SOILI SAARELAINEN

Immune Response to Lipocalin Allergens
IgE and T-cell Cross-Reactivity

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L21, Snellmania building, University of Kuopio, on Friday 15th February 2008, at 12 noon

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ABSTRACT

Although mammalian lipocalin allergens are important respiratory sensitzizers, little is known about the immunological characteristics of these allergens, such as T-cell and B-cell cross-reactivity. The aims of this study were to characterize the murine immune response to the bovine lipocalin allergen Bos d 2 and to elucidate T-cell and B-cell cross-reactivities among lipocalin allergens. Moreover, recombinant dog allergens Can f 1 and Can f 2 were assessed to determine their suitability in diagnosing dog allergy.

Bos d 2, the major respiratory allergen of cow, is known to have a weak stimulatory capacity for human peripheral-blood mononuclear cells. Here, the proliferative response to Bos d 2 was found to be weak in six mouse strains with different major histocompatibility complex haplotypes. Furthermore, only the BALB/c mouse mounted a distinct humoral response to Bos d 2. One immunodominant epitope in Bos d 2, p127-142, was recognized by the BALB/c mouse. The response was of a T-helper type 2. The localization of the epitope in Bos d 2 was similar to that recognized by human T cells. The proliferative and cytokine responses to p127-142 also resembled those found with human T cells. These results support the view that the allergenic capacity of Bos d 2 is associated with the response to its immunodominant epitope.

Human T cells have been shown to recognize a determinant in areas of the rat and dog major allergens, Rat n 1 and Can f 1, corresponding to that of Bos d 2, p127-142. In the BALB/c mouse, p127-142 did not exhibit T-cell cross-reactivity with the 11 lipocalin-derived peptides examined. However, p127-142 did elicit a cross-reactive T-cell response with a bacterial peptide (SP7) from Spiroplasma citri. The cytokine profile induced by SP7 differed from that induced by p127-142, since the former was of a Th0 type. p127-142 and SP7 could reciprocally modulate in vitro the cytokine response of the spleen cells primed by the other peptide. This result suggests that modified allergen peptides can skew the phenotype of primed T cells. The phenomenon may open prospects for allergen immunotherapy.

The IgE cross-reactivity of mammalian lipocalin allergens is also poorly known. Four lipocalin allergens and human tear lipocalin, showed IgE cross-reactivity. IgE inhibition experiments suggest that both common and unique IgE epitopes are found between the IgE cross-reactive pairs (Can f 1 and TL; Can f 1 and Can f 2; Equ c 1 and Mus m 1). Since the sequence identity between the IgE cross-reactive allergens varies from 23% to 61%, it can be speculated that cross-reactivity between these allergens is more associated with their 3-dimensional structures than with the similarity of their amino acid sequences. Furthermore, Can f 1 showed IgE cross-reactivity with human tear lipocalin. It is possible that IgE cross-reactivity among lipocalins, including endogenous lipocalins, may be implicated in sensitization to lipocalin allergens.

The recombinant dog allergens Can f 1 and Can f 2 were found to have high specificity (100%) for the diagnostics of dog allergy. However, sensitivity analyzed by either skin prick tests, IgE immunoblotting and enzyme-linked immunosorbent assay was weak. Immunoblot analyses suggested that other allergens, in addition to Can f 1 and Can f 2, may also be important in sensitization to dog. A 18 kDa protein in a commercial dog epithelial extract was recognized more frequently than Can f 1 by dog-allergic patients. The aminoterminal sequencing of the protein suggested that it is a new member of the lipocalin family.
To Samu and Vesku
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Kuopio, February 2008

Soili Saarelainen
ABBREVIATIONS

3-D 3-dimensional  
Ag antigen  
AE atopic eczema  
AID activation–induced cytidine deaminase  
AKC atopic keratoconjunctivitis  
alum aluminium hydroxide  
APC antigen-presenting cell  
APRIL proliferation-inducing ligand  
ARC allergic rhinoconjunctivitis  
BLys B lymphocyte stimulator protein  
CCD cross-reactive carbohydrate determinants  
CCL chemokine ligand  
CD cluster of differentiation  
cDNA complementary deoxyribonucleic acid  
CFA complete Freund's adjuvant  
cpm count per minute  
CTLA-4 cytotoxic T lymphocyte-associated antigen 4  
DBPCFC double-blind placebo-controlled food challenge  
DC dendritic cell  
IDO indoleamine 2,3-dioxygenase  
IEMA immuno-enzymometric assay  
i.p. intraperitoneally  
ELISA enzyme-linked immunosorbent assay  
ELISPOT enzyme-linked immunospot assay  
FceRI high affinity receptor for immunoglobulin E  
FEIA fluoroenzyme-immunometric assay  
FOXP3 forkhead/winged helix family transcription factor -3  
GM-CSF granulocyte-macrophage-colony stimulating factor  
HEL hen egg lysozyme  
HPLC high–pressure liquid chromatography  
IC_{50} concentration required for 50% inhibition  
IFN interferon  
Ig immunoglobulin  
IL interleukin  
Isaac International Study of Asthma and Allergies in Childhood  
IUIS International Union of Immunological Societies  
kDa kilodalton  
mAb monoclonal antibody  
MHC major histocompatibility complex  
MnSOD manganese superoxide dismutase  
MS multiple sclerosis  
n natural  
NFAT nuclear factor of activated T cells  
nsLTP nonspecific lipid-transfer protein  
p127-142 peptide 127-142 of Bos d 2  
PAC perennial allergic conjunctivitis  
PAMP pathogen-associated molecular pattern
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PAR</td>
<td>protease-activated receptor</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral-blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed death-1</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>r</td>
<td>recombinant</td>
</tr>
<tr>
<td>RAST</td>
<td>radioallergosorbent test</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SAC</td>
<td>seasonal allergic conjunctivitis</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SI</td>
<td>stimulation index</td>
</tr>
<tr>
<td>SP</td>
<td>database search peptide</td>
</tr>
<tr>
<td>SP7</td>
<td>database search peptide from <em>Spiroplasma citri</em></td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
</tr>
<tr>
<td>SOCS1</td>
<td>suppressor of cytokine signaling-1</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th cell</td>
<td>T-helper lymphocyte</td>
</tr>
<tr>
<td>TL</td>
<td>tear lipocalin, von Ebner's gland protein, VEGP</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like reseptor</td>
</tr>
<tr>
<td>Treg cell</td>
<td>regulatory T lymphocyte</td>
</tr>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
</tr>
<tr>
<td>VKC</td>
<td>vernal keratoconjunctivitis</td>
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LIST OF ORIGINAL PUBLICATIONS

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1. INTRODUCTION

In recent decades, the prevalence of allergic diseases and asthma has increased considerably in Finland (Huurre et al., 2004; Isolauri et al., 2004) as well as in other developed countries (Asher et al., 2006). The ISAAC study published in 2006 showed that the prevalence of asthma symptoms in Finnish children aged 13-14 years had increased from 13.1% to 19.0% within five years (Asher et al., 2006). Despite intense study, the mechanisms of allergic diseases are still not fully understood.

Most major, as well as several minor, mammalian respiratory allergens belong to the family of lipocalin proteins. The lipocalins are proteins that can act, for example, as transporters of small hydrophobic molecules (Flower, 1996), modulators of cell growth, and regulators of immune response (Åkerström et al., 2000; Lögdberg et al., 2000). The lipocalin allergens include the major allergens of dog, horse, mouse, rat and, cow (Virtanen et al., 2004). The major allergen of cat, Fel d 1, is a unique allergen among animal-derived respiratory allergens in that it is not a lipocalin (Kaiser et al., 2003). The lipocalins are present both in animals and humans (Åkerström et al., 2000).

Despite the fact that IgE cross-reactivity among pollen and food allergens is common and widely studied (Radauer et al., 2006a), the cross-reactivity among animal allergens is poorly known. For example, few cross-reactivity studies have focused on animal-derived lipocalin allergens (Fahlbusch et al., 2003; Kamata et al., 2007). In addition to IgE cross-reactivities between allergens, the IgE cross-reactivities between endogenous proteins and environmental allergens have also been reported (Budde et al., 2002; Aichberger et al., 2005; Limacher et al., 2007).

IgE cross-reactivity has many potential impacts on allergy. For example, IgE cross-reactive allergens can cause clinical symptoms in patients without prior sensitization (Wensing et al., 2002; Bohle et al., 2003) or lead to misdiagnosis of related allergens (Kochuyt et al., 2005). On the other hand, IgE cross-reactivity between allergens can be useful in the diagnostics of multivalent sensitizations (Tinghino et al., 2002) and immunotherapy of allergy (Niederberger et al., 1998). Moreover, it has been proposed that IgE cross-reactive proteins, especially endogenous proteins that are IgE cross-reactive with exogenous allergens, may be involved in regulating or tolerizing an allergic response (Aalberse et al., 2001), for example, through the high affinity IgE receptor (FcεRI) and indoleamine 2,3-dioxygenase (IDO) (von Bubnoff et al., 2003).
T-cell cross-reactivity is strikingly different from IgE cross-reactivity and much more difficult to predict (Aalberse, 2005). T-cell cross-reactivity between pollen allergens Cha o 1 and Cry j 1 has been shown to contribute to prolonged symptoms even after the pollen seasons of these trees (Sone et al., 2005). Moreover, the activation of pollen-specific Th2 cells by related food allergens, in particular outside the pollen season, may cause deterioration of atopic eczema without a clinical food allergy (Bohle, 2007). Several studies have demonstrated that exogenous allergens can show T-cell cross-reactivity with endogenous counterparts (Fluckiger et al., 2002; Aichberger et al., 2005). It has been further suggested that environmental allergens mimicking a self antigen may represent an important pathomechanism involved in the maintenance and exacerbation of severe forms of allergy (Bünder et al., 2004).
2. REVIEW OF THE LITERATURE

2.1 Allergic diseases

2.1.1 General

Atopy and asthma prevalences have increased in the developed countries (Vartiainen et al., 2002). For example, it has been estimated that among adults 24-30% suffer from allergic rhinitis, 5-8% from asthma, and 10-20% from atopic eczema in Finland (Huurre et al., 2004; Hahtela et al., 2007). Moreover, the birth cohort study of Isolauri et al. showed that the proportion of subjects with detectable IgE antibodies against aeroallergens had consistently increased over the period from 1923-26 to 1990 (Isolauri et al., 2004). In addition to the adverse health effects for the patients, allergic diseases can also pose a significant economic burden. The immediate costs of allergy and asthma were estimated to be as high as 380 million euros in 2001 (http://www.terveyskirjasto.fi/terveyskirjasto/tk.koti?p_artikkeli=suo00033).

Hypersensitivity is a term used to describe objectively reproducible symptoms or signs initiated by exposure to a defined stimulant at a dose tolerated by normal persons (Johansson et al., 2004). Allergy is defined to be a harmful antibody or cell-mediated immune reaction against innocuous antigens, allergens, commonly present in the environment (Johansson et al., 2004). Atopy is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and to produce IgE antibodies on exposure to innocuous antigens. As a consequence, atopic persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema (Johansson et al., 2004). Although atopic diseases have a genetic background, several results suggest that atopic disposition may also be related to differences in lifestyle and environmental factors (von Mutius et al., 1998; Vartiainen et al., 2002; Asher et al., 2006).

2.1.2 Allergic diseases

One of the most common allergic disease is allergic rhinitis. According to a study by Huurre et al., 26% of 32-year-old adults suffer from allergic rhinitis in Finland (Huurre et al., 2004). The early-phase symptoms, itching, sneezing and watery rhinorrhea, are mainly caused by the release of inflammatory mediators of mast cells, of which histamine is the most important (Prussin et al., 2003). The immunological process in both nasal and bronchial tissue involve Th2 lymphocytes and eosinophils (Gelfand, 2004). Moreover, other cell populations, such as
dendritic cells and mast cells, have also been shown to be involved in this process (Rosenwasser, 2007). During the late phase reaction, T cells and mast cells release cytokines, such as IL-4 and IL-5, which stimulate IgE production in B cells and also activate eosinophils. The symptoms of chronic ongoing rhinitis, such as the nasal blockage, loss of smell and “nasal hyper-reactivity”, are due to eosinophils (Gelfand, 2004).

The term allergic conjunctivitis subsumes several types of conjunctivitis, such as seasonal allergic conjunctivitis (SAC) (Bonini, 2004). Patients with allergic conjunctivitis often have a personal or family history of atopy. When accompanied by allergic rhinitis, allergic conjunctivitis is called allergic rhinoconjunctivitis (ARC). Approximately 15% of the children aged 13-14 years suffer from ARC in Finland (Asher et al., 2006). Especially, in atopic and vernal keratoconjunctivitis, clinical sensitizations to common allergens cannot be detected using skin prick tests or IgE immunoassays (Bonini, 2004; Pucci et al., 2003).

The classical type 1 immediate hypersensitivity mechanism (mast cell degranulation induced by the allergen-IgE interaction) is not sufficient to explain all the pathophysiological abnormalities in allergic conjunctivitis (Bonini, 2004). The Th2-type inflammation with mast cells, basophils, eosinophils and polyclonal IgE activation is suggested to have an important role in these disorders (Romagnani, 1994).

The International Study of Asthma and Allergies in Childhood (ISAAC) showed that the prevalence of asthma in children aged 13-14 years was 5-20% in most countries (Asher et al., 2006). Asthma is a chronic inflammatory disease of the bronchial airways with an increased number of activated T cells, eosinophils, mast cells and neutrophils within the airway lumen and bronchial submucosa (Hamid et al., 2003; Marone et al., 2005). Those patients susceptible to asthma often have symptoms associated with inflammation, ranging from bronchial hyperresponsiveness to a variety of inhaled bronchoconstrictor stimuli (Gounni et al., 2005).

