guidelines that are still largely pertinent today (152,153).

According to the EAACI Guidelines (7), allergen-specific immunotherapy is indicated for the treatment of moderate to severe allergic rhinoconjunctivitis and mild to moderate asthma and insect venom allergy, but not for allergic eczema or food allergies. The candidate patients for ASIT need to exhibit a laboratory-confirmed IgE sensitization to the causative allergen and an exacerbation of the symptoms under allergen challenge. Factors further supporting the decision to treat include poor response to pharmacological symptom control, the development of adverse side effects to the medication and/or a frequent,

unavoidable exposure to the sensitizing allergen. Absolute contraindications include a serious immunological disease, a major cardiovascular disease, chronic infections and severe asthma. Allergen-specific immunotherapy requires a period of three to five years. Currently, two types of ASIT are in practice: subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) (154). Both methods have been shown to be effective in reducing symptoms and the use of medications (155,156), inducing long-term (~10 yrs) tolerance (157,158) and preventing disease progression and new sensitizations (159).

The cellular mechanisms underlying the clinical efficacy of allergen-specific immunotherapy are still largely unclear. Previously, it has been demonstrated that the balance between the allergen-specific Th2-biased response and immune tolerance is altered in allergic in comparison to nonallergic individuals (160,161). Therefore, one possibility is that the therapeutic effects of ASIT are linked with the restoration of this balance. In fact, several studies describing the cellular alterations during allergen immunotherapy have demonstrated the deletion of allergen-specific effector Th2 cells or a downregulation of their response (162,163). In addition, enhanced peripheral regulatory activity characterized by an increase in the levels of cytokines IL-10 and TGF-β (164,165) and in the number of allergen-specific regulatory T cells (162,166) have been reported. Both FOXP3+ Tregs and inducible Tr1 cells seem to play a role in the process (167).

Regulatory T cells have also been shown to mediate the protective effects of allergen-specific immunotherapy to other immune cells. For example, they are capable of inducing the production of IgG4 antibodies by B cells (168). IgG4 antibodies are regarded as protective antibodies and their levels generally increase during successful ASIT. The protective effects of IgG4 are thought to be mediated by blocking the IgE-binding of allergens and the IgE-facilitated antigen presentation by B cells (169). Finally, regulatory T cells have been shown to be able to modulate the function of dendritic cells (170,171).

2.6.3 Hypoallergens

The practice of allergen-specific immunotherapy is largely based on empirical experience and may encounter several problems related to the efficacy and safety of the treatment. The use of unstandardized allergen preparations may potentially lead to unpredictable adverse reactions and even new allergic sensitizations (172-174). The long duration of the treatment is a major reason for patient adherence problems (150). Most importantly, allergen-specific immunotherapy is commonly associated with mild to moderate side-effects, but it also involves the risk of developing severe systemic reactions, even anaphylaxis (154). To overcome these problems, it will be necessary to develope innovative allergen products.

An important cause of systemic side effects during allergen-specific immunotherapy is the cross-linking of allergen-bound IgE on the FcεRI-receptors on mast cells (see 2.3). As the IgE binding of an allergen is dependent on its tertiary structure, conformational modifications of the allergen represents one way to reduce this capacity while conserving the T cell reactivity. Several techniques for engineering this type of hypoallergenic products have been introduced. For example, the reduced IgE binding and basophil activation together with stronger T cell stimulatory capacity have been associated with the hypoallergenic derivative of the major cat allergen Fel d 1, which was generated by the duplication of selected T cell epitopes and disruption of the disulphide bonds within the

protein (175). A similar effect was obtained with the introduction of point mutations in the amino acid sequence of the major apple allergen Mal d 1 (176). Moreover, treatment with genetically fragmented or polymerized forms of the major birch allergen Bet v 1 has been shown to be clinically well-tolerated and effective (177-179). Another promising approach to establish allergen-specific T cell tolerance is to use allergen-derived peptide fragments that are capable of stimulating T cells but too short for IgE-cross linking (see 2.6.4; (8,180)).

2.6.4 Peptide immunotherapy

The capability of allergen-derived peptides to induce tolerance to a larger allergen protein was first described with a murine model of cat allergy by Briner et al. (181). Immunization of mice with the peptides derived from the major cat allergen Fel d 1 resulted in a decreased production of IL-2 and allergen-specific IgG. Thereafter, preclinical studies have been performed with various allergens, such as Der p 2 (182), Bet v 1 (183), and the bee venom allergen phospholipase A_2 (PLA_2) (184). The two major cellular outcomes reported by these studies have been the suppression of T cell proliferation and decreased production of the cytokines IL-4, IL-13 and IFN-y. In addition, a decrease in the allergen-specific IgE levels and an increase of the production of the protective IgG4 antibody were seen.

Clinical trials of peptide immunotherapy have been conducted with two allergens; cat Fel d 1 and bee venom allergen PLA_2. Soon after the first murine experiments (181), the peptides derived from the major cat allergen Fel d 1 were developed into a vaccine (ALLERVAX CAT) for clinical testing (185,186). The trials showed modest amelioration of nasal and lung symptoms, however, there were also frequently occurring immediate side effects, evidence that the 27-mer peptides were too long to avoid IgE-cross linking. Subsequently, the use of shorter Fel d 1-derived peptides (16-17 residues) was shown to suppress early phase reactions more effectively (187). In the following years, clinical trials in patients with bee venom allergy were also conducted with peptides derived from PLA_2. In a study where five patients with systemic IgE-reactions to bee stings were treated with a mixture of three immunodominant PLA_2 peptides, three subjects developed clinical tolerance, while the effect was reported to be suboptimal in the other two subjects (188). One of the putative explanations was the limited HLA coverage of the selected T cell epitopes in the vaccine. Indeed, another trial where the number of peptides was increased to cover the whole PLA_2 sequence, i.e. all possible T cell epitopes to match any given HLA-genotype, achieved an improved outcome (189). Similarly, promising results have been reported in recent clinical studies where a mixture of seven immunodominant Fel d 1 T cell epitope-containing peptides of 13 to 17 amino acids was selected based on their wide HLA-binding coverage (190,191). The trials also described persistent improvements of nasal and ocular symptoms and good tolerability of the treatment (190,191). Collectively, these studies suggest that the length of selected peptides and the genotype variety among patients are factors that need to be considered for the safety of peptide immunotherapy.

When the cellular basis of the clinical outcomes was studied, the results were in line with the preclinical studies showing the suppression of Th2-linked effector functions (187,189). In addition, the enhanced production of IL-10 was reported (187,189). The IL-10 secretion in peptide-immunotherapy is claimed to be attributable to the induction of Tr1-like cells (192). They are thought to exert their therapeutic effects by linked epitope suppression, a process

where the treatment with selected epitopes from an allergen resulted in the suppression of responses to other epitopes within the same molecule (193). Another mechanism promoting tolerance induction in peptide immunotherapy is associated with the administration of the peptides, as delivering of peptides in a soluble form without an adjuvant may establish noninflammatory, tolerogenic conditions for T cell recognition (194).

2.6.5 Altered peptide ligands in immunotherapy

The therapeutic potential of structurally modified T cell epitopes (peptide analogues of altered peptide ligands, APLs) in peptide immunotherapy has been evaluated in preclinical models of infectious diseases (195), cancer immunology (196) and autoimmune diseases, such as multiple sclerosis (197,198), type 1 diabetes (199,200) and arthritis (201-203). APLs share the characteristics of the native peptide sequence but, as a result of amino acid substitutions, deliver antagonistic, partial agonistic or superagonistic signals that may induce alterations in the pMHC:TCR binding and modify the subsequent T cell response (204-206). Stimulation with antagonistics/partial agonistic APLs has been demonstrated to induce suppression of T cell proliferation (197,198) and deviation towards the Th2 phenotype assessed by the production of cytokines (197,202) or the expression of transcription factors T-bet and GATA3 (202). Instead, agonistic/superagonistic interactions with APLs have generally increased the proliferation capacity of T cells and the production of IFN-γ (195,199,201). The regulatory mechanisms suggested to be involved in the APL-mediated T cell activation include the induction of anergy (198,200) or apoptosis (203) and the activation of regulatory T cells (197,203).

In the few studies that have evaluated the potential of APLs for use in the immunotherapy of allergy *in vitro*, superagonistic (or heteroclitic) APLs have proved to be therapeutically promising. They seem to be capable of inducing anergy and apoptosis in the responding T cells and shifting the Th2-type response towards the Th1-type response (207-210).

Only a limited number of clinical trials have been conducted with altered peptide ligands. In one study, treatment of multiple sclerosis (MS)-patients with an APL of a myelin basic protein epitope achieved the desired cellular effects, the skewing of the T cell response towards the Th2-phenotype. However, it unfortunately also led to immune hypersensitivity reactions in the patients (211). In another MS study, the expansion of an APL-specific T cell population was demonstrated, but it proved to be Th1-deviated and induced disease exacerbations (212). No improvement in the clinical condition of the patients was observed and both studies were prematurely terminated due to the adverse side effects. A more recent type 1 diabetes trial reported equally disappointing results; the treatment of type 1 diabetes patients with an APL of the B-chain of insulin did not result in any inhibition of autoreactive T cells or any improvement of the clinical status of the patients (213). No clinical trials with allergic patients have been conducted to date.

3 Aims of the Study

The aims of the study were

- To analyze *in vitro* the antigenicity of a dominant epitope of the major dog lipocalin allergen Can f 1 contained in the peptide p105-120 and to evaluate the potential of its single amino acid modified analogues for allergen-specific immunotherapy (I).

- To compare *in vitro* the antigenicity of dog Can f 1 and its human homologue, tear lipocalin (II).

- To compare *in vitro* dog Can f 1 and Can f 4-specific memory T cell responses between allergic and nonallergic individuals (II-III).

- To identify the human T cell epitopes of the dog lipocalin allergen Can f 4 and to assess *in vitro* their potential for use in peptide immunotherapy (III).

4 Materials and Methods

4.1 SUBJECTS

Altogether 25 dog-allergic subjects (I-III) and 19 healthy dog-dust exposed controls (II-III) were recruited into the studies. The allergic subjects were characterized at the Pulmonary and Dermatology Clinics of Kuopio University Hospital (64). They were clinically atopic and reported exacerbation of allergic symptoms upon dog-dust exposure. They exhibited positive dog UniCAP results (FEIA; Pharmacia, Uppsala, Sweden; >0.7 kU/L) and positive skin prick tests (SPT; ≥3 mm) with a commercial dog epithelial extract (ALK Abellò, Hørsholm, Denmark). The sensitization to rCan f 1 (I-II) was verified by SPTs (64) and to rCan f 4 (III) by indirect ELISA (Rytkönen-Nissinen M, manuscript submitted). The control subjects reported no medical history of atopy or allergic symptoms of any kind. The HLA class II genotypes (DR/DQ) of the subjects were determined in the Clinical Laboratory of Finnish Red Cross Blood Service (Helsinki, Finland) or in the Immunogenetics Laboratory of University of Turku (Turku, Finland) by the PCR-SSO and PCR-SSP methods (214,215). The study was approved by the Ethics Committee of the Kuopio University Hospital and written informed consent was obtained from all participants.

4.2 ANTIGENS

4.2.1 Recombinant antigens (I-III)

The recombinant (r) dog allergens rCan f 1 (I) and rCan f 4 (III) were produced in *Pichia pastoris*, and the IgE reactivity of the recombinant products was verified by immunoblotting (60,64,66).

4.2.2 Synthetic peptides (I-III)

The Can f 1 peptide p105–120 (HGRQIRMAKLLGRDPE) and its 16-mer single amino acid variants (I) were synthesized with PerSeptive 9050 Plus automated peptide synthesizer (Millipore) with Fmoc strategy. The peptides were purified by HPLC (Shimadzu) with C18 reverse phase column (Vydac) and verified with a MALDI-TOF mass spectrometer (Bruker)(8). All peptides were readily soluble in water.

The sequences of Can f 1 and tear lipocalin (II) were aligned by the SIB BLAST network service at the Swiss Institute of Bioinformatics (web.expasy.org/blast/). The Can f 1 peptides pC1–pC9 and homologous tear lipocalin peptides pTL1–pTL9 ranging from 16 to 19 amino acids were selected based on the verified T cell epitopes of Can f 1 (8) and the predictions of HLA-DR-binding motifs in Can f 1 and TL (see 4.7). The peptides were synthesized at >80% purity by GL Biochem Ltd., China. For the induction of T cell lines (TCLs), the peptides were arranged in pools, each of the six pools containing three Can f 1 or TL peptides as follows: pC1+pC4+pC7, pC2+pC5+pC8, pC3+pC6+pC9, pTL1+pTL4+pTL7,

pTL2+pTL5+pTL8, and pTL3+pTL6+pTL9. The influenza hemagglutinin (HA) peptide (amino acids 306–318) (216), was also produced by GL Biochem Ltd.
The 48 16-mer Can f 4 peptides (III) overlapping by 13 amino acids covering the entire sequence of Can f 4 were synthesized at >80% purity by GL Biochem Ltd.

4.3 CELL SEPARATION

4.3.1 Isolation of peripheral-blood mononuclear cells (I-III)

Peripheral-blood mononuclear cells (PBMCs) were freshly purified from heparinized blood by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Briefly, whole blood was first diluted 1:1 by phosphate-buffered saline (PBS) and gently layered onto a Ficoll-Paque PLUS solution in a conical tube. After centrifugation (Beckman Coulter Allegra X-12 Centrifuge), the PBMC layer was collected from the diluted plasma/Ficoll interface, then centrifuged once more to reduce platelet/plasma protein contamination and resuspended in the medium appropriate to the further application. The cells were counted after Erythrocin B (Sigma-Aldrich Chemie, Steinheim, Germany) exclusion in a Bürker hemocytometer.

4.3.2 Isolation of CD4+ T cell subsets with magnetic beads (II-III)

Magnetic-activated cell sorting was used for the depletion of $CD4+CD25^{high}$ T cells from PBMCs (II) with anti-CD25-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and for the isolation of memory ($CD4+CD45RA^{-}CD45RO^{+}$) T cells from PBMCs (III) by the depletion of non-CD4+ T cells and naïve CD4+ T cells using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD45RA, CD56, CD123, TCRγ/δ and Glycophorin A with magnetic anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Briefly, the freshly isolated PBMCs were first labelled with the specific antibodies and magnetic beads. After washing, the cells were passed through a column placed in a magnetic field. The magnetically labeled cells were retained in the column. The unlabeled cells passing through the column were collected and washed for further use. To verify the purity of the isolation, a fraction of the collected cells was analyzed simultaneously in parallel with unseparated PBMCs in a BD FACSCanto II flow cytometer (see 4.5.4) after staining with anti-CD4-fluorescein isothiocyanate (FITC) and anti-CD25-phycoerythrin (PE) in study II and anti-CD4-FITC, anti-CD45RA-APC and anti-CD45RO- phycoerythrin-Cy7 (PE-Cy7) in study III (all from BD Biosciences, San Jose, CA). The mean purity of the memory CD4+ T cells (III) was routinely >97%. The cells retained in the column (III) were used as antigen-presenting cells after irradiation (3000 rad).

4.4 T CELL CULTURES

4.4.1 Culture medium (I-III)

The complete culture medium 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 (all from Lonza, Verviers, Belgium) and 5% inactivated human AB serum (Sigma–Aldrich, St. Louis, MO), was used for culturing T cell clones and lines.

4.4.2. Generation of T cell lines and clones (I-III)

For the generation of long-term T cell lines, freshly isolated PBMCs (I), those depleted of CD4+CD25high T cells (II) or purified CD4+CD45RO+ T cells (III) were seeded in 96-well round-bottomed plates (Corning Scientific) in a 150-200 μl volume of complete medium with

Study I	Can f 1 peptide p105-120 or its analogues (2×10^5 PBMCs per well, 20 wells per peptide at 5 μg/ml)
Study II	pooled (see 4.2.2) Can f 1 or TL peptides or the HA peptide (10^5 PBMCs per well, 30 wells per pool, each peptide at 2 μM)
Study III	rCan f 4 (5×10^4 cells per well; 60-90 wells per subject; 10 μg/ml of rCan f 4)

Wells with 1 μM of PPD served as a positive control (II, III). The TCLs were maintained in the complete medium supplemented with 10-20 IU/ml of IL-2 until the split well analysis. In studies I and III, the cultures were restimulated once before the analysis with 1.5×10^5 autologous γ-irradiated (3000 RAD) PBMCs together with 5 μg/ml of the peptides (I) or 10 μg/ml of rCan f 4 (III).

The antigen specificity of the cultures was tested on day 10 (II) or 20 (I, III) by measuring their proliferation with the split well assay. First, half of the cells in each well were split into two new daughter plates and stimulated with or without the antigens together with autologous γ-irradiated (3000 rad) PBMCs (5×10^4 per well) as antigen-presenting cells. After an incubation period of 72 h, [3H]thymidine was added (1 μCi per well; GE Healthcare, Little Chalfont, UK), and after an additional 16 h, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). The radionuclide uptake of the cultures was measured by scintillation counting (Wallac Micro Beta 1540). The results were expressed as counts per minute (CPM). Antigen-specific proliferation was defined based on the stimulation indices (SI; CPM in the presence of antigen divided by CPM in the absence of antigen) and CPM differences between stimulated and unstimulated wells (ΔCPM).

Criteria for positive cultures:

Study I	SI>2
Study II	ΔCPM>2000 and SI>2
Study III	ΔCPM>1000 and SI>2

Positive cultures were next restimulated in 48-well plates with the specific antigens, autologous γ-irradiated PBMCs (10^6/well) and 25-50 IU/ml of IL-2. Thereafter, half of the culture medium was replaced with fresh medium supplemented with IL-2 every 2–3 days during the 14-day culture until they were used in the proliferation assays. As the TCLs established in study II were induced with peptide pools, they were tested after the 14-day culture with each of the three individual peptides in the pool at 10 μM (2.5×10^4 T cells with 5×10^4 autologous γ-irradiated PBMCs as APCs) to determine the peptide specificity. TCLs showing a peptide-specific response (ΔCPM>2000, SI>2) were then restimulated once more with the individual peptide at 2μM and 1.5×10^5 autologous γ-irradiated (3000 RAD) PBMCs before proliferation assays.

The T cell clones 2C9 and 5G2 (I) were isolated from two Can f 1 p105-120-specific T cell lines from a dog-allergic patient by the limiting dilution method basically as previously described (8,10). PBMCs were cultivated on 12-well plates at a density of 6×10^6 cells per well in complete culture medium in the presence of 10 μg/ml of p105-120. On day 6, human IL-2 was added at a final concentration of 5 IU/ml. On day 9, the IL-2 concentration was optimized to 20 IU/ml. On day 14, the cells were restimulated with p105-120 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. The restimulation was completed as described above. The T cell clones were isolated from rCan f 1-specific T cell lines by the limiting dilution method. Specifically, T cells were seeded out into wells of round-bottomed 96-well plates containing allogenic γ-irradiated (6000 rad) PBMCs as feeder cells ($2\text{-}3 \times 10^5$ cells/well), PHA (1 μg/ml) and recombinant IL-2. The cultures were supplied with fresh medium and IL-2 (25 IU/ml) at 2-3 day intervals, and on day 7 with fresh feeder cells ($2\text{-}3 \times 10^5$ cells/well) and IL-2. When growth became visible (on days 12-20), the reactivity of the growing clones to p105-120 was tested in the thymidine incorporation assay (see above). The clonality of the T cell clones was verified by PCR using a panel of TCR Vβ subfamily-specific primers, as described previously (217). The TCR Vβ usage of the clones was Vβ8 for 2C9 and Vβ4 for 5G2.