Asthma can be classified as either allergic or nonallergic asthma. Most cases of asthma in children (80%) and in adults (>50%) have been reported to be allergic and mainly IgE-associated (Johansson et al., 2004). Serum IgE plays an important role in the pathogenesis of smooth muscle hyperreactivity. Recently, human airway smooth muscle (HASM) cells were found to express FcεRI-receptor. IgE sensitization of the cultured cells induced the release of inflammatory mediators, including Th2-type of cytokines IL-5 and IL-13, and chemokines (Gounni et al., 2005).
Eczema, formerly also known as atopic dermatitis or atopic eczema, is an inflammatory, clinically relapsing, noncontagious, and extremely pruritic hereditary skin disease (Allam et al., 2005). Recently, the term ‘atopic eczema’ was defined more precisely to be used only for skin reactions based on IgE sensitization proven by allergen-specific IgE assays and/or skin prick test (Johansson et al., 2004). Atopic eczema is one of the most commonly seen dermatoses, with a prevalence of 22% in children and 13% in young adults in Sweden (Asher et al., 2006). The disease often begins in early childhood (after 3 months of life) and typically affects the face, extensor sides of arms, or legs. The clinical signs of AD may precede the development of asthma and allergic rhinitis, suggesting that AD forms an "entry point" for subsequent allergic disease (Spergel et al., 2003). Eczema is exacerbated by food allergies, especially severe forms of AE in children (Novak et al., 2003). Atopic eczema is characterized by blood eosinophilia and increased IgE production (Breuer et al., 2001). The dominance of Th2-type cells is typically associated with acute-phase skin lesions, whereas chronic-phase lesions also contain Th1 cells (Allam et al., 2005). It has been speculated that cytokines derived from Th2-type cells present in the early phase of atopic eczema may attract eosinophils, stimulate IL-12 expression and thereby cause sequential activation of Th0 and Th1-type cells (Grewe et al., 1998). Furthermore, FcεRI-bearing Langerhans cells and plasmacyoid dendritic cells have an important role in the pathophysiology of AE (Novak et al., 2004; Allam et al., 2005).

Apart from other dermatoses, such as dermatitis, urticaria and angioedema, allergic diseases can also include food and drug allergies. Dermatitis and urticarias are caused by a wide variety of agents, including drugs, food allergens, infections, or systemic diseases (Zuberbier et al., 2007). The mechanisms underlying dermatoses, food and drug allergies, can be mediated by immunological and nonimmunological mechanisms (Johansson et al., 2004; Hennino et al., 2006).
2.2 Allergic sensitization

2.2.1 Cells of the immune system involved in sensitization

2.2.1.1 Antigen-presenting cells

Antigen-presenting cells (APC) are a group of cells, including dendritic cells (DC), monocytes/macrophages and B cells, that can uptake antigens and present them to T lymphocytes via major histocompatibility complex (MHC) I and MHC II molecules (Peppelenbosch et al., 2000; Burgdorf et al., 2007). Moreover, eosinophils can act as APCs and polarize T cells into a Th2 subset in a manner similar to that of DC (Padigel et al., 2006).

DCs are a group of migratory bone-marrow-derived leukocytes that specialize in the uptake, transport, processing and presentation of antigens to T cells (Shortman et al., 2002). DCs are essential for CD4+ T-cell priming and for T-cell differentiation (Eisenbarth et al., 2003). DCs circulate and capture antigens at an immature state in peripheral tissues, such as the skin, the lung and the gut (Wilson et al., 2003). Antigen uptake with the presence of pathogen-associated molecular patterns (PAMPs) (Kapsenberg, 2003) or signals from damaged tissues, e.g., heat-shock proteins, leads to the maturation and migration of DCs to lymph nodes where they are able to stimulate naïve T cells (Shortman et al., 2002). Moreover, DC subsets are found to express distinct patterns of Toll-like receptors (Eisenbarth et al., 2003), such as TLR2 and TLR9, which are important in the polarization of T-cell phenotypes (Redecke et al., 2004). In mucosa, DCs have an immature phenotype and are capable of uptaking and processing allergen, though they lack the capability to stimulate naïve T cells (van Rijt et al., 2005).

2.2.1.2 T-helper cells

The major class of T cells is defined by its surface expression of the αβ T-cell receptor, the antigen receptor of the T cell. The major task of CD8+ T cells is to kill cells infected by intracellular microbes. The CD4+ T cells regulate the cellular and humoral (B-cell help) immune responses and are therefore important cells in allergy. They are divided into several different subtypes (Fig.1). In the blood and secondary lymphoid organs, approximately 65% of T cells are T-helper cells (Th cells) (Chaplin, 2003). T cells undergo maturation in thymus. Fewer than 5% of the developing T cells survive positive and negative selection of the maturation and migrate to the periphery (Chaplin, 2003).
T-helper cells are further polarized into a Th1 or Th2 phenotype during the immune response. It has been suggested that several surface markers, such as TIM-1, TIM-2 and CD62L, can differentiate Th1 cells from Th2 cells. For example, Th1 cells are found to express TIM-3 (Monney et al., 2002) while Th2 cells express TIM-2 (Chakravarti et al., 2005) and CD62L (Matsuzaki et al., 2005). Th1 cells secrete, for example, IL-2, IFN-γ and TNF-β following activation. They are involved in the induction of inflammatory reactions that are often accompanied by tissue damage and destruction (Yssel et al., 2001). However, Th1 cells may also contribute to the chronicity and effector phase in allergic diseases, such as asthma (Taylor et al., 2005). Th2 cells are involved in parasite infections and allergy, and provide optimal help for humoral immune responses, including the class switching of B cells. Moreover, the Th2 cell-produced cytokines IL-4, IL-5, and IL-13 mediate regulatory and effector functions, such as prolonged eosinophil survival and recruitment, as well as tissue homing of Th2 cells (Ying et al., 1997; Romagnani, 2004). Other cytokines secreted by Th2
cells include IL-9, IL-10, and the granulocyte macrophage-colony stimulating factor (GM-CSF) (van Rijt et al., 2005). Furthermore, T cells capable of producing mixed patterns of Th1 and Th2 cytokines are classified as Th0 cells (Abbas et al., 1996). The recently found Th17 cells form a new subset of T-helper cells. They secrete cytokines, such as IL-17 and IL-6. The role of Th17 cells in allergy is largely unclear but experimental models suggest that IL-17 may be important for neutrophilic recruitment in the acute airway inflammation (Fujiwara et al., 2007) and in the initiation of asthma (Scheyder-Candrian et al., 2007).

2.2.1.2 Regulatory T cells

Regulatory T cells (Treg cells), previously also referred to as suppressor T cells, include cell populations of several types (Curotto de Lafaille et al., 2002; Akdis et al., 2004). The main subsets of regulatory cells are CD4+ CD25+ Treg cells and type 1 regulatory T cells (Tr1) (Bacchetta et al., 2005). However, additional regulatory T cells, such as Th3 cells and other cell types with regulatory function, have been reported (Taylor et al., 2005). The regulatory T cells can suppress both Th1 and Th2 cells through multiple mechanisms. These suppressive mechanisms include secretion of immunosuppressive cytokines (e.g., IL-10 and TGF-β) (Akdis et al., 1998; Akdis et al., 2004), and expression of surface molecules (CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) (Read et al., 2000) and PD-1 (programmed death-1) (Nishimura et al., 1999)). It has been shown that CD4+CD25+ T cells and IL-10 have important roles in specific immunotherapy (Akdis et al., 1998; Savolainen et al., 2004) and in healthy immune response (Jutel et al., 2003).

The natural regulatory T cells (nTreg) develop in the thymus. They express CD4 and CD25 molecules on their surfaces, as well as a forkhead/winged helix transcription factor FoxP3 (Rouse, 2007). nTreg cells contribute to the maintenance of immunological self-tolerance and immune homeostasis (Miyara et al., 2007). The suppressive function of nTreg cells is mainly based on cell contact-dependent interactions (Bacchetta et al., 2005). Especially surface molecules, such as CTLA-4, have a central role in the suppressive mechanism of CD4+CD25+ FoxP3 Treg cells (Miyara et al., 2007). It was recently observed that CD4+CD25+ T cells from non-atopic donors suppressed the proliferation and cytokine production of allergen-stimulated CD4+CD25- T cells whereas the inhibition was diminished with the CD4+CD25+ T cells from atopic patients and patients with hayfever (Ling et al., 2004).
Tr1 cells regulate immune responses in transplantation, allergy, and autoimmune diseases (Bacchetta et al., 2005). The suppressive effects of both Tr1 and Th3 cells are based on the high production of immunosuppressive cytokines (Taylor et al., 2005). The major effector cytokine in Tr1 cells is IL-10 and to a smaller degree TGF-β (Rouse, 2007). Th3 cells, on the other hand, produce high amounts of TGF-β and variable amounts of IL-10 and IL-4 (Chen et al., 1994).

2.2.1.4 B cells

B cells are pivotal in the adaptive immune response in that they provide humoral immunity through their secreted antibodies. B cells express the CD19 molecule on their surface and constitute approximately 15% (range 5-21%) of the peripheral-blood lymphocytes (Jentsch-Ullrich et al., 2005). B cells are divided into memory and plasma cells. The latter are terminally differentiated antibody-producing cells. Traditionally, plasma cells have been considered short-lived, end-stage lines of B-cell differentiation. However, recent studies have shown that plasma cells can survive long periods in appropriate survival niches (Radbruch et al., 2006). Furthermore, the development of B-cell memory is thought to be critical in exacerbating allergic syndromes (Chaplin, 2003). It has been suggested that long-lasting IgE responses in both mice and humans are due to long-lived plasma cells (Aalberse et al., 2004; Radbruch et al., 2006), since specific IgE levels may remain high or even rise, despite successful allergen immunotherapy (Kinaciyan et al., 2007).

Because B cells are able to internalize soluble antigens, they can serve as antigen-presenting cells for T cells. B cells capture their cognate antigen, process it intracellularly, and present the peptides on the cell surface associated with MHC class II molecules (Chaplin, 2003). B cells can be activated in a T cell-dependent and T cell-independent manner. T-helper cells that recognize the peptide:MHC complex deliver activating signals to the B cells. One particularly important co-stimulatory molecule in B-cell activation is the T-cell surface molecule CD40L. In contrast to T cell-dependent activation, in which monomeric antigens activate the B cells, T cell-independent activation requires polymeric antigens, such as bacterial lipopolysaccharide, to activate B cells. This activation may occur through the cross-linking and clustering of Ig molecules on the B-cell surface.
2.2.2 Development of the Th2-deviated cellular immune response

2.2.2.1 Allergic sensitization

An allergic reaction requires prior immunization, known as sensitization, to the allergen (Fig. 2). Initial contact with a soluble allergen on mucosal surfaces favors allergen uptake by the potent APC. In the case of respiratory allergies, contact with minute amounts of intact, soluble allergens takes place at the mucosal surface of respiratory tract (Valenta, 2002). The interaction with APCs, such as dendritic cells, initiates the T-cell response and differentiation. Furthermore, sensitization leads to the establishment of allergen-specific, long-lived memory T cells (Chakir et al., 2000). The T cell-B cell interaction in the presence of IL-4 and IL-13 promotes the IgE-class switch (Plebani, 2003). Subsequent allergen contact of B cells in the presence of T-cell help will boost IgE memory B cells to produce increased levels of allergen-specific IgE antibodies (Valenta, 2002).

Although Th2 cells are important in allergic sensitization, the Th1 or mixed Th1/Th2 profile is associated with severe forms of chronic allergic diseases, such as asthma (Bünner et al., 2004) and atopic dermatitis (Aichberger et al., 2005).

Figure 2. Allergic sensitization. Reprinted by permission from Macmillan Publisher Ltd: Nat Rev Immunol, vol:2, Valenta R. The future of antigen-specific immunotherapy of allergy, p.446-5, copyright (2002).
2.2.2.2 Polarization of the cellular immune response

Factors including the route of antigen exposure, the type of antigen-presenting cells, the cytokine milieu at the site of challenge, and the dose of the antigen are all believed to influence the development of T-helper cell phenotypes (McKenzie et al., 1998b). Although dendritic cells have an important role in T-cell polarization, it is becoming evident that several cell types, such as mast cells, basophils and eosinophils, are important in T-cell subset development, as well (Prussin et al., 2003; Adamko et al., 2005; Galli et al., 2005a; Galli et al., 2005b; Gibbs, 2005).

2.2.2.2.1 Cytokines

The key regulator of Th2 polarization seems to be the so-called “early” interleukin-4 (IL-4) (Haas et al., 1999). For example, IL-4-knockout mice do not develop an allergic inflammatory response after airway challenge (Herrick et al., 2003). Although predominantly produced by basophils (Prussin et al., 2003; Gibbs, 2005), IL-4 can also be produced by mast cells (Saarinen et al., 2001; Robinson, 2004; Galli et al., 2005a), eosinophils (Adamko et al., 2005), natural killer T cells, and Th2 cells (Valenta, 2002). Ligation of IL-4R by IL-4 results in the activation of the Janus family of tyrosine kinases and STAT6 activation, which has a central role in the gene regulation of Th2 differentiation. Moreover, STAT6 activation results in the upregulated expression of the Th2 specific transcription factor GATA-3, which may further upregulate the expression of several Th2 cytokines (Haas et al., 1999; Yoshimoto et al., 2007). Apart from IL-4, additional cytokines, such as IL-6, IL-13 and IL-18 may favor Th2 cell polarization (Haas et al., 1999). Interleukin-13 can elicit responses in T cells similar to those in IL-4. McKenzie et al. showed that IL-13-deficient mice produced significantly reduced levels of IL-4, IL-5, and IL-10 and of serum IgE (McKenzie et al., 1998b). Thus, it seems that IL-13 performs a central regulatory role in the development of Th2 cells (McKenzie et al., 1998a; Scales et al., 2007). IL-18 is secreted by activated macrophages (Dai et al., 2007). Although the major action of IL-18 is induction of IFN-γ by Th1 cells, it was shown that IL-18 causes IL-4-dependent Th2 polarization in vitro (Yoshimoto et al., 2000; Shida et al., 2001). However, IL-18 in combination with IL-12 polarizes the response towards Th1-type (Li et al., 2005).
IL-6 is a cytokine produced by a number of cell types, including antigen-presenting cells (macrophages, DCs, and B cells), fibroblasts, Th2 cells, and endothelial cells (Mowen et al., 2004). IL-6 is a proinflammatory cytokine that, for example, activates endothelial cells (Romano et al., 1997) and induces the synthesis of acute phase proteins (Xing et al., 1998). It elicits Th2 differentiation in two independent molecular mechanisms (Diehl et al., 2002). IL-6 promotes Th2 differentiation by activating transcription through the nuclear factor of activated T cells (NFAT), thus leading to production of IL-4 by naïve CD4+ T cells (Diehl et al., 2002). Moreover, IL-6 inhibits Th1 differentiation by inducing SOCS1 (suppressor of cytokine signaling–1) expression, which interferes with IFN-γ signaling (Diehl et al., 2000). Nevertheless, it seems that IL-6 is not essential for Th2 development (Blum et al., 1998; La Flamme et al., 1999).