4.5 ANALYSES OF LYMPHOCYTE FUNCTION

4.5.1 T cell proliferation assays (I-III)

The T cell proliferation assays were set up in 96-well round-bottomed plates in triplicates or duplicates for the T cell clones (I) and T cell lines (I-III), respectively. After a 72h incubation of 2.5×10^4 T cells with 5×10^4 autologous γ-irradiated PBMCs with and without the respective antigen up to five 10-fold dilutions, the proliferation of the T cells was assessed by [3H]thymidine incorporation (see 4.4.2). Dose-response curves were generated for each TCC (I) and TCL (I-III) to determine the concentration of the peptide needed to induce a half-maximal proliferative response (effective concentration 50, EC_{50}) as an indicator of the functional TCR avidity. The frequencies of antigen-specific T cells (II, III) were estimated by dividing the number of antigen-specific TCLs in the proliferation assays by the number of cells seeded per subject, assuming that each of them represented a monoclonal expansion of a specific CD4+ T cell.

The Can f 4 epitope mapping (III) was conducted by stimulating rCan f 4-specific TCLs with 48 overlapping 16-mer Can f 4 peptides (5 μg/ml) in duplicate wells. Wells containing PHA (1 μg/ml) and those without stimulant were included as positive and negative controls. A proliferation response of SI>2 to at least two adjacent peptides was regarded as specific.

4.5.2 HLA class II restriction analyses (I, III)

The HLA class II restriction of the TCCs (I) and TCLs (III) was determined by assessing the capabiliity of monoclonal antibodies (mAbs) specific for HLA-DR (L243), DP (B7/21) or DQ (SPVL3) to inhibit the peptide-specific proliferative responses of the cultures *in vitro*. 5 x 10^4 APCs were first incubated in duplicate for 1 h with 10 μg/ml of the antibodies. Next, 2.5 x 10^4 T cells together with 10 μg/ml of the specific peptide were added to the wells. Inhibition of the proliferative response by at least 50% in the [3H]thymidine incorporation test was considered statistically significant. In addition, individual restricting alleles were determined by using allogenic, partially HLA-matched PBMCs and/or EBV-transformed B lymphoblastoid cell lines with known HLA genotypes as APCs.

4.5.3 Cytokine production assays (I-III)

For the cytokine assays, T cell culture supernatants (100 μl/well) were collected from the 96-well proliferation assay plates after a 72 h stimulation with a specific antigen. Supernatants from replicate test wells were pooled and stored at −70°C until analyzed. The production of IL-4, IL-5, IFN-γ, IL-10 and IL-17 was measured by using the commercial Bioplex system (I; Bio-Rad, Hercules, CA) or ELISA kits (II,III; DuoSet; R&D Systems; Minneapolis, MN) according to the manufacturers' instructions. To assess the cytokine phenotypes of TCLs (III), an IFN-γ/IL-5 ratio was calculated (Th1: >5; Th2: <0.01; Th0: 0.01-5).

4.5.4 Flow-cytometric analyses (I-III)

For the analysis of the T cell receptor Vβ subtypes expressed by the TCLs (I), the IOTest Beta Mark kit (Immunotech, Marseille, France) was used according to the manufacturer's instructions with a BD FACSCanto II flow cytometer, as previously described (9). Briefly, the cells were incubated with anti-CD4-PE-Cy5 antibody together with 24 fluorochrome-labeles anti-Vβ antibodies (Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ10, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, Vβ23), washed twice with PBS and analyzed by flow cytometry In the test kit, three TCR Vβ-specific antibodies were combined in a single test. One antibody was conjugated to FITC, another one to PE, and the third to both FITC and PE. TCR Vβ expansions were considered statistically significant when more than 10% of the CD4+ T cells expressed a particular Vβ subtype and the level of expression was at least two times greater than that on PBMCs.

The expression of a panel of cell surface markers (CD45RO, CD45RA, CD62L, CCR7, CD25, CTLA-4, CXCR3, CCR4 and CCR6) by TCLs (II, III) was analyzed by flow cytometry using monoclonal antibodies labeled with PE, PE-Cy7 or allophycocyanin (APC; all from BD Biosciences) or corresponding isotype control mAbs. The results were expressed as mean fluorescence indices (MFI).

4.6 HLA CLASS II PEPTIDE-BINDING ASSAYS

A panel of common HLA-DR (I-III) and HLA-DP4 (I-II) molecules was first immunopurified from homologous EBV cell lines by affinity chromatography using the monomorphic mAbs L243 and B7/21, respectively. The binding affinity of the Can f 1 peptide p105-120 and its analogues (I), Can f 1 peptides pC1-pC9 and TL peptides pTL1-pTL9 (II), and the peptides covering the immunodominant regions of Can f 4 (III) to the HLA molecules was assessed by competitive ELISA, as described elsewhere (218-220). Briefly, the HLA molecules were incubated with different concentrations of the competitor peptide and an appropriate biotinylated peptide, and the bound peptide was assessed in a fluorescence assay. The concentration of the competitor peptide that prevented binding of 50% of the labeled peptide (IC_{50}) was calculated. To assess the validity of independent experiments, unlabeled forms of the biotinylated peptides were used as reference peptides. The results were expressed as IC_{50} ratios (ratio between the IC_{50} value of the tested peptide and that of the reference peptide) in order to take into account the disparity of the binding sensitivity between different HLA molecules. Lower IC_{50} values correspond to higher binding affinities. An IC_{50} ratio of < 20 was considered as strong binding affinity, IC_{50} ratio between 20-100 as moderate binding affinity and IC_{50} ratio > 100 as low binding affinity in these studies.

4.7 PREDICTIONS OF HLA CLASS II BINDING SEQUENCE MOTIFS

The HLA DR binding motifs of the Can f 1 and TL sequences (II) were predicted with the ProPred program (http://www.imtech.res.in/raghava/propred; (221)) and the top 3% binders were selected. The IEDB resource Consensus tool (222,223) was used for the HLA DR, DP and DQ binding predictions for the sequence of Can f 4 (III). Alleles expressed by the study population were included in the analysis and the top 3% percentile rank of each loci were selected.

4.8 STATISTICAL ANALYSES

The statistical analyses were conducted using the commercial GraphPad Prism software (Graphpad Software, San Diego, CA). The Mann-Whitney *U* test was used for the analysis of differences in the T cell frequencies, proliferation responses, cytokine production and surface marker expression between TCLs stimulated with different antigens (I-II) or TCLs obtained from different subject groups (II-III). The Fisher's exact test was used to assess the distribution of TCLs into specific avidity (II) or cytokine phenotype categories (III), and to analyze the association between the atopic status of the donors and the number of TCLs obtained (III). The Wilcoxon signed rank test was used to evaluate the binding affinity between Can f 1 peptides and their TL homologues to a set of HLA molecules (II), and to compare the HLA-binding of the peptides covering the immunodominant regions of Can f 4 (III). *p* values of 0.05 or less were considered statistically significant.

5 Results

5.1 HUMAN T CELL RESPONSE TO THE PEPTIDE P105-120 CONTAINING A T CELL EPITOPE OF DOG CAN F 1 (I)

5.1.1 Characteristics of the p105-120-specific T cell clones

Prior to undertaking the analysis of the human CD4+ T cell responses to the peptide p105-120 containing a dominant T cell epitope of Can f 1(8), two T cell clones (TCCs) specific to the peptide, 2C9 and 5G2, were isolated from the T cell lines of a Can f 1-allergic individual (HLA-DRB1*1101/1501 and DQB1*0301/0602). The characteristics of the TCCs were assessed by measuring their proliferation, HLA-restriction and capability of secreting cytokines. In the proliferation assays, the concentration of p105-120 needed to induce a half-maximal response (EC_{50}) was 0.05 μM (corresponding to 0.1 μg/ml) for clone 2C9 and 1.3 μM (2.4 μg/ml) for clone 5G2, indicating a higher functional TCR avidity for the former clone. Clone 2C9 was found to be restricted by HLA-DQB1*0602 (I, Fig. 1). The restricting locus of clone 5G2 was HLA-DR (I, Fig. 1), but the restricting allele could not be reliably confirmed due to the weak proliferative capacity of the clone (data not shown). Both clones secreted high levels of IL-5 and IFN-γ upon stimulation with p105-120. Based on the calculated IL-5/IFN-y ratios, clone 2C9 was regarded as Th2-biased (ratio >10) and clone 5G2 as Th0/Th2-biased (ratio ~5; I, Fig. 1).

5.1.2 Recognition of p105-120 and its analogues by p105-120-specific T cell clones

For the assessment of the antigenicity of p105-120 on human T cells, the Can f 1-specific T cell clones were stimulated with p105-120 or its analogues (altered peptide ligands: APLs) containing single amino acid substitutions as compared with the natural peptide.

For the generation of the peptide analogues, the core sequences of the T cell epitopes recognized by the p105-120-specific clones were first determined. Based on the recognition of a panel of alanine- (or serine-) substituted peptide analogues (I, Fig. 2), the epitope core was mapped between the residues 113 and 118 for the clone 2C9 and the residues 107 and 115 for the clone 5G2. The result with the DR-restricted clone 5G2 coincided with the bioinformatic (IEDB) prediction of potential HLA-DRB1*1101 binding motifs (aa 108-116 and aa 109-117). Surprisingly, the epitope core of the HLA-DQB1*0602-restricted clone 2C9 did not overlap with the predicted binding motifs for DQB1*0602 (aa 108-116 and aa 109-117; IEDB, accessed 8/28/2014).

Next, additional peptide analogues were generated with conservative and semi conservative amino acid substitutions between the residues 109-116 overlapping the experimentally verified epitope cores of both TCCs. While some of the amino acid substitutions were not tolerated by the clones (e.g. substitutions for K113 by 2C9 and substitutions for R110 and A112 by 5G2; I, Fig. 3), a total of 4 and 10 heteroclitic (i.e. more optimal than the natural peptide) APLs were identified for the clones 2C9 and 5G2, respectively (I, Fig. 3). These APLs induced a stronger proliferative response and a ≥10-fold

decrease in the EC_{50} values in comparison to the original peptide (I, Fig. 4). These results indicate that the natural Can f 1 peptide p105-120 is recognized suboptimally by human T cells.

5.1.3 Stimulatory capacity of the heteroclitic analogues of p105-120 on polyclonal T cells

The stimulatory capacity of the Can f 1 peptide p105-120 and its heteroclitic analogues was further assessed by generating long-term TCLs with p105-120 and five of its most potent analogues (R110K, K113R, L114I, L115F and G116T; I, Fig. 4) from the PBMCs of six Can f 1-allergic subjects with different HLA genotypes. Only a total of four T cell lines specific to the natural p105-120 peptide were obtained from two out of six Can f 1-allergic subjects (I, Table 1). Instead, the heteroclitic APLs proved to be more potent than p105-120 in inducing specific TCLs. R110K, K113R and G116T induced a total of 7 to 11 specific TCLs from five different subjects. L115F was found to be the most potent APL, inducing significantly more TCLs than the original peptide (19 vs. 4, respectively, $p=0.002$). One to eight L115F-specific TCLs were obtained per subject (I, Table 1). L114I was the only APL to induce a lower number of specific TCLs (2) than the natural peptide.

Importantly, when the APL-induced TCLs were stimulated with p105-120, the majority, 31 of the 46, TCLs recognized the natural peptide. However, with the exception of 2 TCLs, none of the cross-reactive responses were of higher functional avidity than the response to the peptide used in the induction of the TCLs (I, Table 2). Importantly, a total of 13 TCLs cross-reactive with p105-120 were induced with the most stimulatory APL, L11F, demonstrating that L115F could expand p105-120-reactive T cells more effectively than the natural peptide itself (13 vs. 4, $p=0.04$).

Next, the binding capacities of p105-120 and the heteroclitic APLs to a panel of common HLA-DR and DP molecules were assessed to determine the role of HLA binding for the differential stimulatory capacities by the APLs. p105-120 was found to bind to several HLA alleles (DRB1*0301, DRB1*1101, DRB1*1301, DRB4 and DRB5) with strong affinity (I, Table 4). Only minor alterations (less than 6-fold) in the IC_{50} ratios were observed in the binding affinities of the APLs R110K, K113R, L114I, L115F and G116T as compared with p105-120. L115F was found to bind to DRB4 with a slightly improved affinity in comparison to the natural peptide (a 12-fold difference). However, this finding is not sufficient to explain the increased frequency of TCLs induced with L115F, since only one of the subjects (subject F) expressed the DRB4 gene. The results emphasize the role of TCR recognition of the peptides rather than that of HLA binding as the source for the stronger APL-specific T cell responses.

5.1.4 Phenotype of the T cell lines specific to the analogues of p105-120

The expression of the TCR Vβ subtypes of the p105-120 and APL-specific TCLs was analysed by flow cytometry. It was observed that the TCLs expressed a variety of Vβ subtypes, and a total of 13 out of 24 subtypes tested were expanded in at least one of the TCLs (data not shown). These findings suggest that the T cell responses specific to p105-120 and the APLs are not restricted by a specific TCR Vβ subtype.

Generally, the production of the cytokines IL-4, IL-5, IFN-γ, IL-10, IL-17 by the TCLs induced with p105-120 was very weak, and only a tendency was seen towards a slightly

stronger cytokine secretion in the APL-induced TCLs ($p>0.05$; I, Table 3). IL-5 and IFN-γ were the predominant cytokines produced by all TCLs. IL-17 was not detected in the supernatant of any TCL. The TCLs induced with K113R and L114I were observed to be Th2-polarized with the predominant production of IL-5 and the TCLs induced with R110K, L115F and G116T appeared to be of the Th0 phenotype with the balanced production of both IL-5 and IFN-γ. In all, stimulation with the APLs did not result in a significantly different cytokine profile compared to the natural peptide.

5.2 ANALYSIS OF HUMAN T CELL RESPONSES TO CAN F 1 AND ITS HUMAN HOMOLOGUE TEAR LIPOCALIN (II)

5.2.1 Frequencies of Can f 1 and tear lipocalin-specific T cells in peripheral blood

In study II, the antigenic properties of Can f 1 were assessed by generating T cell cultures from 14 Can f 1-sensitized and 15 healthy subjects with nine Can f 1 peptides (pC1-pC9) containing dominant epitopes of the allergen (8). In parallel, T cell cultures were generated with nine Can f 1-homologous peptides present along the sequence of human tear lipocalin (TL; pTL1-pTL9). The immunodominant epitope of influenza hemagglutinin (HA; aa 306-318) served as a microbial control peptide. A specific expansion to at least one Can f 1 peptide was observed in the cultures of 13 subjects from both subject groups. On average, 1.85±0.32% and 1.47±0.31% of the cultures seeded per allergic and per nonallergic subject, respectively, were found to be specific to one of the nine Can f 1 peptides tested (II, Fig. 1). The respective figures upon stimulation with the nine homologous TL peptides were about half lower, 0.74±0.17% and 0.84±0.23% per subject. The frequencies of the lipocalin-specific TCLs, however, clearly contrasted to those obtained upon stimulation with the influenza HA peptide (12.68±10.98% per allergic and 8.60±13.37% per nonallergic subject; II, Fig. 1).

When 10^5 PBMCs were seeded per well, assuming that PBMCs contain approximately 30% of CD4+ T cells and that each peptide-specific TCL represents a monoclonal expansion of an antigen-specific CD4+ T cell, the average frequency of the Can f 1 peptide-specific T cells in the circulating CD4+ T cell pool was estimated to be around 0.6 per 10^6 (0.0185 per 0.3 x 10^5) CD4+ T cells in allergic subjects and around 0.5 per 10^6 in nonallergic subjects. The respective frequencies of TL peptide-specific CD4+ T cells were estimated to be in the same range, around 0.3 per 10^6 in both subject groups. In contrast, the value for the HA peptide-specific TCLs was higher, around 3.5 per 10^6 CD4+ T cells per subject.

Collectively, the estimated frequency of T cells specific to Can f 1 was found to be low, in the range of 1 per one million CD4+ T cells, or even lower in both allergic and nonallergic subjects. Importantly, it was only twice higher than that of the self-TL peptide-specific T cells, but considerably lower than the frequency of the T cells specific to the influenza HA peptide (II, Fig. 1).

5.2.2 Characteristics of the CD4+ T cell lines specific to Can f 1 and tear lipocalin

In line with the TCL frequency data (see 5.2.1), the proliferative responses of Can f 1-peptide-specific TCLs within allergic and nonallergic subject groups were found to be

equally strong as the TL peptide-specific TCLs, but significantly weaker than those specific to the influenza HA peptide (II, Fig. 2A), upon stimulation with the respective peptides. In the analysis of the functional TCR avidities, the generated TCLs were categorized into groups of low (EC_{50}>1.0 μM), intermediate (EC_{50} = 0.1–1.0 μM) and high avidity (EC_{50}<0.1 μM). Only up to 1.4% of Can f 1 and up to 3.6% of TL peptide-specific TCLs were of high avidity (II, Fig. 2B), whereas a substantially greater proportion (25%) of HA peptide-specific TCLs were of high avidity (II, Fig. 2B).

pC4 was the most frequently recognized Can f 1 peptide by the TCLs of both allergic and nonallergic subjects (5.0±1.15% and 4.2±1.61%, respectively). Based on the IEDB binding prediction, the sequence between residues 76 and 84 (YTAYEGQRV) contained in pC4 is the motif that binds to HLA class II molecules in the most promiscuous way. This epitope has also previously been demonstrated to be capable of inducing T cell responses in multiple Can f 1-allergic subjects (54). Within the TL peptides, it was found that pTL3 was recognized the most frequently (1.89±0.57% and 4.2±0.68% in allergic and nonallergic subjects, respectively).

In order to determine whether direct T cell cross-reactivity exists between homologous Can f 1 and TL peptides, the peptide-specific TCLs were stimulated with the counterpart Can f 1 or TL peptide. It was found that only six Can f 1 peptide-specific TCLs (induced with pC1 or pC4) and one TL peptide-specific TCL (induced with pTL4) recognized the counterpart peptide in the proliferation assay. Five of the TCLs were obtained from allergic subjects and two from nonallergic subjects (II, Fig. S2).

Both Can f 1 and TL peptide-specific TCLs were found to exhibit a Th2-biased cytokine phenotype with the predominant production of IL-4 and IL-5 and weak production of IFN-γ (II, Fig. 3). The phenomenon was stronger within the allergic subjects in comparison to the nonallergic subjects. However, no significant differences in the production of these cytokines between the Can f 1 and TL peptide-specific TCLs were observed in either of the subject groups. Instead, the Th1-bias of the T cell responses of both allergic and healthy subjects to the HA peptide was obvious, as the HA peptide-specific TCLs produced significantly more IFN-γ than the Can f 1 or TL peptide-specific TCLs. In accordance with the cytokine data, the Th2-associated chemokine receptor CCR4 was found to be expressed to a greater extent whereas that of the Th1-associated chemokine receptor CXCR3 was less expressed on the Can f 1 and TL peptide-specific TCLs than on the HA peptide-specific TCLs (II, Figs 4 and S6). The expression level of the surface molecules CD45RO, CD45RA, CD62L, CCR7, CTLA-4, CCR4 and CCR6 did not differ in terms of the antigen specificity of the TCLs.

Collectively, the results demonstrate that both the strength and phenotype of the T cell responses induced with Can f 1 resemble those induced with the self-lipocalin TL but clearly differ from the T cell responses induced with the microbial antigen HA.

5.2.3 Binding of Can f 1, tear lipocalin and influenza hemagglutinin peptides to HLA class II molecules

Differences in the binding affinities of antigenic peptides to HLA class II molecules can potentially affect their stimulatory capacity and the subsequent T cell response. To address this possibility, the binding of Can f 1, TL and HA peptides to a repertoire of common

HLA-DR molecules was measured. The results were expressed as relative binding ratios (IC_{50} ratios).

When the binding of Can f 1 and TL peptides by individual HLA-DR molecules was assessed, no significant differences were observed between them (II, Fig. 5). When the binding of homologous Can f 1 and TL peptide pairs to the HLA alleles was compared, only one Can f 1 peptide, pC7, was found to exhibit a significantly higher affinity for the alleles than the counterpart peptide, pTL7. Interestingly, the binding affinity of one of the TL peptides, pTL3, was also significantly higher than that of the counterpart peptide, pC3. The HA peptide was observed to be bound with high affinity to five HLA-DR molecules, which is a value not atypical for some of the Can f 1 or TL peptides (II, Fig. 5). When the binding affinities of all HLA alleles were compared between the HA peptide and individual lipocalin peptides, the HA peptide bound to HLA-DR significantly more strongly than one of the Can f 1 peptides, pC9, and three of the TL peptides, pTL2, pTL8 and pTL9.