Th2 polarization can be favored by the inhibition of Th1 polarization. Recently, Traidl-Hoffmann et al. have shown that inhibition of IL-12, the important interleukin for Th1 differentiation, enhanced Th2 polarization (Traidl-Hoffmann et al., 2005). In parasitic infections, IL-4 (Yamakami et al., 2002) and IL-10 can suppress IL-12 production and Th1 response development, thereby ensuring Th2 polarization (Yamakami et al., 2002; McKee et al., 2004).

2.2.2.2 Role of antigen-presenting cells
Dendritic cells (DCs) are essential in CD4+T-cell priming and T-cell differentiation (Eisenbarth et al., 2003). Contrary to what had been previously thought, the Th1/Th2 sensitzations induced by myeloid and plasmacytoid DCs are not confined to one subset (Cabanas et al., 2000; Shortman et al., 2002; van Rijt et al., 2005). The maturation of DCs represents a key regulatory step in T-cell activation and polarization (Eisenbarth et al., 2003; van Rijt et al., 2005). Maturation of DCs mainly relies on recognition of pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) belonging to the group of pattern recognition receptors (PRRs) (Kapsenberg, 2003). Furthermore, TLR2 seems to be important in Th2 deviation, whereas TLR9 is important in Th1 deviation (Redecke et al., 2004). The maturation of DCs induces their migration to draining lymph nodes. Without this migration of DCs, the Th2 sensitization to inhaled allergens can not occur (Eisenbarth et al., 2003). The recognition of PAMPs was thought to be involved only in Th1 responses. However, it seems that they have a role in inducing Th2 responses, as well. It has been observed that without a
PAMP protein, allergens do not activate APCs in vivo nor in vitro, but instead further induce Th2 sensitization (Eisenbarth et al., 2003).

Polarization of T-helper cells requires three dendritic cell-derived signals. The first signal is the antigen-specific signal mediated through the MHC class II/peptide complex coupled with TCR. The production of Th2 cytokines up-regulate MHC II expression on APCs, which reciprocally stimulate Th2 cells, favoring a Th2 disease pathway due to selective down-regulation of IL-12 (Powe et al., 2004). The second, co-stimulatory signal is mainly mediated by the triggering of CD28 by CD80 and CD86 (Kapsenberg, 2003). Induction of the Th2 response also involves other important co-stimulatory signals, such as OX40/OX40L (Hoshino et al., 2003) and CD40/CD40L (MacDonald et al., 2002). The absence of a co-stimulatory signal leads to anergy and possibly to tolerance (Kapsenberg, 2003). The recognition of PAMPs by DCs mediates the third signal, in addition to signals 1 and 2 (van Rijt et al., 2005). The activation of particular PRR by PAMPs defines the profile of T cell-polarizing factors (Kapsenberg, 2003). Furthermore, Th2 polarization can be influenced by CCL2 (chemokine ligand), PGE$_2$ (prostaglandin E$_2$), and histamin via polarizing dendritic cells (Kapsenberg, 2003).

In addition to dendritic cells, other cells such as eosinophils are also known to affect the polarization of Th2 cells. Eosinophils express co-stimulatory molecules essential for interaction with lymphocytes (Adamko et al., 2005).

### 2.2.2.2.3 Role of the antigen

Much experimental research has demonstrated that Th2 priming is preferentially favored by low-dose antigen exposure, whereas higher doses favor Th1 development (Holt et al., 2005). The nature of the antigen can also instruct DCs to influence the polarization of T cells (van Rijt et al., 2005). For example, the eggs rather than the worms of *Schistosoma mansoni* induce the maturation of DCs into a subtype which promotes the development of the Th2 phenotype (Kapsenberg, 2003). Moreover, *Aspergillus fumigatus* conidae induce a Th1 type of response, whereas the hyphae cause a Th2 response (Bozza et al., 2002). Anderson et al. showed that birch allergens have an intrinsic Th2-polarizing ability on neonatal cells by suppressing DC maturation (Andersson et al., 2004). The characteristics of the antigen are discussed below in more detail.
2.2.3 Immediate allergic reaction

2.2.3.1 Synthesis of immunoglobulin E (IgE)

IgE class-switch recombination is accomplished through 2 pathways (Geha et al., 2003). The classical or T cell-dependent pathway of IgE synthesis involves the presence of IL-4, IL-13, or both during ligation of the CD40-CD40 ligand. The ligation leads to the induction of Cε germline transcription and of activation-induced cytidine deaminase (AID) (Poole et al., 2005). The second pathway requires no T-cell interaction but does require IL-4. The T cell-independent IgE class-switch takes place when the B lymphocyte stimulator protein (BLys) and the proliferation-inducing ligand (APRIL), both expressed on monocytes and dendritic cells, bind to their respective receptors on the B cells in the presence of IL-4 (Litinskiy et al., 2002). Moreover, it has been shown recently that IgE synthesis can be induced by IL-4 and IL-13, which are produced by adenosine-activated mast cells (Ryzhov et al., 2004). Since IL-4 and CD40L are also expressed by basophils, the role of basophils in IgE class-switch has been discussed as well (Prussin et al., 2003). IgE class-switch, on the other hand, is negatively regulated by several mechanisms, including cytokines (e.g., IFN-γ and IL-21), various B-cell surface receptors (CD45, CD23), and several transcription factors (Poole et al., 2005).

2.2.3.2 Type I hypersensitivity

Mast cells and basophils have been regarded as key effector cells in IgE-associated immediate hypersensitivity and allergic disorders (Galli et al., 2005a). Atopic diseases are also classically associated with eosinophilia (Adamko et al., 2005). Release of mediators by mast cells and basophils, as well as by eosinophils, begins within minutes of antigen challenge in a sensitized individual. In such individuals, preformed IgE specific for the allergen is bound on the surface of the effector cells by the type I high-affinity Fc-receptor of IgE (FceRI). The cross-linking of the FceRI requires that an allergen binds to at least two antibody molecules on the surface of the effector cell. Therefore, for antibody binding to occur, an allergen must contain at least two IgE binding sites (epitopes), each of which will be a minimum of approximately 15 amino acid residues in length. Antibody cross-linking of FceRI on effector cells elicits changes in the cell membrane, causing degranulation. Release of mediators, such as histamin, leukotrienes and prostaglandins, increases vascular permeability, smooth muscle contraction, tissue edema and excretion of mucus. The pattern of the mediators depends on the signal, activation site and cytokine milieu. Activation of effector cells also leads to
cytokine secretion. For example, after allergen challenge, basophils are the predominant IL-4-producing cells in the human asthmatic airways (Prussin et al., 2003). On the other hand, in the upper dermis of lesional atopic dermatitis skin mast cells are one of the major sources of IL-4 (Saarinen et al., 2001). Recently, it has become apparent that effector cells, like mast cells and basophils, are active in maintaining a state of inflammation (Powe et al., 2004).

2.3 Allergens

2.3.1 General

Despite several attempts to find out, it seems that allergens do not have one single unifying structural or functional feature (Aalberse, 2000; Radauer et al., 2006a). The properties of protein structure most likely to be relevant for allergenicity are solubility, stability, size, and the compactness of the overall fold. For airborne allergens, size and solubility are important (Aalberse, 2000).

In addition to structural characteristics, it is believed that the functional characteristics of some allergens may also play a role in sensitization. Proteins such as lipocalins, nonspecific lipid-transfer proteins (nsLTPs) and calcium-binding proteins show increased stability and resistance to proteolysis (Breiteneder et al., 2005). Moreover, increased thermal stability and stability to digestion have been claimed as characteristics of some food allergens (van Ree, 2002), e.g., for peanut allergen Ara h 1 (Maleki et al., 2000).

Several allergens, such as Api m 1, Equ c 1, Equ c 4 and Equ c 5, have surfactant properties. Hydrophobic interactions may take place when proteins come into contact with membrane phospholipids or hydrophobic surfaces, such as a pulmonary surface, favoring allergen penetration into the tissue (Goubran Botros et al., 2001). It has recently been suggested that a common denominator for allergens would be the lack of bacterial homologs (Emanuelsson et al., 2007).

The allergenicity of respiratory animal lipocalins is hypothesized as arising from the similarity between exogenous allergens and endogenous lipocalins (Virtanen et al., 1999). It seems that T-cell epitopes in lipocalin allergens are clustered in a few regions along the molecules (Zeiler et al., 1999; Jeal et al., 2004; Immonen et al., 2007). Furthermore, experimental data and epitope predictions suggest that endogenous lipocalins and allergenic lipocalin allergens may have T-cell epitopes in the corresponding parts of the molecule (Zeiler et al., 1999; Immonen et al., 2005). Therefore, it can be further speculated that T-cell
reactivity against lipocalin allergens may be modified by endogenous lipocalins, for instance, by preventing strong T-cell reactivity against lipocalin allergens (Virtanen et al., 1999). Weak T-cell reactivity is known to favour Th2-type responses (Pfeiffer et al., 1995; Janssen et al., 2000; Foucras et al., 2002). Furthermore, the IgE cross-reactivity between the major dog allergen Can f 1 and human tear lipocalin is also in line with this hypothesis (Saarelainen et al., 2007).

Antibody response to pollen, arthropod or fungal allergens appears to differ from that of animal allergens (Platts-Mills, 2007). For example, the specific IgE titers of mite allergens can be very high, whereas specific IgE titers to animal allergens are generally low (Erwin et al., 2007; Reininger et al., 2007). Recently, it has been suggested that one reason for the difference in antibody response between arthropod and animal allergens may be the methylation of DNA associated with the allergens (Platts-Mills, 2007). Unmethylated DNA, such as DNA of mite (Platts-Mills, 2007), contains immunostimulatory sequence motifs that are recognized by TLR9 (Horner, 2006).

2.3.2 Posttranslational modifications

Posttranslational modifications, such as intramolecular disulfide bonds and glycosylation, have been proposed to enhance the allergenicity of a protein by increasing its uptake and detection by the immune system (Huby et al., 2000). Deletion of disulfide bonds from Asp f 2 has been shown to reduce the binding of IgE from allergic patients (Banerjee et al., 2002). However, intramolecular disulfide bonds per se do not make a protein an allergen, nor does their absence preclude allergenicity (Huby et al., 2000).

Since many allergens, especially in the plant kingdom, are glycosylated it is possible that the glycosyl groups could contribute to their allergenicity. It is clear that IgE binds to N- and O-linked oligosaccharides, especially N-glycans with 1-3 linked fucose and β1-2 linked xylose, in plant and invertebrate allergens (Wilson et al., 1998; van Ree et al., 2000; Fotisch et al., 2001) and can activate basophils (Bublin et al., 2003). However, oligosaccharides are minor IgE epitopes (Bublin et al., 2003; Okano et al., 2004) and the clinical significance of carbohydrate determinants is controversial (Fotisch et al., 2001).
2.3.3 Enzyme activity

Extracellular endogenous proteases, as well as exogenous proteases from mites and molds, react with cell-surface receptors in the airways to generate leukocyte infiltration and to amplify the immune responses to allergens (Reed et al., 2004). For example, the mite allergens Der p 1, Der p 3, Der p 6 and Der p 9 are proteases (Chapman et al., 2007). Protease-activated receptors (PARs) are widely distributed on the cells of the airway, where they contribute to inflammation, an important characteristic of allergic diseases (Reed et al., 2004). PAR stimulation of epithelial cells opens tight junctions, causes desquamation, and induces the production of cytokines, chemokines, and growth factors. Proteases of mites and molds appear to act through similar receptors. They amplify IgE production to allergens, degranulate eosinophils, and generate inflammation, even in the absence of IgE (Asokananthan et al., 2002; Miike et al., 2003). Kheradmend et al. has found that fungal protease allergens delivered to the airways elicited a direct Th2-type immune response (Kheradmand et al., 2002). It has been suggested that proteases from mites and fungi growing in damp, water-damaged buildings might form the basis for the increased prevalence of symptoms of rhinitis, asthma, and other respiratory diseases among individuals residing in these buildings.

2.3.4 Lipocalin allergens

Lipocalins comprise a large group of proteins that are found in vertebrates, invertebrates, plants and bacteria (Åkerström et al., 2000; Bishop, 2000; Hieber et al., 2000). The lipocalin family is divided into two subgroups, based on the presence of the conserved sequence motifs. The kernel lipocalins have three and outlier lipocalins one or two of these motifs (Flower et al., 2000). The sequence similarity is low, typically below 35% (Akerstrom et al., 2000). On the other hand, high sequence similarities also exist. For example, human tear lipocalin (TL) and dog Can f 1 have 61% sequence identity (the SIB BLAST network service at the Swiss Institute of Bioinformatics, April 5, 2007) and the sequence identity between horse Equ c 1 and cat Fel d 4 is 67% (Smith et al., 2004) (the SIB BLAST network service at the Swiss Institute of Bioinformatics, April 5, 2007). Despite the low similarity in their sequences, lipocalins share a similar three-dimensional (3D) structure; for example, the structures of TL and cow Bos d 2 are very close, even though their sequence identity is only 18% (Rouvinen et al., 1999; Breustedt et al., 2005). Studies using X-ray crystallography have shown the
lipocalin structure to consist of an eight-stranded antiparallel β-barrel that forms a ligand-binding pocket and one α-helix (Flower et al., 2000).