Taken together, these data suggest that the HLA-DR binding is an unlikely factor to account for the observed differences in the induction and stimulatory capacities of T cell lines by Can f 1, TL or HA peptides.

5.3 COMPARISON OF DOG LIPOCALIN ALLERGEN-SPECIFIC T CELL RESPONSES BETWEEN ALLERGIC AND NONALLERGIC SUBJECTS

5.3.1 Frequency and functional characteristics of Can f 4-specific memory CD4+ T cells in allergic and nonallergic subjects (III)

The comparison of *in vivo*-induced allergen-specific T cell responses between allergic and healthy subjects may help in elucidating the pathogenesis of allergy. In study III, Can f 4-specific T cell lines were induced by stimulating highly purified memory CD4+ T cells from 13 dog-allergic and 12 nonallergic subjects with recombinant Can f 4 protein. A variety of HLA class II alleles were expressed by both allergic and healthy subjects (III, Table E1) and the distribution of the alleles did not differ statistically between the subject groups.

Can f 4-specific memory TCLs were obtained from 12 out of 13 allergic subjects (92%, 86 TCLs) but only from 3 out of 12 nonallergic subjects (25%, 11 TCLs, p=0.001). The frequencies of Can f 4-specific memory CD4+T cells were estimated by the number of specific cultures obtained per seeded memory CD4+ T cells (3-4.5 x 10^6) per subject, assuming that each of them represented a monoclonal T cell growth. An 8-fold difference was observed in the calculated frequencies of Can f 4-specific memory CD4+ T cells between the allergic and healthy subject groups (2.46±2.45 vs. 0.31±0.59 per 1 million memory CD4+ T cells, respectively; III, Fig. 1A). Moreover, the functional avidity of Can f 4-specific memory CD4+ TCLs from allergic subjects, as assessed by EC_{50} values, was observed to be considerably higher than that of TCLs from nonallergic subjects, (III, Fig. 1B).

Collectively, the results demonstrate that allergic individuals harbour a higher frequency of Can f 4-specific CD4+ T cells of memory origin and that these cells recognize the allergen with higher avidity than the T cells of healthy subjects.

5.3.2 Phenotype of Can f 1 and Can f 4-specific T cells in allergic and healthy subjects (II, III)

The production of the cytokines IL-4, IL-5, IFN-γ, IL-10 and IL-17 by the Can f 1 (II) and Can f 4-specific (III) TCLs from allergic and nonallergic subjects was compared. The Th2-type cytokines IL-4 and IL-5 were observed to be produced more efficiently by the Can f 1-specific TCLs of allergic subjects than by their nonallergic counterparts (II, Fig. 3). A similar finding was observed in study III, with Can f 4-specific memory TCLs from allergic subjects producing more IL-4 and IL-5 than those from nonallergic individuals (III, Fig. 2A)

The production of IFN-γ or IL-10 by the Can f 1-specific T cells did not differ between the allergic and nonallergic groups (II, Fig. 3). However, the Can f 4-specific memory TCLs of nonallergic subjects were observed to secrete more IFN-γ and IL-10 than those of allergic subjects (III, Fig, 2A), indicating a possible Th1/Tr1 deviation of the *in vivo*-induced T cell response in these nonallergic subjects. When each Can f 4-specific TCL was categorized phenotypically by calculating an IFN-γ/IL-5 ratio (III, Fig. 2B), 64% of the TCLs from allergic subjects and 27% of those from healthy subjects were observed to represent the Th2 phenotype ($p=0.03$). In contrast, only 4% of the TCLs from allergic subjects but 36% from healthy subjects were Th1-biased ($p=0.003$). In line with the cytokine data, the expression of the Th1-associated chemokine CXCR3 was lower in the Can f 4-specific memory TCLs from allergic subjects (III, Fig. 2C). Moreover, the activation status of the Can f 4-specific TCLs, as assessed by the expression of the IL-2 receptor alpha-chain (CD25), was found to be higher in the TCLs from allergic subjects (III, Fig. 2C).

5.4 T CELL EPITOPES OF CAN F 4 (III)

5.4.1 Epitope mapping

In an attempt to identify the T cell epitope regions along the sequence of Can f 4, 88 specific TCLs obtained with the allergen protein from the memory CD4+ T cells of 12 (out of 13) allergic and 3 (out of 12) nonallergic subjects were stimulated with overlapping 16-mer Can f 4 peptides. The epitope-containing regions recognized by the allergic subjects were found to be scattered throughout the allergen sequence (III, Table I and Table E4 in the Online Repository). The few Can f 4-specific TCLs obtained from subjects without allergy recognized the same epitopes as the TCLs from allergic subjects, indicating that the T cells of allergic and nonallergic subjects do not differ substantially in terms of their patterns of epitope recognition. The average of 3.3±0.62 and 2.3±0.88 epitopes was recognized per allergic and per nonallergic subject, respectively.

The four most prevalently recognized Can f 4 regions were located between amino acids (aa) 13-31, 43-67, 91-127 and 124-148 (III, Table I). These regions were recognized by the Can f 4-specific TCLs of at least three subjects. They were also observed to be more stimulatory than the other Can f 4 regions, as assessed by the mean stimulatory indices (46.4±66.5 vs. 29.6±27.6, $p=0.007$). The region aa 43-67 was found to be recognized most frequently, as the peptides covering it stimulated TCLs from 10/11 (90%) of allergic and 1/3 (33%) of nonallergic subjects (III, Table I). Three allergic subjects and one nonallergic subject harbored T cells specific to this region only. The amino acid sequence shared by adjacent

Can f 4 peptides able to stimulate a T cell line was defined as the core sequence of an epitope region. The minimal core sequence of the most stimulatory region aa 43-67 was the 19-mer sequence PRLKMLFNFYVKVDGECVE (aa 46-64; III, Fig. 3).

Next, the HLA class II restriction elements of 34 Can f 4-specific TCLs from eight allergic subjects and one nonallergic subject were analysed (III, Table E5). It was discovered that 74% of the T cell responses were restricted by HLA-DR, 21% by HLA-DQ and 4% by HLA-DP. Importantly, T cell responses to the four most stimulatory regions of Can f 4 were not exclusively restricted by either HLA-DR, -DP or -DQ molecules. A similar variability was also observed in the analysis of allelic restrictions (III, Table E5). Collectively, the results suggest that Can f 4 epitopes are presented by a wide array of HLA class II alleles.

5.4.2 HLA-binding capacity of Can f 4 peptides

Bioinformatic predictions of the HLA class II peptide binding motifs in Can f 4 were performed with the IEDB Analysis Resource program. Altogether, 38 DR-binding motifs, 6 DP-binding motifs and 7 DQ-binding motifs in Can f 4 were predicted. When assessing the correlation of the predictions and the experimentally identified epitope regions, 38 out of 51 predicted motifs were experimentally confirmed, i.e. the prediction specificity was 75% (III, Fig. 3). In addition, eight core sequences were experimentally discovered outside the HLA binding predictions, indicating a prediction sensitivity of 84%. Importantly, most (37/51; 73%) of the predicted HLA-binding sites were found to be contained in the four most stimulatory epitope regions of Can f 4, each of them containing at least four distinct binding motifs (III, Fig. 3), suggesting that the promiscuous HLA-binding capacity is linked with the antigenicity of Can f 4 epitope regions.

To experimentally validate the HLA binding predictions, the binding affinities of 15 selected peptides covering the immunodominant epitope regions to a panel of prevalent HLA-DR molecules were measured. In line with the predictions, promiscuous HLA binding was observed, as three of the four regions contained peptides that were capable of binding to a minimum of three different HLA class II alleles with a moderate to high affinity (III, Table II). Notably, the peptides aa 43-58 and 46-58 from the most antigenic epitope region of Can f 4 bound to the HLA class II alleles in the most prevalent way (strong binding affinity to 5/6 and 4/6 HLA alleles tested, respectively; III, Table II).

5.4.3 Co-localization of epitopes in lipocalin allergens and human tear lipocalin

The amino acid sequence of Can f 4 was aligned with the sequences of other lipocalin allergens with previously mapped T cell epitopes (8,10,12,13) as well as with the sequence of the human tear lipocalin to assess the degree of co-localization of the epitopes. Generally, it was observed that there was high variability between the epitope regions (data not shown). However, one region was found to co-localize with all of these proteins (Fig. 3). Interestingly, this epitope region partly overlaps with the first of the three structurally conserved regions of lipocalin proteins, which is characterized by the signature motif glycine-X-tryptophan (see 2.2.3). Despite the co-localization, the region was not considered immunodominant with any of the proteins studied.

Figure 3. Co-localization of the lipocalin T cell epitope cores (blue lines). The first structurally conserved region of the lipocalins is indicated with a box. The signature motifs are shown in red.

6 Discussion

6.1 ALLERGENICITY OF DOG LIPOCALIN ALLERGENS (I-II)

6.1.1 Suboptimal T cell recognition of lipocalin allergens by human T cells

Only a limited number of environmental proteins are allergenic, i.e. capable of inducing a specific Th2-deviated CD4+ T cell response and the subsequent production of IgE-antibodies by B cells in susceptible individuals (70). Therefore, it is conceivable that allergens possess specific properties that contribute to the allergic sensitization mounted against these agents. As reviewed in 2.3.3, most of the significant mammal-derived respiratory allergens belong to the lipocalin family of proteins. The allergenicity of lipocalin allergens has been proposed to be associated with their evolutionary relatedness with human lipocalins (224). According to the hypothesis, the presence of endogenous lipocalins in humans should have resulted in the elimination of strongly responding cells from the lipocalin allergen-specific T cell repertoire. The allergic immune response would therefore arise from the low-avidity interactions between the allergen and remaining T cells, which could predispose the immune response towards being Th2-dominated (129,225,226). The theory is based on the uniform observations that PBMCs from clinically allergic subjects proliferate very weakly upon stimulation with lipocalin allergens (9,10,13,227), and on the observation that lipocalin allergen-specific T cell lines are capable of cross-recognizing endogenous lipocalins (228).

The results from study I support this hypothesis. It was observed that the peptide p105-120 containing an epitope of the dog lipocalin allergen Can f 1 is recognized suboptimally by human T cells. A total of 14 heteroclitic APLs were identified that were able to induce T cell proliferation at 10-30-fold lower concentrations than the natural peptide p105-120. Suboptimal recognition of p105-120 was found not to be limited to the T cell clones alone, as it was also demonstrated with polyclonal TCLs derived from the peripheral blood of dog-allergic subjects. Four out of five heteroclitic APLs (R110K, K113R, L115F and G116T) were more frequently capable of inducing specific TCLs from the PBMCs of six Can f 1-allergic subjects with heterogeneous HLA backgrounds than the natural peptide (II, Table 1). The total number of specific TCLs induced with one of the APLs, L115F, was statistically significantly higher than that induced with p105–120 (19 TCLs vs. 4 TCLs, $p=0.002$). Importantly, 13 of the 19 TCLs induced with L115F were cross-reactive with p105−120 demonstrating that L115F could expand p105-120-reactive T cells more efficiently than the natural peptide *in vitro* (13 TCLs vs. 4 TCLs, $p=0.04$). Since most of the L115F-induced TCLs recognized p105-120 with a lower avidity, the increased frequency of p105−120-reactive TCLs may be explained by the enhanced capacity of L115F to activate low-avidity p105−120-specific T cells. Moreover, the heteroclitic activity of the APLs over p105-120 is likely to be attributable to the improved TCR recognition rather than to the HLA binding, as the HLA-binding affinities of p105-120 and the APLs were observed to be largely similar.

The strength of TCR stimulation has been acknowledged as an important regulator of the quality of the T cell response, with strong stimulation favoring Th1 differentiation and weak stimulation leading to Th2 dominance (84,129,226). The exact mechanisms by which the strength of TCR recognition drives the phenotypic differentiation of the cells are not clear, but it has been associated with changes in the levels of endogenously produced IL-4 that is regulated by several different TCR downstream signaling routes (131,229,230). Although the phenotypic differences between the TCLs induced with p105-120 and its analogues were minimal, the APLs were observed to exhibit some immunomodulatory capacity, as the most stimulatory L115F, for instance, induced a balanced Th0-type response but the less stimulatory APL L114I induced Th2-biased responses (I, Table 3). Shaping of the allergic T cell response *in vitro* has also been demonstrated previously with the heteroclitic APLs of the immunodominant epitope of the cow lipocalin allergen Bos d 2. The analogues of Bos d 2 have been found to induce greater Th1/Th2 ratios in the cytokine secretion by Bos d 2-specific T cell clones than the natural epitope (231). Moreover, TCLs induced from multiple patients with one of the analogues exhibited a more Th1-deviated phenotype than those induced with the natural epitope (232). Collectively, both the previous as well as the current results indicate that the poor antigen recognition can be a factor promoting weak Th2-deviated T cell responses against lipocalin allergens.

6.1.2 Human CD4+ T cell responses to the dog allergen Can f 1 and its human homologue tear lipocalin resemble each other

The identification of heteroclitic APLs, and thereby the suboptimal T cell recognition of the natural epitopes of lipocalin allergens, is not a unique feature of these proteins. Several self antigens, such as myelin basic protein (233) and proteolipid protein (234), have been demonstrated to be recognized suboptimally by human T cells. Instead, this feature does not appear to be a characteristic of true foreign antigens, such as influenza virus hemagglutinin (HA) (235). Lipocalin allergens also resemble self proteins in terms of their amino acid sequence (see 2.2.3). For example, Can f 1 and the human tear lipocalin (TL) share a sequence identity of about 60%. On the basis of sequence homology, lipocalin allergens, proteins of exogenous origin, may be immunologically close to endogenous antigens. In other words, they may exist at the borderline of self and non-self.

This idea was further examined in study II in a setting where the antigenicity of Can f 1 on CD4+ T cells was compared *in vitro* with that of tear lipocalin, a homologous self protein and influenza HA, an exogenous microbial antigen. Based on the estimation by the split-well method, the frequency of Can f 1 peptide-specific CD4+ T cells proved to be low, at the level of 1 per 10^6 CD4+ T cells. Previous estimations on the frequency of lipocalin allergen-specific CD4+ T cells, analyzed with the same method, have been in the same range (83,228,236). It is possible that the *in vitro* -protocol used in the current study may underestimate the antigen-specific CD4+ T cell frequencies to some extent. However, only slightly higher frequencies of lipocalin allergen-specific CD4+ T cells have been reported in a study utilizing another approach, the HLA class II tetramer technique (236). Importantly, in the study II, the frequency of Can f 1-specific CD4+ TCLs was observed to be very close to that of the CD4+ TCLs specific to TL. This contrasted clearly with the frequency of the

CD4+ TCLs specific to the influenza hemagglutinin, known to contain a potent epitope (216). In accordance with the frequency data, the strength of the *in vitro* proliferative responses of peptide-specific TCLs did not differ between Can f 1 and TL in the comparison of allergic and nonallergic subject groups but they were clearly weaker than the responses of TCLs specific to the HA peptide (II, Fig. 2A). A further analysis suggested that the phenomenon was attributable to the low functional TCR avidity of the lipocalin peptide-specific TCLs in comparison to those specific to HA (II, Fig. 2A). The HLA class II binding affinity or the peptides did not appear to be able to explain the results, as it was found to be very similar for all of the antigens examined in the study (II, Fig. 5), and the distribution of HLA class II alleles did not differ between allergic and healthy subjects (II, Table S1). In line with the current results, a study analyzing Can f 1-specific CD4+ T cell proliferation with the CFSE dilution assay revealed that the proliferative response of Can f 1-reactive memory CD4+ T cells of allergic subjects was clearly weaker than that for streptokinase (SK) and purified protein derivative (PPD), antigens of microbial origin (83). Several other studies have also reported that T cell responses specific to autoantigens generally exhibit lower TCR avidity than those specific for the exogenous microbial antigens, including influenza HA (233,237-239).

The Can f 1 and TL peptide-specific TCLs also resembled each other phenotypically. The production of the Th2 cytokines IL-4 and IL-5 predominated with both Can f 1 and TL-specific TCLs, whereas the HA-specific TCLs mostly produced the Th1 cytokine IFN-γ (II, Fig. 3). A similar phenotypic difference was also seen in the analysis of the T cell surface markers, as a higher expression of CCR4 and a lower expression of CXCR3 by the Can f 1 and TL-specific TCLs was observed in comparison to the HA-specific TCLs (II, Fig. 4). In all, the functional avidities and phenotypic deviations of the lipocalin and HA peptide-specific TCLs are in line with the concept that low TCR avidity favors Th2-development whereas high avidity promotes Th1-development, respectively (84,129,226).

6.1.3 The multiple layers of allergenicity

The current results suggest that the human CD4+ T cell responses to Can f 1 and TL resemble each other in a surprising manner, with Can f 1 appearing to be only slightly more antigenic on human T cells than its self homologue *in vitro*. This observation is in line with the findings of the suboptimal stimulatory capacity of Can f 1 (I). This feature is likely to affect the development of Th2-type immune response and could therefore be considered as a factor promoting the allergenicity of Can f 1. However, as demonstrated with many other allergens (see 2.2.2), it is probable that there are also other mechanisms associated with Can f 1 and other lipocalins that contribute to their allergenic capacity (240).

These mechanisms include at least physical properties, i.e. the efficient dispersion capability of lipocalins from animal dander and excretions in the environment and inhaled air (5). Although the ability to promote the activation of the innate immune system has been postulated as a mechanism of allergenicity for a wide and diverse group of allergen proteins (76,243,244), it has remained somewhat unclear with lipocalin allergens (245). Instead, the adjuvantic effect provided by bacterial lipopolysaccharide (LPS), a substance commonly coexisting with animal dander, has been shown to favor the Th2 differentiation of the adaptive immune cells (241,242). Another factor possibly promoting the allergenicity

of lipocalins may be enzymatic activity. For example, the human tear lipocalin has been reported to function as an endonuclease (246) and a cysteine protease inhibitor (247). In view of its structural similarity with Can f 1, the enzymatic activity could also be a property of Can f 1 (248), although this motif is only partially conserved in the allergen. Moreover, ligation to the mannose receptor (MR) on dendritic cells by the carbohydrate structures of glycoprotein allergens has been shown to play a role in the induction of Th2 cells (76,249), and might also be involved in the allergenicity of Can f 1, which is a glycosylated protein. Finally, one interesting characteristic of lipocalins in terms of their allergenicity not examined in detail here is their lipid binding capacity. Bioactive lipid cargo could enhance the allergenic response via Toll-like receptors (TLR) or CD1 pathways (250,251). Considering all these factors, the basis for the allergenicity of lipocalins is most probably a multidimensional phenomenon with many aspects yet to be unraveled.

6.2 T CELL EPITOPES OF CAN F 4 (III)

6.2.1 The region aa 43-67 of Can f 4 is highly stimulatory on human CD4+ T cells

Detailed characterization of allergen epitopes is a premise for understanding the allergic sensitization process and for the development of better preparations for both the diagnostics and the immunotherapy of allergy. The epitopes of Can f 4 were found to cluster in several regions of the allergen (III, Table I). Four highly immunogenic epitope regions (aa 13-31, 43-67, 91-127 and 124-148) were identified. On average, three and two epitope regions were recognized by an allergic and a nonallergic subject, respectively. The most frequently recognized region, aa 43-67, stimulated the Can f 4-specific TCLs in 90% of the allergic subjects tested. The significance of this region for the allergenicity of Can f 4 was further emphasized by the observation that three of the allergic subjects reacted exclusively to this epitope (III, Table I).