Lipocalins have several different functions. They can act as transporter proteins of small, principally hydrophobic molecules, such as retinoids, steroids, odorants and pheromones (Flower, 1996; Åkerström et al., 2000). Moreover, they can function as modulators of cell growth and metabolism or regulators of immune response (Åkerström et al., 2000; Lögdberg et al., 2000). Since lipocalins are proteins that are present both in animals and humans it has been hypothesized that the allergenicity of lipocalins is caused by misrecognition between endogenous lipocalins and exogenous lipocalin allergens (Virtanen et al., 1999).

Most of the major as well as minor mammalian respiratory allergens included in the Official Nomenclature of the International Union of Immunological Societies (IUIS) are lipocalins (Table 1). The major allergen of cat, Fel d 1, is a unique allergen among other animal-derived respiratory allergens since it is not a lipocalin (Kaiser et al., 2003). However, the recently identified cat allergen, Fel d 4, belongs to the lipocalins (Smith et al., 2004). Furthermore, a food allergen beta-lactoglobulin found in cow’s milk (Bos d 5) is also lipocalin. Other lipocalin allergens are from invertebrates, Bla g 4 from the cockroach, Tri a p 1 from the California kissing bug (Virtanen et al., 2004), and Arg r 1 from the pigeon tick (Hilger et al., 2005).

Lipocalin allergens are found in both monomeric and dimeric forms (Virtanen et al., 2004). Half of the inhalant lipocalin allergens are glycosylated or have a putative glycosylation site.
Table 1. Mammalian respiratory lipocalin allergens

<table>
<thead>
<tr>
<th>Animal</th>
<th>IgE prevalence (%)</th>
<th>Glycosylation</th>
<th>Oligomeric state</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>Bos d 2 &gt;90</td>
<td>no</td>
<td>m</td>
<td>1-2</td>
</tr>
<tr>
<td>Dog</td>
<td>Can f 1 50-75</td>
<td>putative</td>
<td>m+d</td>
<td>3-5</td>
</tr>
<tr>
<td></td>
<td>Can f 2 25-28</td>
<td>yes</td>
<td>m+d</td>
<td>3-5</td>
</tr>
<tr>
<td></td>
<td>Can f 4 1)</td>
<td>60</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Cav p 1 1)</td>
<td>70</td>
<td>m+d</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Cav p 2 1)</td>
<td>55</td>
<td>-</td>
<td>6-7</td>
</tr>
<tr>
<td>Horse</td>
<td>Equ c 1 80</td>
<td>yes</td>
<td>d</td>
<td>8-9</td>
</tr>
<tr>
<td></td>
<td>Equ c 2 1)</td>
<td>50</td>
<td>-</td>
<td>10-11</td>
</tr>
<tr>
<td>Cat</td>
<td>Fel d 4 63</td>
<td>putative</td>
<td>m</td>
<td>12</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mus m 1 50-66</td>
<td>no</td>
<td>m</td>
<td>13-15</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Ory c 1 1)</td>
<td>-</td>
<td>yes</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Ory c 2 1)</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>Rat</td>
<td>Rat n 1 9.7</td>
<td>yes</td>
<td>m</td>
<td>14.17</td>
</tr>
</tbody>
</table>

1) only the N-terminus is known, 2) m monomer, d dimer

2.3.5 Recombinant allergens

Since the use of natural allergen extracts poses several problems not existing with recombinant proteins, the recombinant allergens could provide essential tools for the diagnostics and immunotherapy of allergy, as well as for investigating the cellular mechanisms of immediate hypersensitivity and the molecular basis of inflammatory reactions (Chapman et al., 2000). The key advantage of recombinant allergens compared with allergen extracts is standardization. Even in so-called standardized preparations, allergen composition and content can vary. Moreover, irrelevant proteins can even cause new sensitizations (Moverare et al., 2002a). Natural products are also at high risk of being contaminated with
allergens from other sources and thus containing proteolytic enzymes. The enzymes could be allergenic or nonallergenic, but in either case, they can lead to degradation and loss of potency when administered together with other allergens during immunotherapy. Furthermore, most food allergens can easily be degraded by physical and/or chemical strain during the extraction process (Bohle et al., 2004).

Another advantage of recombinant allergens is that they can be produced in large milligram or gram quantities with high purity. Additionally, they can be easily standardized in mass units (Bohle et al., 2004). Recombinant allergens are produced in bacterial, yeast, or insect cells without biological or batch-to-batch variation in the product (Chapman et al., 2000; Slater, 2004). In contrast, the final amount of an allergen in an extract depends on the raw material used and the methodology applied to extract the protein.

Recombinant allergen-based diagnostic tests have been shown to improve sensitivity compared to extract-based tests (Ballmer-Weber et al., 2002; Bohle et al., 2004; van Hage-Hamsten et al., 2004). For example, the sensitivity of SPTs for diagnosing cherry allergy has been shown to be higher with a panel of recombinant cherry allergens than with the commercially available cherry extract (Ballmer-Weber et al., 2002). In the study of Ballmer-Weber et al., all the patients had a positive result to cherry in a double-blind placebo-controlled food challenge (DBPCFC). Furthermore, 96% of the patients had a positive SPT result with the panel of recombinant proteins, whereas the cherry extract produced a positive SPT result in only 20% of the patients (Ballmer-Weber et al., 2002).

Moreover, recombinant allergens would make it possible to precisely identify patients’ IgE reactivity profile. Therefore, an optimal combination of the allergens can be selected for immunotherapy (van Hage-Hamsten et al., 2004). When recombinant allergens are used in microarray testing of allergen-specific IgE, a larger amount of information can be achieved with a smaller amount of serum (Jahn-Schmid et al., 2003).

A problem in using recombinant proteins produced in E.coli would be the improper folding or lack of posttranslational modifications of the allergen. For example, Lol p 1 and Lol p 5 produced by E.coli have been shown to have lower binding capacity than their natural counterparts, thus giving rise in false negative results in RAST (van Ree et al., 1998). These problems, however, can be solved by using eukaryotic expression systems, such as that of the yeast Pichia pastoris, which enables posttranslational modifications. It is for this reason that Pichia pastoris has today become the major system for expressing recombinant allergens (Macauley-Patrick et al., 2005).
2.4 Cross-reactivity

2.4.1 Antibody cross-reactivity

2.4.1.1 General

Antibody cross-reactivity is the ability of an antibody to bind to an antigen (or allergen) that is different from the one that has induced its synthesis. Two types of antibody cross-reactivity occur: symmetric and asymmetric (Aalberse, 2007). When the multivalent allergens share epitopes, the allergens can inhibit each others’ specific IgE binding in a similar manner and the cross-reactivity is symmetric (Weber, 2001). Typical examples include plant profilins (Radauer et al., 2006b). However, the usual finding with the birch allergen Bet v 1 is that the allergen inhibits IgE binding to an apple allergen in a similar or even better way than the apple allergen (Vanek-Krebitz et al., 1995; Kinaciyan et al., 2007). The apple allergen only partially inhibits IgE binding to Bet v 1. In this case, the cross-reactivity is asymmetric, and the epitopes are partly the same (Weber, 2001). The cross-allergenicity seems to reflect the taxonomy in the great majority of cases with pollen allergens (Weber, 2003). IgE cross-reactivity has also been observed among allergens of taxonomically related animals (Savolainen et al., 1997).

Cross-reactivity has been considered to result from the high sequence similarity. Pan-allergens, such as lipid transfer proteins (about 95%) and polcalcins (64%-92%), share a high degree of sequence identity (Sankian et al., 2005; Radauer et al., 2006a) and are thus highly IgE cross-reactive (Radauer et al., 2006a). However, it seems that a high structural homology of pan-allergens plays an important role in IgE-mediated poly-sensitization (Fluckiger et al., 2002). This is reasonable, since IgE-epitopes are known to be conformational (Limacher et al. 2007). The view that similarity at the three dimensional (3-D) level may be more important than similarity at the sequence level is also supported by recent studies of profilins (Sankian et al., 2005), the Bet v 1 family, and an nsLTP subfamily of prolamin (Jenkins et al., 2005). However, high structural similarity does not always result in cross-reactivity (Aalberse, 2000). Furthermore, the sequence identity of at least 50% of most pollen allergens seems to be prerequisite for allergenic cross-reactivity (Radauer et al., 2006a).
2.4.1.2 Plant and food allergens

IgE cross-reactivity among allergens is common and has widely been studied, especially in pollen and food allergies (Radauer et al., 2006a). Among those patients with allergies to birch pollen who suffer from such clinical syndromes as hay fever and asthma, up to 80% also show hypersensitivity to fresh fruits and vegetables (Neudecker et al., 2001; Ferreira et al., 2004). In the pollen–food allergy syndrome, the pollens and foods are not usually botanically related but nonetheless do contain conserved homologous proteins, such as profilins. Although only 10% to 20% of the patients with pollen allergies are sensitized to profilins (Wensing et al., 2002), they are responsible for cross-reactivity, for example, among mugwort pollen-celery-spices and among grass pollen-celery-carrots (Ferreira et al., 2004; Weber, 2001). Approximately 30 to 50% of latex-allergic persons are also allergic to specific plant foods (Wagner et al., 2004). Class I chitinases containing a small N-terminal hevein domain are described as the most important pan-allergens associated with a latex-fruit syndrome (Diaz-Peral et al., 2002; Karisola et al., 2005). Moreover, consistent with the pollen-food syndrome, the IgE cross-reactivity of profilins has been shown to be one cause for latex-fruit syndrome, as well (Wagner et al., 2004).

It seems that in pollen-food and latex-fruit syndromes, the patients are primarily sensitized to pollen or latex allergens and subsequently react to food allergens. This is also supported by the finding that the IgE from patients with latex allergy bound with much higher efficiency to hevein and prohevein than to proteins from fruit (Karisola et al., 2005). Moreover, allergy to fresh fruits and vegetables is much more common among patients with pollinosis than among those without pollen allergy (Egger et al., 2006).

The inhibitory capacity of the cross-reactive plant and food allergens can be very strong. For example, a study of peanut allergen rAra h 8 and Bet v 1 showed that they similarly inhibited (80-100%) IgE binding to a peanut allergen extract (Mittag et al., 2004). In the study of Radauer et al., in which Phl p 12-specific IgE binding was inhibited with several profilins, most of the inhibitions were over 70%, and half of the inhibitions were over 90% (Radauer et al., 2006b).

2.4.1.3 Animal allergens

Even though IgE cross-reactivity among plant, food and arthropod allergens has received much attention, cross-reactivity among mammalian animal allergens remains poorly
understood. Only a few studies have focused on animal allergen extracts, e.g., dog, cat and cow extract, (Spitzauer et al., 1995; Savolainen et al., 1997; Cabanas et al., 2000; Ferrer et al., 2006; Reininger et al., 2007) and purified allergens (Fahlbusch et al., 2003; Kamata et al., 2007).

Since animal serum albumins have a highly conserved sequence similarity and 3-D structure, it is not surprising that they have been studied most often. According to the Swiss-Prot protein database, the sequence identities among albumins range from 75% to 83%. Dog and cat albumins have been demonstrated to be IgE-cross-reactive (Spitzauer et al., 1995; Cabanas et al., 2000; Pandjaitan et al., 2000). Cabanas et al. reported that dog albumin inhibited IgE binding to cat albumin at a high percentage (71-100%). However, inhibition of the binding of dog albumin-specific IgE with cat albumin was lower (49-99%) (Cabanas et al., 2000). Therefore, it was thought that cat and dog albumins share some common (Spitzauer et al., 1995), though protein-specific determinants (Cabanas et al., 2000). Furthermore, dog albumin has been shown to have IgE cross-reactivity with the albumsins of mouse, chicken, rat (Spitzauer et al., 1994), guinea pig (Spitzauer et al., 1995), horse (Cabanas et al., 2000) and man (Pandjaitan et al., 2000).

In addition to albumins, the IgE cross-reactivity has been described for a few purified mammalian respiratory allergens. Characterization of the major lipocalin allergens of guinea pig revealed that Cav p 1 and Cav p 2 have IgE-cross-reactive epitopes (Fahlbusch et al., 2003). Moreover, Cav p 1 showed IgE cross-reactivity with a guinea pig allergen with a molecular mass of 14 kDa (Fahlbusch et al., 2003). Lipocalin allergens of dog, Can f 1 and Can f 2, have also been shown to be IgE-cross-reactive (Kamata et al., 2007). It was recently observed that IgE binding to the cat lipocalin allergen Fel d 4 can be blocked by an allergen extract from cow and to a lesser degree by extracts from horse and dog (Smith et al., 2004). Recently, the major cat allergen Fel d 1 has been shown to be IgE-cross-reactive with an allergen in dog dander (Reininger et al., 2007).

2.4.1.4 Exogenous allergens and their endogenous human homologs

Interestingly, several allergens have been shown to IgE cross-react with their human counterparts. Valenta et al. reported for the first time in 1991 about the cross-reactivity between plant profilins and human profilin (Valenta et al., 1991). Thereafter, human serum albumin (Pandjaitan et al., 2000), human acidic ribosomal P2 protein (Mayer et al., 1999), human cyclophilins CyP A and CyP B (Fluckiger et al., 2002), and calcium-binding protein
Hom s 4 (Aichberger et al., 2005) have been shown to be IgE cross-reactive with exogenous allergens. It has been suggested that molecular mimicry leading to cross-reactivity between environmental allergens and their human counterparts is due to the primary sensitization to environmental allergens (Budde et al., 2002; Aichberger et al., 2005; Limacher et al., 2007). Furthermore, a study with human manganese superoxide dismutase (MnSOD) and MnSOD of Aspergillus fumigatus and Malassezia sympodialis confirmed recently that IgE-mediated autoreactivity against a human protein is one mechanism involved in the exacerbation of AD (Schmid-Grendelmeier et al., 2005).