The T cell epitopes of four other lipocalin allergens, Can f 1(8), Bos d 2 (10), Equ c 1 (12) and Rat n 1 (13), have similarly been observed to be localized in specific regions along the allergen sequences. Interestingly, when the sequences of Can f 4 and these allergens were aligned, the first structurally conserved region of lipocalins containing the sequence motif G-x-W (252) was found to be stimulatory for T cells in all of these allergens (Fig. 3). The corresponding region of the human endogenous tear lipocalin was also shown to be antigenic for human T cells (peptide pTL1 in study II).

The co-localization of T cell epitopes has similarly been reported within the allergens of the pathogenesis-related (PR)-10 family of plant proteins such as birch (Bet v 1) (253), apple (Mal d 1) (254), celery (Api g 1) (255) and hazelnut (Cor a 1) (256) allergens. In these allergens, the co-localizing epitopes were observed to focus on the regions with a high level of amino acid sequence homology. Consequently, T cells recognizing these regions also showed a high degree of cross-reactivity (253). The T cell cross-reactivity of lipocalin allergen epitopes appears to be less prominent, probably due to their lower sequence identity (generally around 20-40%). However, it is noteworthy that a few TCLs induced with peptides from Can f 1 and TL, i.e. those exhibiting a sequence homology of about 60%, demonstrated cross-reactivity with their counterparts in study II (Fig. S2) and also in a

previous study (228). As T cell cross-reactivity of endogenous and exogenous proteins has also been reported in autoimmune diseases (257-259), it is conceivable that the homology of self and non-self structures plays a role in the pathogenesis of immunological diseases.

6.2.2 The promiscuous HLA-binding of the T cell epitopes of Can f 4

In autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis or type 1 diabetes, the disease susceptibility is strongly associated with certain HLA class II genes (260). In allergic diseases, HLA-restriction elements have been revealed for example with the ragweed allergen Amb a 5 that contains a single T cell epitope exclusively presented by HLA-DRB1*1501/02 (261). Associations have also been reported with the mugwort allergen Art v 1 (HLA-DRB1*01 and HLA-DRB1*16) (262) and the bee venom PLA_2 (DRB1*0103/ DQB1*0501) (263). However, these cases represent a minority of allergens, and generally multiple epitopes with multiple restriction elements are involved in the response.

The majority of the Can f 4-specific T cell responses were found to be restricted by HLA-DR (III, Table E5). In line with this observation, the T cell responses to Timothy grass (264) ragweed (265) and horse (266) allergens have also been observed to skew towards HLA-DR restriction. The findings are not surprising since HLA-DR molecules are expressed more abundantly than -DP or -DQ molecules on professional APCs (267). Importantly, the T cell response to each of the four dominant Can f 4 epitope regions identified was restricted by at least two different types of HLA molecules (III, Table E5). This promiscuous HLA presentation was confirmed by the finding that epitope-containing peptides were commonly observed to bind to several HLA-DR alleles with moderate to high affinity (III, Table II). Moreover, the bioinformatically predicted HLA-binding motifs were found to cluster in these regions (III, Figure 3). Of special interest, the most stimulatory Can f 4 region, aa 43-67, was predicted to contain a total of 16 HLA-DR, -DP and -DQ binding motifs (III, Fig. 3). Peptides covering this region also exhibited a high binding affinity to most of the HLA alleles tested (see Table II). These data collectively suggest that the capacity of peptides to bind to different HLA class II molecules is a factor associated the immunodominance of Can f 4 epitopes. Promiscuous HLA binding has also been associated with the immunodominance of the epitopes of Timothy grass allergens (264) as well as those of microbe-derived proteins (268,269).

6.2.3 Bioinformatic predictions of T cell epitopes of allergens

When the IEDB analysis of the HLA-binding motifs of Can f 4 was compared to the experimentally discovered Can f 4 epitope regions (III, Fig. 3), the predictive sensitivity of 75% was achieved. Previously, the coverage of bioinformatic predictions has been shown to be lower, around 50% of the experimentally discovered epitopes (264,270). This can partially be attributable to methodological differences between the studies. In addition, it was observed with Can f 4 that 17% of the experimentally revealed epitope regions were not covered by the prediction algorithms. Based on these results, it appears that although the algorithms could be useful in the selection of likely T cell epitope candidates within a protein, they may be insufficient to gather a comprehensive and detailed characterization of allergen epitopes. Furthermore, the apparent inaccuracy of the bioinformatics approach to predict T cell epitopes demonstrates that the HLA-binding capacity can not solely explain

the antigenicity of an allergen epitope. Instead, other mechanisms are likely to be involved in the process. These include at least the regulation of antigen processing in dendritic cells that contributes to the epitope density on APCs (271,272).

6.3 DOG ALLERGEN-SPECIFIC T CELL RESPONSES IN INDIVIDUALS WITH AND WITHOUT ALLERGY (II-III)

6.3.1 Can f 4-specific memory CD4+ T cells exist at higher frequencies in allergic subjects in comparison to nonallergic subjects

Understanding the allergen-specific CD4+ T cell responses in both allergic and nonallergic individuals is essential for elucidating the pathogenesis of allergy. Moreover, characterizing the mechanisms of the natural, unharmful responses in healthy individuals can be helpful in developing specific immunotherapeutic approaches. Therefore, the functional and phenotypic properties of the T cell response to the dog lipocalin allergens Can f 1 and Can f 4 in allergic and healthy subjects were compared in studies II and III.

When the frequencies of allergen-specific CD4+ T cells were assessed in the PBMC cultures induced with the Can f 1 peptides pC1-pC9, no significant differences were observed between the two subject groups (II). Instead, an eight-fold higher frequency of Can f 4-specific CD4+ T cells of memory origin was demonstrated in allergic than in nonallergic subjects (III). These observations are in concordance with several other studies in which the characterization of unpurified cell populations has yielded only marginal differences in the frequency of allergen-specific CD4+ T cells between allergic and nonallergic subjects (228,266,273,274). Instead, substantial differences in the frequency of isolated antigen-specific memory ($CD45RA^{-}CD45RO^{+}$) CD4+ T cells between the diseased and healthy subject groups have been reported *in vitro* and *ex vivo* in both allergic (83,275-278) and autoimmune diseases (279,280). This discrepancy is likely to be attributable to the activation and preferential expansion of naïve T cells in unpurified PBMC cultures *in vitro*, as allergic and nonallergic subjects appear to harbor naïve allergen-specific CD4+ T cells at comparable frequencies (275).

Further characterization of the allergen-specific T cell responses revealed that Can f 4-specific T cells from allergic subjects were of higher functional avidity than those from healthy subjects (III, Fig 1B). Based on the predominant IL-4 and IL-5 cytokine production and the low expression of the surface marker CXCR3, the Can f 1 and Can f 4-specific T cell lines of allergic subjects can be regarded as Th2-biased (II, Fig. 3; III, Fig. 2A & 2B). In contrast, the Can f 4-specific memory CD4+ T cells of nonallergic subjects responded to allergen stimulation with stronger IFN-γ and IL-10-biased cytokine production indicative of a Th1/Tr1-deviated phenotype (III, Fig. 2A & 2B).

6.3.2 The expansion capacity of allergen-specific T cells in nonallergic subjects is limited

The exact mechanisms accounting for the frequency difference of allergen-specific memory CD4+ T cells between allergic and healthy individuals are largely unclear. It is likely, however, that they reflect the differential *in vivo* activation and expansion capacities of allergen-specific T cells in allergic and nonallergic subjects in response to allergen

challenge. It is known that T cells with a strong TCR affinity for their antigen can outcompete lower affinity cells and begin to dominate the immune response under antigen stimulation (281-283). The observation that Can f 4-specific memory T cell responses in the allergic subjects exhibited higher functional avidity (III, Fig. 1B) is in line with this concept. Moreover, it was commonly found that several individual TCLs from an allergic subject recognized exactly the same epitope regions of Can f 4 (III, Table E4). Although not analyzed at the TCR sequence level, this observation suggests that they likely represent clonal expansions of the same allergen-specific precursor. Together with the higher expression of CD25 on the Can f 4-specific TCLs of allergic subjects (III, Fig. 2C), these findings indicate that the T cells of allergic subjects exhibit more effective *in vivo* activation capacity than the Can f 4-specific T cells of nonallergic individuals.

Several factors can contribute to the restricted capacity of nonallergic subjects to generate Th2-type responses to allergens. One possibility could be that allergic and nonallergic subjects differ in terms of their naïve T cell repertoires. In support of this view, a study characterizing the T cell repertoires of allergic and nonallergic subjects specific to an immunodominant epitope of the cow lipocalin allergen, Bos d 2, reported functional and phenotypic differences not only in the allergen-specific memory T cell repertoire but also in the naïve repertoire (275). As the composition of the naïve antigen-specific T cell repertoire has been demonstrated to directly affect the diversity and quality of a primary CD4+ antigen-specific response (284,285), differential naïve repertoires could theoretically provide one explanation for the differential responses between allergic and nonallergic subjects.

As the Can f 4-specific responses of nonallergic subjects were generally very weak, low in terms of their functional avidity and only weakly Th1 or Tr1-polarized, it is possible that they are not *bona fide* allergen-specific T cells, but rather represent a marginally cross-reactive population primed *in vivo* with an unrelated, high-affinity antigen, for example, a microbial antigen. This hypothesis is supported by a recent report that demonstrated the existence of memory-phenotype CD4+ T cells specific to numerous foreign and autoantigens without any preceding exposure. The cells displayed cross-reactivity with homologous epitopes from environmental antigens (286). The reported frequencies of these cross-reactive CD4+ T cells in the peripheral blood were in the same range as those observed with Can f 1 (II) and Can f 4 (III) in nonallergic subjects.

Another mechanism affecting the differences in the T cell responses between subjects with and without allergy may be the differential recognition of allergen epitopes. For example, it has been reported that the cat allergen Fel d 1 contains tolerogenic DR7-restricted epitopes associated with IL-10 and IFN-γ production in PBMC cultures. Recognition of those epitopes could provide a protective effect in subjects expressing the DR7 allele (287). However, when a comparison was made of the recognition of dog lipocalin allergen epitopes by the specific TCLs of allergic and nonallergic subjects in study II, it was found that the same individual Can f 1 peptides were recognized by the TCLs of both subject groups (II, Fig. 1B). The Can f 1 peptide pC4 was found to be the peptide most frequently recognized by the individuals in both of the groups (II, Fig. 1B). In study III, the Can f 4 epitope regions recognized by TCLs from allergic subjects were also observed to stimulate Can f 4-specific TCLs obtained from subjects without allergy (III, Table I). With respect to

the T cell phenotype, no polarization was observed in relation to the epitope specificity of the Can f 1 and Can f 4-specific TCLs (data not shown). Collectively, the observations suggest that the T cell epitope recognition patterns are unlikely to determine the susceptibility to develop allergic sensitization to Can f 1 or Can f 4. Similar findings have also been reported with many other allergens (163,288,289).

There is also evidence that active peripheral regulatory mechanisms, especially regulatory T cells, would play a role in the suppression of harmful allergen-specific T cell responses in nonallergic individuals. In one study in which the T cell responses specific to the birch allergen Bet v 1 and house dust mite allergen Der p 1 were compared between allergic and nonallergic subjects, it was observed that the healthy subjects harbored significantly higher frequencies of IL-10-secreting Tr1 cells than allergen-specific Th2-type cells, whereas the situation with allergic subjects was the opposite (160). Another study indicated that the suppressive function of atopic subjects' CD4+CD25+ regulatory T cells is compromised (290). The induction of regulatory T cells appears to be one of the central mechanisms in allergen-specific immunotherapy (see 2.5.5). The finding that IL-10 was produced predominantly by the Can f 4-specific memory TCLs of nonallergic individuals (III, Fig. 2A) suggests that regulatory T cells (Tr1) may also be involved in the development of tolerance to lipocalin allergens, although opposite results have also been presented (83).

In conclusion, possible mechanisms differentiating the allergen-specific CD4+ T cell responses in allergic and nonallergic subjects include the composition of naïve T cell repertoires, the function of regulatory T cells and HLA associations of allergen epitopes. Since also many other cell types, for example those of innate immunity (291), appear to function differently between individuals with and without allergy, it is evident that the allergenic polarization of the T cell response is the overall result of a complex interplay between the cells of the immunological network.

6.4 IMMUNOTHERAPEUTIC POTENTIAL OF CAN F 1 AND CAN F 4 EPITOPE-CONTAINING PEPTIDES (I, III)

6.4.1 Can f 4_{46-64} – a candidate peptide for dog allergen-specific peptide immunotherapy?

The principle of peptide immunotherapy is the use of preparations composed of linear peptides containing the T cell epitopes of a native allergen, which is an attractive option for conventional allergen immunotherapy alternative using crude allergen extracts due to the reduced risk of IgE-mediated adverse systemic reactions (292,293). The administration of peptides containing a single or a few T cell epitopes of an allergen has been demonstrated to induce hyporeactivity to the entire protein in numerous murine experiments (181,182,193,294) and clinical trials (187,190), probably by linked epitope suppression (193) via the induction of T cells with regulatory or immunosuppressive function (187,190,193,294).

For the development of population-wide peptide immunotherapy, the identification of promiscuous allergen epitopes, i.e. those with a capacity to bind to a variety of commonly expressed HLA class II molecules, are of special interest. In study III, the subjects included in the Can f 4 epitope mapping were found to express the most common HLA-DRB1 alleles

in the Finnish population (295), with the exception of DRB1*0301. The T cells of 90% of these subjects were capable of recognizing the region aa 43-67 of Can f 4. Moreover, the region was predicted to contain a total of 14 different HLA class II binding motifs (III, Fig. 3). Based on these results, the peptide Can f 4_{46-64} containing the minimal core of the region (III, Fig. 3) could be considered as a potential candidate for Can f 4-specific peptide immunotherapy.

However, it is noteworthy that the individual reactivity to allergen epitopes can vary considerably, not only as a result of differential HLA class II restrictions of epitopes but also due to mechanisms associated with antigen processing and presentation, as discussed above (see 6.2.3). For example, it was observed in study III that individuals sharing the same HLA class II genotype, e.g. the dog-allergic subjects A-2, A-5 and A-8 (DRB1*0101/1501, DQB1*0501/0602; III, Tables 1 and E1), displayed variability in their epitope recognition patterns. In view of this heterogenity of the T cell response, the use of a single peptide, pCan f 4_{46-64}, containing the immunodominant epitope may be an insufficient approach for designing peptide immunotherapy to Can f 4 allergy. Instead, using a combination of several epitope-containing peptides could be functional. In support of this view, it has been shown that a mixture of six peptides of Japanese cedar pollen allergens Cry j 1 and Cry j 2 stimulated human PBMCs *in vitro* more efficiently than a single epitope-containing peptide (296). As an average of 2.6 of the four immunodominant Can f 4 epitope regions were recognized per allergic subject, a pool of peptides derived from the most antigenic regions of Can f 4 (aa 13-31, 43-67, 91-127 and 124-148) could be considered as an ideal combination for the induction of tolerance in the vast majority of Can f 4-allergic individuals.

Another problem linked with the treatment of dog allergy with Can f 4 preparations is that only 35-60% of dog allergic subjects are sensitized to the allergen. Moreover, most dog allergic subjects are sensitized to several of the six known dog allergens, most commonly to Can f 1 (63) or Can f 5 (69). Only a minority of the subjects reacts exclusively to Can f 4 (65). Therefore, it is reasonable to assume that the treatment of dog allergy with Can f 4-derived peptides alone is an inefficient approach. Instead, a more feasible option might be to select cocktail of immunodominant T cell epitopes from the most commonly recognized dog allergens. Another option could be the generation of a patient-tailored immunotherapy vaccine based on the IgE reactivity profiling of an individual by allergen microarrays. This would allow the precise identification of the disease eliciting allergens (297).

6.4.2 Therapeutic potential of altered peptide ligands

Hypothetically, the use of altered peptide ligands could be another way to further develop the efficacy of peptide-based immunotherapy. APLs that exhibit cross-reactivity with the T cells specific to the natural T cell epitope of an allergen and which mediate tolerogenic or Th1-deviating outcomes would be of special interest (298). Previously, a heteroclitic analogue of the peptide containing the immunodominant epitope of the cow lipocalin allergen Bos d 2 was observed to induce apoptosis, hyporesponsiveness and increased Th1/Th2 cytokine ratios in the allergen-specific T cell clones *in vitro*. Similar effects have also been reported with the analogues of the house dust mite Der p 1 (299,300) or the bee

venom PLA2 (301) allergen epitopes. In study I, the APLs of the Can f 1 peptide p105–120 exhibited a capacity to functionally and qualitatively modify the T cell response (I, Tables 2 & 3). Two of the analogues were observed to induce a Th2-deviated response with IL-5 production predominating, whereas the other three induced a more balanced Th0-type response. Cross-recognition of the natural epitope was observed with the majority of the TCLs induced with the APLs. In particular, the analogue L115F could be considered therapeutically potential, as it was capable of inducing strong T cell responses that were highly cross-reactive with the peptide containing the natural epitope.

Despite these promising preclinical studies, however, evidence for the safety and efficacy of altered peptide ligands in a clinical setting are lacking. Based on the reports from clinical trials of autoimmune diseases (211,212), the major problem associated with the APL-mediated immunotherapy appears to be the unpredictability of the subsequent T cell response. For example, it has been shown that an antagonistic APL for a T cell clone *in vitro* may act as an agonist for another autoreactive T cell *in vivo* (302). Thus, taking into account the broadness of the human T cell repertoire, the development of APL-based immunotherapy may be hampered by the risk that they may evoke harmful cross-reactive responses (198,303).

7 Conclusions

This study aimed to dissect the mechanisms behind allergy against the dog lipocalin allergens Can f 1 and Can f 4 by comparing CD4+ T cell responses against them in allergic and nonallergic individuals. In addition, T cell epitopes within Can f 1 and Can f 4 were characterized and the potential of selected epitopes for peptide immunotherapy of dog allergy was evaluated.

Can f 1 was found to be weakly antigenic on human CD4+ T cells *in vitro*, as the cellular responses to Can f 1 resembled those to the endogenous homologue, tear lipocalin. The responses clearly contrasted with those to a microbial antigen, influenza hemagglutinin. The weak and Th2-deviated T cell responses against lipocalin allergens may be attributable to the suboptimal recognition of their T cell epitopes. This was demonstrated here by the identification of several heteroclitic APLs for the Can f 1 epitope-containing peptide p105-120. The basis for the low antigenicity and suboptimal recognition of dog lipocalin allergens by human T cells may reside in their structural resemblance with self antigens. These features can affect the development of a Th2-type immune response and could therefore be considered as a factor promoting the allergenicity of Can f 1.

Dog lipocalin allergen-specific CD4+ T cell responses between allergic and nonallergic individuals differ both functionally and phenotypically. Importantly, although allergen-specific memory CD4+ T cells exist in both allergic and nonallergic subjects, their frequency appears to be lower in nonallergic subjects in comparison to allergic subjects. Here, an eight-fold difference was observed in the frequency of Can f 4-specific memory CD4+ T cells between these subject groups. Active peripheral regulation is one possible mechanism accounting for the phenomenon.

The binding capacity of Can f 4 epitope-containing peptides to a variety of HLA class II molecules proved to play a major role in their capacity to induce T cell responses *in vivo*. In therapeutic terms, an interesting target for further investigations would be the 19-mer peptide Can f 4_{46-64} containing the core sequence of the most immunogenic Can f 4 epitope region. This peptide was found to contain multiple HLA class II-binding motifs and it was recognized by the memory CD4+ T cells of 90% of the allergic subjects.

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Human T cell Response to Dog Lipocalin Allergens

Prospects for Allergen Immunotherapy

The aim of this study was to analyze human CD4+ T cell responses to the dog lipocalin allergens Can f 1 and Can f 4 in individuals with and without dog allergy.

In general, these allergens were found to be only weakly stimulatory on human T cells. However, substantial differences were observed in the allergen specific memory CD4+ T cells between the allergic and nonallergic subjects.

Importantly, a peptide derived from the Can f 4 allergen, Can f $4_{46\text{-}64}$, was found to be immunodominant and could be considered as a candidate for the development of peptide immunotherapy of dog allergy.

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