2.4.2 T-cell cross-reactivity

2.4.2.1 Antigen recognition by T cells

It has been known for almost ten years that a T cell can recognize more than one ligand (Wucherpfennig, et al., 1995; Hemmer et al., 1997). The peptides that associate with MHC Class II are 12-20 amino acids in length (Godkin et al., 2001). The peptide has several amino acid positions that are more occupied than others for the binding into MCH and TCR. The positions 1, 4, 6 and 9 of the minimal peptide epitope are the anchor amino acids for MHC II while the residues in positions 2, 3, 5, 7, and 8 are available to interact with the TCR (Sant'Angelo et al. 2002). Moreover, the MHC II anchor residues are often hydrophobic in the peptide (Yassai et al., 2002). Initial studies demonstrated that one or a few amino acids of a peptide in MHC anchor positions could be replaced without loss of T-cell recognition as long as peptide binding to the MHC is maintained (Stern et al., 1994). Subsequently, this concept was refined so that no single residue was strictly required for recognition as long as the available residues provided enough binding energy for MHC and TCR (Hemmer et al., 1998). Hemmer et al. further demonstrated that antigen recognition by CD4+ T cells is defined by several different factors. One of them is the baseline affinity of the TCR for the MHC (Hemmer et al., 2000). Moreover, the antigenic recognition by T cells is also influenced by the individual contribution of each amino acid residue but also by the synergistic effects of certain amino acid combinations of the antigenic peptide (Hemmer et al., 2000). Recently, Sant’Angelo et al. have shown that no specific interactions occurred between peptide flanking residues and TCR (Sant'Angelo et al. 2002). However, the peptide flanking residues contribute substantially to MHC binding (Sant'Angelo et al. 2002).
Wucherphennig et al. hypothesized that TCR recognition is characterized by a considerable degree of cross-reactivity and that a TCR could recognize a number of different peptides that might be rather distinctive in their sequence (Wucherpfennig, 2004). This flexibility in T cell recognition is proposed to be caused by several factors (Holler et al. 2004), such as conformational changes of a single TCR (Reiser et al. 2003). Based on observations with a subset of T-cell clones that can be activated by combinatorial peptide libraries, it has been postulated that a single TCR can recognize $10^6$ different peptide ligands (Mason, 1998). Thus, T-cell cross-reactivity may be much more difficult to predict than B-cell cross-reactivity, in which the sequence homology should typically be over 50% (Radauer et al., 2006a). Although a T cell can recognize a large repertoire of ligands, most of the peptides are recognized with low-affinity interaction (Zhou et al., 2004). Furthermore, despite the fact that longer peptides are involved in the Th-cell-MHC class II interaction compared to the cytotoxic T-cell-MHC class I interaction, the specificity of the former interaction is even lower than that of the latter (Aalberse, 2005). TCR cross-reactivity appears to be a general property of T-cell recognition as well as a critical aspect of T-cell development in the thymus (Wucherpfennig, 2004).

2.4.2.2 Exogenous allergens and their endogenous human homologs

T-cell cross-reactivity, e.g., between pollen and related food allergens occurs independently of IgE cross-reactivity (Bohle, 2007). The major birch allergen Bet v 1 has T-cell cross-reactive epitopes, for example, with Api g 1, the major allergen in celery (Bohle et al., 2003), as well as Cor a 1 and Dau c 1, the major allergens in hazelnut and carrot, respectively (Jahn-Schmid et al., 2005). The proliferative and cytokine response to the group 1 and 7 allergens of Dermatophagoides pteronyssinus and D. farinae indicates a large degree of T-cell cross-reactivity between the purified allergens from each species (Hales et al., 2000). Moreover, human T-cell cross-reactive epitopes have been demonstrated, for example, between the Japanese cypress pollen allergen Cha o 1 and the Japanese cedar allergen, Cry j 1 (Sone et al., 2005) and between the profilin allergens Bet v 2 and Phl p 12 (Burastero et al., 2004). The results suggest that a modulation of the response to one sensitizing allergen can occur following natural exposure or following immunotherapy with another allergen (Burastero et al., 2004).

Molecular mimicry is characterized by an immune response to an environmental agent that cross-reacts with a host antigen, resulting in a disease. Molecular mimicry, also called epitopic and antigenic mimicry, is one of the leading theories that attempts to explain why the
immune system turns against its own body in autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes. For example, the autoantigen of the pancreatic beta cell GAD65 has been shown to cross-react with cytomegalo viruses (Roep et al., 2002). Furthermore, T cells or T-cell clones from multiple sclerosis (MS) patients have been shown to cross-react with virus peptides, such as peptides of an influenza virus (Markovic-Plese et al., 2005). Zhou and Hemmer have discussed that the probability of autoimmunity increases with the increasing affinity of TCRs for the cross-reactive antigen. Therefore, cross-reactivity that leads to autoimmunity is more likely to occur if the affinity of the mimicking peptide approaches the affinity of the initial microbial antigen (Zhou et al., 2004). This hypothesis is supported by the results of several studies (Hennecke et al., 2001; Zhou et al., 2004).

It has also been suggested that an environmental allergen mimicking the self may represent an important pathomechanism involved in the maintenance and exacerbation of severe and chronic forms of allergy (Bünder et al., 2004). In atopic dermatitis, T-cell mediated autoimmunity against manganese superoxide dismutase (MnSOD) may be due to primary sensitization through fungal MnSOD (Schmid-Grendelmeier et al., 2005). Moreover, it has been discussed that the chronic manifestations of atopic diseases, such as chronic skin lesions of atopic eczema, are accompanied by a mixture of Th1 and Th2 cytokine production (Hamid et al., 1994; Truyen et al., 2006; Wang et al., 2007). This is in line with the study of Bünder et al. showing that sensitization with a foreign antigen mimicking self can induce an allergic immune response of a mixed Th2 and Th1 cytokine profile (Bünder et al., 2004).

2.4.3 Significance of the IgE and T-cell cross-reactivity

2.4.3.1 Clinical significance

Demonstration of IgE cross-reactivity in vitro is not always reflected in cross-reactivity in vivo (Aalberse et al., 2001). For example, IgE cross-reactivity of cross-reactive carbohydrate determinants (CCD) seems to have limited clinical relevance (Wensing et al., 2002). Kochuyt et al. have shown that patients allergic to hymenoptera stings have specific IgE to venom glycoproteins which cross-reacts with CCD in pollen. This IgE cross-reactivity of CCD may cause false positive IgE antibody results with pollen allergens in up to 16% of hymenoptera-allergic patients, thus leading to the possibility of a misdiagnosis of multivalent pollen sensitization (Kochuyt et al., 2005). Moreover, Wensing et al. have suggested that
monosensitization to profilins can also be accompanied by several positive RAST results without any clinical relevance (Wensing et al., 2002).

IgE cross-reactivity may be exploited in allergy diagnostics. For example, the grass-pollen allergen rPhl p7 which belongs to calcium-binding (EF-hand) pollen allergens, contains the most IgE epitopes present in allergens of other calcium-binding allergen families (Tinghino et al., 2002). Therefore, it could serve as a useful diagnostic marker to identify patients who have become sensitized to several IgE-cross-reactive EF-hand allergens (Tinghino et al., 2002). Furthermore, Niederberger and co-workers have shown that the recombinant pollen allergens Bet v 1 and Bet v 2 contain most of the IgE epitopes present in birch and other members of the Fagales family (Niederberger et al., 1998). Consequently, it may be possible to use these allergens as representative molecules for the diagnostics and therapy of birch-related pollen and food allergies (Niederberger et al., 1998).

In some situations, such as with major allergens of botanically-related grasses, it is impossible to determine the sensitizing allergen without information on allergen exposure (Aalberse, 2007). Highly IgE-cross-reactive allergens, such as profilins (Wopfner et al., 2002; Radauer et al., 2006b), can elicit clinical symptoms in patients sensitized to one of them (Wensing et al., 2002; Radauer et al., 2006b). Moreover, pollen-associated food allergy is usually caused by IgE-cross-reactivity between birch pollen allergen Bet v 1 and certain food allergens (Bohle, 2007). For example, Bet v 1 was found to initiate sensitization to the major allergen in celery, Api g 1 (Bohle et al., 2003). In addition to IgE cross-reactivity, T-cell cross-reactivity may have clinical implications. The activation of Bet v 1-specific Th2 cells by related food allergens, in particular outside the pollen season, may cause ‘visible’ clinical symptoms, e.g., deterioration of atopic eczema, without immediate clinical symptoms of food allergy (Bohle, 2007). Ingestion of birch-pollen related food could also maintain perennially increased allergen-specific IgE levels (Bohle et al., 2003; Bohle, 2007). This view is supported by the study of Burastero et al. whose study showed that a significant proportion of IgE cross-reactivity can result from the T-cell help to B cells provided by Th2 cells which are activated by pan allergens (Burastero et al., 2004).

2.4.3.2 Immunological significance

Discussion on the significance of T-cell cross-reactivities between self antigens and microbial antigens has been controversial. Although the role of cross-reactivity in initiation of the autoimmune diseases or allergy is uncertain, it may be important in regulating and
exacerbating the disease. This view is in line with the finding that autoreactive T cells are not confined to MS patients but are also found in healthy donors (Tejada-Simon et al., 2001). The result further suggests that autoreactive T cells are part of the normal T-cell repertoire and not necessarily harmful (Tejada-Simon et al., 2001).

The high affinity IgE receptor, FcεRI, has a central role in the initiation and control of atopic allergic inflammation (von Bubnoff et al., 2003). It is expressed on the surface of effector cells, such as mast cells and basophils (Kay, 2001), but more importantly, it is also expressed on the surface of antigen-presenting cells, such as dendritic cells from atopic (Allam et al., 2003; Foster et al., 2003) and nonatopic patients (Allam et al., 2003). It has been speculated that IgE-cross-reactive proteins, especially endogenous proteins that are IgE-cross-reactive with exogenous allergens, could regulate or tolerize an allergic immune response (Aalberse et al., 2001), e.g., through this receptor (von Bubnoff et al., 2003).

After FcεRI cross-linking, the production of tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) might comprise part of a mechanism to suppress unwanted T-cell responses (von Bubnoff et al., 2003). Tryptophan is critical to the generation of T-cell responses (Gordon et al., 2005). Furthermore, IDO-driven tryptophan consumption by APCs can lead to deletion of the contacting T cells through the induction of apoptosis (von Bubnoff et al., 2002; Gordon et al., 2005). Since IDO is a rate-limiting enzyme, T-cell suppression can be prevented by addition of tryptophan or by inhibition of IDO (von Bubnoff et al., 2002). Several reports have shown that monocytes and dendritic cells can inhibit T-cell proliferation (Grohmann et al., 2001; Mellor et al., 2004) and tolerize Th2 responses both in vitro and in vivo through IDO (von Bubnoff et al., 2003; Gordon et al., 2005). Recently, it has been suggested that in addition to DCs, eosinophils may also express IDO (Odemuyiwa et al., 2004).
3. AIMS OF THE STUDY

As the knowledge of the immunological characteristics of animal-derived lipocalin allergens is limited the purpose of the study was to clarify the lipocalin allergen-specific immune responses of mice and humans. Furthermore, since the recombinant allergens provide an essential tool for the research and diagnostics of allergy the suitability of recombinant dog allergens for dog allergy diagnostics was assessed.

The aims of the study were

1. To analyze immune response to Bos d 2 and to its immunodominant epitope p127-142 in a mouse model (I-II).

2. To examine T-cell and IgE-cross-reactivities among lipocalin allergens and endogenous lipocalins (II, IV).

3. To elucidate the suitability of recombinant dog allergens Can f 1 and Can f 2 for the diagnostics of dog allergy (III).
4. MATERIALS AND METHODS

4.1 Immunization of mice (I-II)

For study I, six- to eight-week-old female mice (A.SW (H-2^s), A/J (H-2^s), BALB/c (H-2^d), B10.M (H-2^f), C57BL/6 (H-2^b), CBA (H-2^k)) were obtained from The National Laboratory Animal Center (Kuopio, Finland). For study II, mice (BALB/c (H-2^d), C57BL/6 (H-2^b), CBA (H-2^k)) of the same age were obtained from Taconic M&B A/S (Ry, Denmark). The mice were maintained under pathogen-free conditions throughout the study. In study I, the mice were injected at two-week intervals intraperitoneally (i.p.) or subcutaneously (s.c.) at the base of the tail, up to four times with antigens (25µg-500µg). Alum (prepared as described in manuscript I), Imject Alum (Pierce, Rockford, USA), incomplete Freund's adjuvant (IFA; Sigma; F5506) or complete Freund's adjuvant (CFA; Sigma; F5881) were used as adjuvants. In study II, the mice were injected s.c. at the base of the tail with peptides (0.005µmol) in complete Freund's adjuvant. The control mice were treated with phosphate-buffered saline (PBS), CFA or tetanus toxoid in CFA. The blood, spleen and inguinal lymph node samples were collected 10 days after the last immunization. Experiments were performed with the permission of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg 18 March 1986, adopted in Finland 31 May 1990). The study was approved by the Animal Care and Use Committee of the University of Kuopio.

4.2 Subjects (III-IV)

Subjects with dog (III-IV), cow, horse or mouse allergy (IV) diagnosed at the Department of Pulmonary Diseases of Kuopio University Hospital, subjects with self-reported symptoms of mouse allergy (IV), healthy nonatopic subjects (IV) and healthy nonatopic dog owners (III-IV) were recruited for the studies (Table 2). Partly the same subjects participated in studies III-IV. A subject was classified as allergic if the result of UniCAP fluoroenzyme-immunometric assay (FEIA) (Pharmacia, Uppsala, Sweden) was >0.7kU/l or the result of skin prick test (SPT; epithelial preparations from ALK Abelló, Horsholm, Denmark) was ≥3 mm with the allergen extract. SPTs were performed according to European recommendations (Dreborg 1993) in duplicates on the backs of the allergic and/or control subjects. In SPT the rCan f 1, rCan f 2 (III), rEqu c 1 and rMus m 1 were used at ten-fold dilutions from 250µg/ml
to 0.025µg/ml (IV). The dilutions were made in 0.9% NaCl immediately before the test. Histamine hydrochloride (10 mg/ml) and diluent (0.9% NaCl) were included as positive and negative controls, respectively. After 15 min, the wheals were marked and documented by direct tracing onto strips of tape. The means of the longest and the midpoint orthogonal diameters of the duplicates were calculated and the mean values of ≥3mm were considered positive. The total serum IgE of dog-allergic and healthy dog owners was measured by immuno-enzymometric assay (IEMA) using chemiluminescence substrate (Diagnostic Products Corporation, Los Angeles, CA, USA) (III). The studies were approved by the Ethics Committee of the Kuopio University Hospital.

Table 2. The subject in studies III-IV

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Sex</th>
<th>Age (years)±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Female /Male</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Dog-allergic patients</td>
<td>13 / 12</td>
<td>39±9</td>
</tr>
<tr>
<td></td>
<td>Healthy nonatopic dog owners</td>
<td>8 / 3</td>
<td>43±13</td>
</tr>
<tr>
<td>IV</td>
<td>Dog-allergic patients</td>
<td>15 / 16</td>
<td>41±9</td>
</tr>
<tr>
<td></td>
<td>Cow-allergic patients</td>
<td>8 / 10</td>
<td>39±9</td>
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<tr>
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<td>Horse-allergic patients</td>
<td>12 / 9</td>
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<tr>
<td></td>
<td>Healthy nonatopic subjects</td>
<td>14 / 7</td>
<td>38±13</td>
</tr>
</tbody>
</table>

4.3 Allergen preparations

4.3.1 Cloning and production of recombinant proteins in *Pichia pastoris* yeast (I, III-IV)
The expression of recombinant Can f 1, Can f 2, (III-IV), Bos d 2 (I, IV), Equ c 1 (IV), Mus m 1 (IV) and human tear lipocalin (TL) (IV), was done in *P. pastoris* (Invitrogen, CA, USA). For rCan f 1, total RNA was isolated from the tongue tissue, for rCan f 2 from the parotid gland of a male dog, and for Equ c 1 from the sublingual tissue of horse. The isolation was made with the RNAgents® total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. First-strand cDNAs were synthesized using random
hexanucleotides. A plasmid containing TL cDNA (a kind gift from Dr. Bernhard Redl, Institut für Molekulare Biologie, Innsbruck, Austria) was used as a template for generating the rTL construct.

To generate the DNA constructs encoding Can f 1, Can f 2, Equ c 1, and TL, specific primers were designed based on the published nucleotide sequences of the proteins (Gregoire et al., 1996; Holzfeind et al., 1996; Konieczny et al., 1997). For the 3’ primers, polyhistidine tag sequences were included. The amplificational products were subcloned into the pPIC9 (Invitrogen, CA, USA) (Can f 1, Equ c 1, and TL) and pHLs1 (Invitrogen, CA, USA) (Can f 2) expression vectors. All generated vector constructions were verified by DNA sequencing using Thermo Sequence CY5 Dye Terminator Kit and A.L.F Express DNA Sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). *P. pastoris* was transformed with the vector constructs, and the proteins were produced following the manufacturer’s instructions (Invitrogen, CA, USA).

The *P. pastoris* expression clone pHIL-D2MUP for rMus m 1 was a kind gift from Dr. Elena Ferrari, Istituto di Chimica Biologica, Facolta di Medicina e Chirurgia, Universita di Parma, Italy. Recombinant Bos d 2 was constructed as previously reported (Rautiainen et al., 1998; Rouvinen et al., 1999).

Histidine-tagged recombinant proteins (Can f 1, Can f 2, Equ c 1 and TL) were purified with the HisTrap Kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer’s instructions. The rMus m 1 was purified using Äktapurifyer (Amersham Pharmacia Biotech AB, Uppsala, Sweden) with the Resource Q column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and NaCl gradient in Tris-HCl buffer, basically as described by Ferrari et al. (Ferrari et al., 1997). The subsequent gel filtration to recombinant proteins was performed with Superdex 75 media (Amersham Pharmacia AB, Uppsala, Sweden) with Natrosteril 0.9 (Orion OY, Espoo, Finland) or PBS as an eluent. Recombinant psoriasin which was used as a control protein in immunoblot inhibition assays was produced and purified, as described previously (Porre et al., 2005).

The purities of the preparations were verified by SDS-PAGE and IgE immunoblotting with monoclonal Anti-His6 (Roche, Mannheim, Germany) (Can f 1, Can f 2, Equ c 1, TL), rabbit immune sera (Mus m 1 and TL) or patient sera (Can f 1, Can f 2, Equ c 1, Bos d 2). Recombinant Equ c 1 was expressed as a dimer. rTL was expressed in both dimeric and monomeric forms, and the rest of the proteins in monomeric forms. The recombinant proteins were subjected to in-gel digestion, as described previously (Rautiainen et al., 1995), with slight modifications. The sequences of the tryptic digests of the proteins were analyzed by
HPLC-electrospray ionization mass spectrometry, as described previously (Rautiainen et al., 1998). Protein concentrations were determined by the method of Bradford using the BioRad (Hercules, CA, USA) protein assay or by absorbance spectroscopy using the molar absorption coefficient (Curr. prot. in protein science). Sterile-filtered preparations were stored at -70°C.

4.3.2 Synthetic peptides (I-II)

For the epitope mapping of Bos d 2 (I), the 16-mer peptides overlapping by 14 amino acid residues and covering the Bos d 2 sequence were synthesized using a simultaneous multiple-peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt, Germany) by Fmoc (N-[9-fluorenyl]methoxycarbonyl) chemistry, as reported previously (Kinnunen et al., 2003). The peptides were desalted and purified by reverse phase high-pressure liquid chromatography (HPLC).

The Bos d 2 peptide p127-142 (ELEKYQLNNSERGVPN) (I), its 16-mer analogs, truncated derivatives, and other peptides (II) were synthesized using PerSeptive 9050 Plus automated peptide synthesizer (Millipore, Bedford, MA) with Fmoc strategy. The peptides were purified by HPLC (Shimadzu, Tokio, Japan) with a C_{18} reverse phase column and acetonitrile as eluent (0.1% TFA in H_{2}O/0-60% acetonitrile gradient for 60 min). All the peptides (I-II) were verified with the MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). No signs of impurities, e.g., endotoxin, were observed in the analyses. The sequences (Table 3) for the peptides SP1-SP9 homologous to p127-142 were obtained from the Swiss-Prot protein sequence database using the program FindPatterns. The sequences for the peptides SP10-SP20 corresponding to p127-142 were obtained by the sequence homology alignments with lipocalin proteins (from dog, horse, cow, cockroach, mouse, rat and human) from the same database using the program BestFit, as described previously (Kinnunen et al., 2003). Sterile filtered or γ-irradiated peptides were stored frozen at 70°C.
Table 3. The sequences of database search peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>127-142</td>
<td>Natural peptide</td>
<td>ELK YQLNSER GPVPN</td>
</tr>
<tr>
<td>SP1</td>
<td>KIAA1224 (Human)</td>
<td>DLESYLQNCERGTPWR</td>
</tr>
<tr>
<td>SP2</td>
<td>RBBP-4 (Human)</td>
<td>SNQCOQLTSSRFDSP</td>
</tr>
<tr>
<td>SP3</td>
<td>YTRE (Bacillus subtilis)</td>
<td>VLELIOQLNRERGIFTF</td>
</tr>
<tr>
<td>SP4</td>
<td>5-HT-2C (Human)</td>
<td>PRGTMQAINNERKASK</td>
</tr>
<tr>
<td>SP5</td>
<td>Nestin (Human)</td>
<td>VRLELQQLQAERGGNL</td>
</tr>
<tr>
<td>SP6</td>
<td>DNAE (Chlamydia pneumoniae)</td>
<td>GKKDFQMEQERKEFC</td>
</tr>
<tr>
<td>SP7</td>
<td>SPV1 ORF14 (Spiroplasma citri)</td>
<td>HVIEVQINSERSWFF</td>
</tr>
<tr>
<td>SP8</td>
<td>NF-H (Human)</td>
<td>YQEAIQQLDALRNKT</td>
</tr>
<tr>
<td>SP9</td>
<td>R51HD (Human)</td>
<td>TRL1IQYLDSSERQRIL</td>
</tr>
<tr>
<td>SP10</td>
<td>Can f 1 (Dog)</td>
<td>ALEDFFERFSRAGLNL</td>
</tr>
<tr>
<td>SP11</td>
<td>Can f 2 (Dog)</td>
<td>DFLPAFESVCEDIGLH</td>
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<tr>
<td>SP12</td>
<td>Epc c 1 (Horse)</td>
<td>IKEEFVKIVQKRGIVK</td>
</tr>
<tr>
<td>SP13</td>
<td>Bos d 5 (Cow)</td>
<td>ALEKFDFKALKALPMHI</td>
</tr>
<tr>
<td>SP14</td>
<td>Bla g 4 (Cockroach)</td>
<td>YNDKGKAFSAPYSVLA</td>
</tr>
<tr>
<td>SP15</td>
<td>Mus m 1 (Mouse)</td>
<td>IKERFAQLCEEHHGIŁR</td>
</tr>
<tr>
<td>SP16</td>
<td>Rat n 1 (Rat)</td>
<td>IKKEFKACLCEAHGİTR</td>
</tr>
<tr>
<td>SP17</td>
<td>A1AG (Human)</td>
<td>LGEFYEALDCRLIPKS</td>
</tr>
<tr>
<td>SP18</td>
<td>TL (Human)</td>
<td>ALEDFFEKAAGARGLST</td>
</tr>
<tr>
<td>SP19</td>
<td>APD (Mouse)</td>
<td>TITYLKDLITSNIGID</td>
</tr>
<tr>
<td>SP20</td>
<td>NGAL (Mouse)</td>
<td>LKERFTFRFAKSGLKLD</td>
</tr>
</tbody>
</table>

4.3.3 Other allergen preparations (I,III-IV)

Natural forms of the proteins were used in studies I (nBos d 2) and IV (nMUP). Both of the proteins were purified as their recombinant counterparts described above. The production of *Escherichia coli*-produced recombinant Bos d 2, its N- and C-terminal fragments (amino acids 1-115 and 65-156, respectively) and the fusion part glutathione S-transferase (GST) used in study I is described elsewhere (Mäntyjärvi et al., 1996; Zeiler et al., 1997). For sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analyses, the same commercial dog (III) and horse (IV) epithelial extracts, were same as for SPTs (AKL Abelló, Hørsholm, Denmark).
4.4 Immunochemical tests

4.4.1 Western blot and Western blot inhibition analyses (III-IV)

The IgE reactivity of dog (III) and horse-allergic patients (IV), as well as control patients, to the commercial SPT preparations was detected by SDS-PAGE immunoblotting as described previously (Rautiainen et al., 1997; Zeiler et al., 1997) with a few modifications. The IgE reactivity of mouse-allergic patients (IV) to purified natural and recombinant MUP was detected in the same way. In brief, SDS-PAGE-separated proteins were transferred to nitrocellulose membranes and blocked with 1% BSA in PBS. The membrane strips were incubated overnight with patient and control sera (1:10), washed and then incubated with monoclonal horseradish peroxidase-labeled mouse anti-human IgE (1:2000; Southern Biotechnology Associates, Inc, Birmingham, AL, USA). After washing, the reactive bands were visualized with ECL detection reagents (Amersham International, Buckinghamshire, England) according to the manufacturer’s instructions.

The inhibition of immunoblotting was performed essentially as described previously (Rautiainen et al., 1997). Human sera reactive to the recombinant allergens under study, diluted at 1:12.5 (III) or 1:15 (IV), were mixed with equal volumes of recombinant allergen dilutions (Can f 1, Can f 2, Equ c 1, and Mus m 1) to obtain a final protein concentration of 100µg/ml (III) or 200 µg/ml (IV). Recombinant psoriasin was used as a control protein. After incubation for two hours at +37°C, IgE reactivity against the epithelial dog and horse preparations or natural MUP was visualized as described above.

4.4.2 Measurements of murine antibodies (I)

Specific murine antibody responses were measured by indirect ELISAs, as described previously with a few modifications. For measuring the specific IgG levels, the plates were coated with natural Bos d 2 at a concentration of 1µg/ml overnight at 4°C in the coating buffer. After blocking, the plates were stored frozen at -70°C. Serum samples were diluted 1:250. The bound IgG was detected by goat anti-mouse IgG (Biomakor, Israel; 1:1000, 0.5 h, room temperature (rt)), followed by biotinylated rabbit anti-goat IgG (Cappel, Turnhout, Belgium; 1:2000, 0.5h, rt). The colour reaction was developed using ABC Elite kit (Vector, Burlingame, CA, USA), as described previously.

The IgG subclass levels were detected as described above with minor modifications. ELISA plates were coated with nBos d 2, HEL or TT at a concentration of 1µg/ml (IgG1) or
50µg/ml (IgG2a). Serum samples were 10-fold diluted 1:100-1:1000 000, IgG1 and 1:10-1:100 000, IgG2a. The bound IgG was detected by biotinylated anti-IgG1 (1:1000) or anti-IgG2a (1:100) antibodies (Caltag, Burlingame, Ca, USA) at 37°C for 1h. The colour reaction was developed using ABC Elite kit (Vector, Burlingame, CA, USA), as described previously.

4.4.3 Measurements of human IgE (III-IV)

To measure the lipocalin-specific IgE levels of human serum samples (Can f 1, Can f 2 in III-IV, Bos d 2, Equ c 1 and TL in IV), indirect ELISAs were used as described previously (Virtanen et al., 1996; Zeiler et al., 1997) with small modifications. In brief, in study III, the plates for IgE detection were coated with 5 µg/ml of rCan f 1 and rCan f 2. Sera were diluted at 1:10. The bound IgE was detected by rabbit anti-human IgE (1:500; Dako A/S, Glostrup, Denmark) combined with biotinylated goat anti-rabbit immunoglobulins (1:2000; Dako A/S, Glostrup, Denmark). The color reaction was developed using the ABC Elite kit (Vector, Burlingame, CA, USA), as described previously.

In study IV, the plates for IgE measurements were coated with 5 µg/ml of rCan f 1 or rCan f 2 and 10µg/ml of Equ c 1, Bos d 2, Mus m 1 or TL. Sera were diluted at 1:10. The bound IgE was detected by biotinylated rabbit anti-human IgE (1:1000; Southern Biotechnology Associates, Inc, Birmingham, AL, USA) combined with streptavidin-HRP (1: 10 000; Amersham International, Buckinghamshire, England). The color reaction was developed by the commercial reagent (Zymed laboratories Inc. South San Francisco,CA, USA) according to the manufacturer’s instructions.

In studies III and IV, the cut-off for a positive reaction was defined as the mean OD of the control subjects + 3 SD.

4.4.4 IgE Elisa inhibition tests (IV)

The ELISA inhibition was performed essentially in the same way as the indirect ELISA measurement of human IgE described above, except for an additional preincubation of the serum pools with inhibitor proteins. Serum pools contained sera from 4-5 allergic patients reactive to the recombinant proteins under study. In the preincubation, the final serum dilution was 1:10-1:50 and the lipocalin concentration from 0.6 to 200 µg/ml. After an incubation of one hour at +37°C, samples were added to allergen or TL-coated plates and the bound IgE was detected as described above.
4.5 In vitro analysis of lymphocytes (I-II)

4.5.1 Isolation and the proliferation assays of murine lymph node and spleen cells (I-II)

The spleens or lymph nodes were removed from the mice and prepared as a single-cell suspension. The red blood cells were removed from the spleen cell suspension by lysing cells with 0.017 M Tris-0.75% NH₄Cl. Alternatively, the spleen cells were isolated by the Lympholyte M (Cedarlane, Hornby, Ontario, Canada) density gradient centrifugation according to the manufacturer’s instructions. The cells were suspended in culture medium: DMEM (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 50 μM 2-ME, 1 mM sodium pyruvate (BioWhittaker, Walkersville, MD, USA), 10 mM HEPES (BioWhittaker, Walkersville, MD, USA), 100 IU/ml penicillin (Orion, Espoo, Finland), 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA), and 10% inactivated fetal calf serum (Biological Industries, Beit Haemek, Israel). Proliferation tests were performed, as described in study I. In brief, 2x10⁵ cells/well were incubated in triplicate in 96-well round-bottomed microtiter plates (Corning, Acton, MA) with proteins at 12.5-100 μg/ml or peptides at 0.15-50 μM for 3 days in a humidified 5% CO₂ incubator at 37°C and then pulsed for 16 h with 0.5 μCi/well [³H] thymidine (Amersham, Little Chalfont, UK). The radionuclide uptake was measured by scintillation counting and the results expressed as count per minute (cpm) or as stimulation indices (SI: ratio between the mean cpm in cultures with stimulant and the mean cpm without stimulant).

4.5.2 Determination of restriction by major histocompatibility complex (II)

CD4+ T cells were separated from spleen cell suspension by SpinSep™ Cell Enrichment Method (StemCell Technologies, Vancouver, Canada) according to the protocol of the manufacturer. The purity of CD4+ T cells determined by flow cytometry was >95% after two purification cycles. The CD4+ cells (1x10⁵ cells/well) were cultured in triplicate in 96-well round-bottomed microtiter plates with γ-irradiated (6000 rad) I-A^d- or I-E^d-transfected fibroblasts (LA(d) and LE(d)) (Texier et al., 1999) as feeder cells (0.5 x10⁵ or 1x10⁵ cells/well). The p127-142 concentration ranged from 0.5 μM to 50 μM. The response was measured by the proliferation assay.
4.5.3 MHC class II-peptide binding assay

The competitive MHC II-peptide binding assay was performed as previously described (Texier et al., 1999). The purified I-A\textsuperscript{d} and I-E\textsuperscript{d} molecules were incubated with serial dilutions of competitor peptides and the biotinylated competitor peptides MYO 106-118 and HA 306-318 peptide, respectively. The samples were incubated for 24 or 72 h for I-Ad or I-E\textsuperscript{d}, respectively. After neutralization, the samples were incubated for 2 h at room temperature on plates coated with 10 µg/ml MKD6 (mAb for I-A\textsuperscript{d}) or 14.4.4S (for I-E\textsuperscript{d}). The bound biotinylated peptide was detected using a streptavidin-alkaline-phosphatase conjugate (Amersham, UK) and 4-methylumbelliferyl phosphate substrate (Sigma, France). Emitted fluorescence was measured at 450 nm upon excitation at 365 nm. Maximal binding was determined by incubating the biotinylated peptide with the MHC II molecule in the absence of a competitor. Results were expressed as the peptide concentration which prevented binding of 50% of the labeled peptide (IC\textsubscript{50}).

4.5.4 Measurement of cytokine production by murine lymph node and spleen cells

Mouse spleen or lymph node cells (I) were stimulated with nBos d 2, HEL or TT at a concentration of 100 µg/ml at an optimal density of 3x 10\textsuperscript{6} cells/ml in 4-ml test tubes in a volume of 1.5 ml for 48h (IL-2 and IFN-γ) or 96h (IL-4 and IL-5) in a humidified 5% CO\textsubscript{2} incubator at 37°C. Positive (Con A at 5µg/ml) and negative controls (cells with culture medium only) were included. Stimulations of the spleen or lymph node cells from mice immunized with a peptide were performed at two peptide concentrations, 3 and 30µg/ml. An irrelevant peptide served as a control. After stimulation, the culture supernatants were collected, aliquoted and stored at –70°C until examined. The IL-4, IL-5, and IFN-γ levels of the supernatants were measured by ELISA in duplicate using commercial reagents (all reagents from PharMingen, San Diego, USA) according to the manufacturer’s instructions. The color reactions were developed as described for antibody measurements. For measuring IL-2, the CTLL-2 cell line was used as previously described with minor modifications. The mAb against murine IL-4 (R&D Systems, Abingdon, UK) at a neutralizing concentration (1µg/ml) was included in the assay. A semiquantitative estimate of IL-2 production was determined from a standard curve of rIL-2 (Strathmann Biotech, Hamburg, Germany). The limit of detection for IL-2 was 0.05U/ml.
4.5.5 Enumeration of cytokine-producing murine spleen cells

The ELISPOT analyses (II) of cytokine production by spleen cells were performed as described previously (Immonen et al., 2003). Briefly, ELISPOT plates (Cellular Technology Ltd., Reutlingen) were coated with anti-mouse IL-4 or IFN-γ monoclonal antibodies (Pharmlingen, San Diego, USA) at 4 µg/ml in PBS overnight at +4°C. The medium used was HL-1 (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The spleen cells at a density of 10⁶ cells/well (0.2 ml) were added in duplicate in plates with peptides at concentrations ranging from 0.5 to 50 µM. The cells were incubated for 24 h (IFN-γ) or 48 h (IL-4) in a humidified 5% CO₂ incubator at 37°C. The produced IL-4 or IFN-γ (Pharmlingen) was detected by biotinylated monoclonal antibodies (4 µg/ml) at 4°C overnight followed by streptavidin-HRP conjugate (diluted 1:2000, Dako A/S, Denmark). The color reaction was allowed to develop using a solution containing 3-amino-9-ethyl carbazole (Sigma, dissolved in N-N-dimethylformamide (Merck, Darmstadt, Germany)), 0.1 M sodium acetate, pH 5.0, and 30% H₂O₂ (Riedel-de Haën, Seelze, Germany). The spots were counted under a stereomicroscope.

4.6 Statistical analyse (I, III-IV)

Statistical differences between groups were determined with the Kruskal-Wallis test and post hoc comparisons with the Mann-Whitney U-test. For determining correlations, the Spearman Rank Correlation test was used. Concordances for results classified as positive or negative were determined by calculating the Phi coefficient and the Fisher’s Exact P-value (III).
5. RESULTS

5.1 Validation of recombinant lipocalins (III-IV)

The *Pichia pastoris*-produced recombinant lipocalins were purified by chromatography. After gel filtration, rCan f 1, rCan f 2 (III) and rMus m 1 (IV) were eluted as monomers. In contrast, rEqu c 1 (IV) was eluted as a dimer and rTL (IV) at two approximate molecular sizes (20 and 40 kDa), compatible with being monomeric and dimeric forms of the protein. SDS-PAGE revealed rCan f 1 (III), rEqu c 1, rMus m 1 and rTL (IV) as single bands with molecular masses of 23 kDa, 26 kDa, 18 kDa, and 22 kDa, respectively, whereas rCan f 2 (III) was revealed as two bands (23 and 25 kDa). The sequences of the tryptic digests of the recombinant proteins obtained by HPLC-electrospray ionization mass spectrometry also matched those reported for the proteins (Gregoire et al., 1996; Holzfeind et al., 1996; Ferrari et al., 1997; Konieczny et al., 1997). In study III, a further mass-spectroscopic analysis of Can f 2 showed that the higher molecular weight peptide was glycosylated with two N-acetyl glucosamines and 8-14 hexoses, while the smaller molecular weight peptide was unglycosylated.

The IgE-binding capacities of natural and recombinant Can f 1, Can f 2 (III), rEqu c 1 and rMus m 1 (IV) were verified with patient sera in immunoblotting (III, Figs. 1 and 2 and IV, Fig. 1). The immunoblotting, skin prick test and ELISA results of studies III-IV are summarized below in Table 4. In study III, 72% of the dog-allergic patients had IgE reactivity against allergens in the dog epithelial SPT preparation (III, Fig.2) and 52% against Can f 1. IgE reactivity against Can f 2 was 28%. All the patients recognizing Can f 2 also recognized Can f 1 (III-IV). In study IV, the sera of horse- and mouse-allergic patients reactive to the proteins corresponding to natural Equ c 1 and Mus m 1, evaluated by the molecular size (23 kDa and 20 kDa, respectively), also recognized the recombinant allergens (IV, Fig. 1). Control sera did not show IgE binding to the recombinant allergens.

Fig. 1 (III) and Fig. 1A (IV) show that preincubation of a single serum (III) or serum pool (IV) with the appropriate recombinant allergens (Can f 1, Can f 2 or Equ c 1) resulted in the elimination of IgE binding to the 22 kDa, 25 kDa or 23 kDa components in the allergen extract, respectively. In addition, no IgE binding to the natural Mus m 1 was observed after prior incubation with the recombinant allergen (IV, Fig. 1B).
5.2 Antibody and skin prick test reactions to lipocalins (I, III-IV)

5.2.1 Human IgE immunoblotting to dog epithelial SPT preparation (III)

In study III, the immunoblot analysis showed that dog-allergic patients had IgE reactivity against allergens other than Can f 1 and Can f 2 in dog epithelial extract. The IgE reactivity was mostly seen against the 18 kDa (60%), 40 kDa (44%) and 70 kDa (48%) molecules. Further analysis of the 18kDa protein suggested that it is a new member of the lipocalin family. The aminoterminal sequence (LPNVLTQVSGPWK) which exhibits no identity with any known dog or other allergens, contains the triplet glycine-x-tryptophan which is characteristic for the first structurally conserved region of lipocalins.

5.2.2 Human IgE responses measured by ELISA (III-IV)

The IgE reactivity of dog-, cow-, horse-, and mouse-allergic patients to dog rCan f 1 and 2, cow rBos d 2, horse rEqu c 1 and mouse rMus m 1, respectively, was measured by indirect ELISAs (Table 4). The prevalence of dog-allergic patients’ sensitization to Can f 1 was 44% (III) and 42% (IV) and to Can f 2 20% (III) and 16% (IV).

A significant correlation was found between the IgE levels of dog-allergic patients and Can f 1 and Can f 2 (IV, r=0.514, p=0.003). The sensitization prevalence of horse-allergic patients to Equ c 1 was 76%, whereas 66% of the mouse-allergic patients had specific IgE to Mus m 1. Among the cow-allergic patients, 83% were sensitized to Bos d 2. All control subjects were negative. The differences in the IgE levels between control subjects and allergic patients were statistically significant (III, Fig. 4 and IV, Fig. 2).

In study IV, the measurement of specific IgE levels to an endogenous lipocalin, rTL, showed that seven out of 42 allergic patients had specific IgE to the protein (IV, Fig. 3). It is noteworthy that six of the rTL IgE-positive patients were Can f 1 IgE-positive, while all seven patients were dog-allergic (IV, Fig. 3). The rTL-specific IgE level of Can f 1-positive patients differed significantly (p=0.014) from that of Can f 1-negative patients. Moreover, a high correlation (r=0.81, p=0.007) was seen between the rCan f 1 and rTL-specific IgE levels of Can f 1-allergic patients.
Table 4. IgE and skin prick test reactivities of allergic patients

<table>
<thead>
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<th></th>
<th>Frequency (n,%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Skin prick test</td>
<td>Immunoblotting</td>
<td>ELISA</td>
<td>study</td>
</tr>
<tr>
<td>Can f 1</td>
<td>13 (52)</td>
<td>13 (52)</td>
<td>11 (44)</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13 (42)</td>
<td>IV</td>
</tr>
<tr>
<td>Can f 2</td>
<td>8 (32)</td>
<td>7 (28)</td>
<td>5 (20)</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 (16)</td>
<td>IV</td>
</tr>
<tr>
<td>Equ c 1</td>
<td>11 (52)*</td>
<td>16 (76)</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Mus m 1</td>
<td>10 (66)</td>
<td>10 (66)</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Bos d 2</td>
<td>15 (83)</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL</td>
<td>7 (17)</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of patients allergic to dog: 25 (III) 31 (IV), to horse: 21, to mouse: 15, to cow: 18, total number of allergic patients 41 (IV). * Determined with sera of 11 patients

5.2.3 Skin prick test reactivity to Can f 1 and Can f 2 and its association to IgE immunoblot and ELISA results (III)

In study III, the relationships were analysed for the results obtained with IgE immunoblotting, IgE ELISA and SPT. Negative and positive SPT and immunoblotting results with Can f 1 showed perfect concordance (Table 5). The results between ELISA and immunoblot or SPT were also highly concordant. As for the reactivity to Can f 2, the highest concordance was obtained between the SPT and IgE immunoblotting results. The concordance between ELISA and SPT results was lower than that between ELISA and immunoblot results.

Table 5. The concordances for positive and negative results between the results obtained with SPTs, IgE-immunoblotting and IgE-ELISA

<table>
<thead>
<tr>
<th></th>
<th>SPTs (Φ coefficient)</th>
<th>Immunoblotting (Φ coefficient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can f 1</td>
<td>Immunoblot 1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ELISA 0.88</td>
<td>0.88</td>
</tr>
<tr>
<td>Can f 2</td>
<td>Immunoblot 0.92</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ELISA 0.75</td>
<td>0.82</td>
</tr>
</tbody>
</table>

P<0.001 in all analyses
The magnitude of SPT reactions at 2.5µg/ml and the level of rCan f 1-specific IgE (all subjects, n=36) exhibited a high correlation (r =0.82, P<0.001). The correlation for rCan f 2 was lower (r =0.66, P<0.001). The sizes of SPT reactions showed no difference between Can f 1-single positive and Can f 1&Can f 2-positive patients. However, the results differed significantly from those of the control and Can f 1-negative patients (III, Fig. 3). The SPT results of the control subjects were negative.

The sensitivity was highest (52%) in SPT with rCan f 1, but 44% for rCan f 1-specific IgE measurements. The sensitivities in tests with rCan f 2 remained lower: 32% for SPT and 20% for the IgE measurement.

5.2.4 Specific IgG and IgG-subclass responses of different mouse strains to Bos d 2 (I)

To examine the murine immune responses to natural Bos d 2, mouse strains with different H-2 (i.e. murine MHC) haplotypes were immunized i.p. twice at two-week intervals with nBos d 2 (25µg/mouse) in alum. Out of the 6 mouse strains, only BALB/c mice clearly showed an elevated level of nBos d 2-specific IgG (OD>1.9; I, Fig. 1). Although A.SW, B10.M and CBA/s mice showed clearly lower levels of Bos d 2-specific IgG (OD<0.65) immunization with tetanus toxoid (25µg/mouse) induced a high TT-specific IgG response (OD>2) in all the mouse strains.

The IgG subclass analysis of nBos d 2-immunized BALB/c mice (50µg/mouse) showed that specific IgG1 levels in the serum were elevated after the second immunization. The Bos d 2-specific IgG1 levels increased up to the fourth immunization (I, Fig.2). In contrast, no specific IgG2a was observed. When the mice were immunized with HEL or TT, the specific IgG2a was detected after the second immunization. The level of TT-specific IgG2a increased up to the fourth immunization. In contrast to Bos d 2 and HEL immunizations, TT-specific IgG1 was seen immediately after the first immunization (I, Fig. 2). After the second immunization, the antibody-specific IgG1-levels differed statistically (P<0.05).

When the BALB/c mice were immunized with recombinant Bos d 2 (50µg/mouse), low levels of antigen specific IgG2a were detected after the second immunization (OD 0.17±0.04 (SEM)). The immunization with a higher dose of rBos d 2 (500µg/mouse) slightly increased the Bos d 2-specific IgG2a level (OD 0.28±0.05 (SEM)). In parallel, Bos d 2-specific IgG1 levels tended to be higher with the high dose immunization than with the low dose
immunization. However, the difference in the IgG2a or IgG1 levels between the groups with high or low dose immunizations was not statistically significant (P>0.05 with both). As illustrated in Fig.3 (I), the differences in IgG1/IgG2a-ratios between the control protein groups (HEL, TT) and the low dose rBos d 2 group were statistically significant (P< 0.05).

5.3 Responses of murine spleen and lymph node cells to rBos d 2 (I)

5.3.1 Proliferative responses (I)

To examine proliferation responses of spleen cells, 6 different mouse strains were immunized i.p. with 25 µg of nBos d 2/mouse in alum. In vitro responses of spleen cells with nBos d 2 at 50µg/ml turned out to be very low (stimulation indices <1.7, medium background range 600-2900c.p.m.) after two immunizations. Additional immunizations, an increased dose of rBos d 2 (50µg/mouse), usage of CFA or IFA or an increase in the dose of rBos d 2 in vitro did not enhance the spleen cell response of BALB/c, CBA/s or B57BL/6 mice (I, data not shown). Comparison of the proliferative responses of spleen cells of rBos d 2, HEL or TT-immunized BALB/c mice showed that the in vitro response induced by rBos d 2 was the weakest (I, Fig.4). The second immunization increased the responses in all immunization groups, although the increase in the response to rBos d 2 was negligible. The differences in proliferative responses between rBos d 2, HEL and TT-immunized groups were statistically significant after the second immunization (I, Fig.4).

However, when the mice were immunized s.c. with rBos d 2 or HEL in CFA the proliferative responses of BALB/c mice lymph node cells were observed to be stronger than those of the spleen cells in i.p immunized mice, SI 3.5±0.7 (SEM) and 1.4±0.2 (SEM), respectively.

5.3.2 Cytokine responses (I)

To characterize cytokine production of BALB/c mouse spleen cells, the mice were immunized i.p. with 50µg of rBos d 2, HEL or TT /mouse in alum up to two times at a two-week interval. After the first immunization, rBos d 2 induced only a weakly detectable level of IL-4, which did not notably increase after the second immunization. The differences in IL-4 production were statistically significant between rBos d 2 and the other immunization groups (HEL and TT) after the second immunization (P<0.05), whereas the IL-5 production
showed no statistical difference (P>0.05). Although TT and HEL were stronger inducers of IL-2 than rBos d 2 (P<0.05), IL-2 production was very weak in all groups. IFN-γ production was not detected upon stimulation with the antigens. Con A stimulation induced substantial secretion of the four cytokines in all groups.

To verify the influence of the immunization protocol on the outcome of the response, BALB/c mice were immunized s.c. with rBos d 2 and HEL in CFA. Immunization of the mice with rBos d 2 s.c. increased the production of IL-4 and IFN-γ of rBos d 2-stimulated lymph node cells. On the other hand, the IL-4 and IL-5 responses of the cells in HEL immunized mice were weaker than those of mice with i.p. immunization. No statistical differences were seen between rBos d 2 and HEL in the production of IL-5 and IFN-γ.

5.4 Immunodominant epitope of Bos d 2 (I-II)

5.4.1 Proliferative spleen and lymph node cell responses to the immunodominant epitope p127-142 (I-II)

The epitope mapping of Bos d 2 with overlapping 16mer peptides showed that the spleen cells of BALB/c mice recognize one immunodominant epitope in Bos d 2 (I, Fig. 3), the p127-142. The epitope is localized at the C-terminus of the protein and is almost identical to that recognized by human T cells. Proliferative responses of spleen cells from mice immunized with p127-142 (100 µg/mouse) in IFA or CFA seemed to be stronger than those obtained with alum as an adjuvant (I, Fig. 6, P > 0.05). As observed with Bos d 2, the magnitude of proliferative response to p127-142 stimulation was dependent on the cell type. Immunization with p127-142 in CFA followed by in vitro stimulation of the lymph node cells with the peptide, induced a stronger proliferative response than that with spleen cells. The stronger proliferative response was observed with lymph node cells in spite of the ten-fold lower immunization dose (10µg/mouse s.c. and 100 µg/mouse i.p.) (I, Table 4).

In study II, the spleen cells of PBS-treated BALB/c mice or mice primed with database search peptide (SP) were found to proliferate when stimulated with p127-142 (50 µM) (II, Fig. 4, Fig. 5G, and data not shown). The phenomenon was further studied with BALB/c, CBA and C57BL/6 mouse strains. In repeated experiments, the spleen cells from PBS-treated BALB/c mice proliferated dose-dependently upon stimulation with the peptide in vitro (II, Fig. 6). Interestingly, the spleen cells of PBS treated C57BL/6 mice recognized p127-142, although this strain was observed to be a nonresponder to rBos d 2 in study I. CBA, a strain
which was found to be a nonresponder to nBos d 2 in study I, did not mount any spleen cell response to p127-142.

5.4.2 Cytokine responses of murine spleen and lymph node cells to p127-142 (I-II)

In the BALB/c mouse, the cytokine response to p127-142 varied depending on the immunization route and the cells stimulated. The cytokine response of i.p. primed spleen cells in BALB/c mice was of the Th2-type (I, Table 3, II, Fig.5B) regardless of the adjuvant. For example, with alum as the adjuvant, IL-5 production was high (332±160 pg/ml), whereas IL-2 and IL-4 secretions were at a low level (2.3±1.0 U/ml and 7.8±1.5 pg/ml, respectively). No IFN-γ production was detected. In study II, in which cytokine production was measured by ELISPOT, the cytokine profile upon stimulation with p127-142 was IL-4-dominated at low stimulation doses (II, Fig.5B). The profile of p127-142 was more balanced at a stimulation dose of 100µM.

In contrast to spleen cells, lymph node cells from BALB/c mice immunized s.c. with CFA as the adjuvant, mounted a Th1-type response with strong IFN-γ production (I, Table 4, 734.8±231.1 pg/ml) and low level of IL-5 production (I, Table 4, 48.5±11.6 pg/ml). No IL-4 was observed.

5.4.3 Characteristics of the core region of p127-142 (I-II)

The epitope core sequence was estimated to be between E129 and V140 (EKYQQNLNSERGV) with overlapping peptides (I). Additional analyses with truncated peptides of p127-142 suggested that the critical amino acids for recognition by T cells of BALB/c mouse were between K130 and G139 (II, Fig.2). The spleen cell responsiveness of p127-142-primed mice was very sensitive to the deletion of the terminal amino acids of the peptide. Removal of four amino acids from the N-terminus (including K130) or four from the C-terminus (including G139) strongly decreased the proliferative response. Furthermore, the response to a peptide truncated at both ends (K130-G139) remained at a background level.

For further characterization of p127-142, the primed spleen cells were stimulated in vitro with the alanine-substituted analogs of the peptide (II, Fig. 1). In the central part of the peptide, the residues Q132, Q133, N135, E137 and R138 did tolerate notably less alanine or other amino acid substitutions, classified as conservative or semiconservative, (Tangri et al., 2001) (data not shown) suggesting the importance of these amino acids in T-cell recognition.
Furthermore, the probable MHC anchor amino acids, Y131, L134, S136, and G139 (see below), were less sensitive to substitution, since their replacement with conservative amino acids did not abrogate the response.

The MHC restriction of the immunodominant epitope was characterized with purified spleen T cells and I-A<sup>d</sup> or I-E<sup>d</sup>-transfected fibroblasts as antigen presenting cells. I-A<sup>d</sup>-transfected cells were able to present the peptide to T cells more efficiently than were the I-E<sup>d</sup>-transfected cells (II, Fig. 3). This result was confirmed by measuring the binding of p127-142 and its 34 analogs to I-A<sup>d</sup> (II, Table 1) and I-E<sup>d</sup> molecules (data not shown). The peptide bound to I-A<sup>d</sup> molecule, whereas no binding with the peptide was observed in the I-E<sup>d</sup> molecule. The binding results suggest that Y131 is the MHC anchor amino acid in position 1. The amino acids in position 1 and 4 of the peptide are in accordance with the motif reported for I-A<sup>d</sup> molecule (Database Syfpeithi, http://www.syfpeithi.de/).

**5.5 T-cell and IgE cross-reactivity of lipocalin proteins (II, IV)**

**5.5.1 Cross-reactivity of mouse spleen cells recognizing the immunodominant epitope**

(II)

To examine the potential cross-reactivity of p127-142-recognizing T cells, the spleen cells of p127-142-primed BALB/c mice were stimulated in vitro with p127-142, database search peptides SP1-SP9 or lipocalin peptides SP10-SP20 at 50 µM. Only SP3 (*Bacillus subtilis*) and SP7 (*Spiroplasma citri*) were able to give rise to a proliferative response, albeit at a low level (SI 2.4±0.3 and 2.1±0.2, respectively) compared to p127-142 (SI 11.8±2.5) (II, Fig.4). In contrast to other SP-primed murine spleen cells, in vitro stimulation of the cells of SP7-primed mice with p127-142 induced a strong cross-reactive response (Figs. 4 and 5D).

For analyzing the capacity of different peptides to modulate the Th1-Th2 balance of an immune response, the IFN-γ and IL-4 secretion induced by SP7 and SP12 (lipocalin allergen peptide from Equ c 1) was compared with that induced by p127-142 (Fig. 5). ELISpot analysis revealed that the cytokine responses to p127-142 and SP12 induced a similar IL-4-dominated cytokine profile (II, Figs. 5B and 5H), although their capacity to induce lymphocyte proliferation was different (II, Figs. 5A and 5G). SP7 induced a Th0-type response and elicited the strongest proliferation (II, Figs. 5D and 5E).

The peptides p127-142 and SP7 could prime spleen cells reciprocally for cytokine production (II, Figs. 5C and 5F). The spleen cells of p127-142 primed mice stimulated in vitro
with SP7, mounted a Th0-type response (Fig. 5C). Whereas, the stimulation of spleen cells from SP7-primed mice in vitro with p127-142 resulted in a IL-4 dominated response (Fig. 5F).

5.5.2 Human IgE cross-reactivity among lipocalin proteins (IV)

The IgE cross-reactivity among lipocalins was determined by indirect ELISAs. Three to five sera with strong reactivity to pure recombinant allergens were pooled and preincubated with the allergens, rTL or the control proteins before ELISA assays with lipocalin-coated plates. The cross-reactivity was seen dose-dependently with four serum pools specific to rCan f 1, rTL, rCan f 2 and rMus m 1 (IV, Fig. 4). The preincubation of the rCan f 1-specific serum pool with rTL could inhibit IgE binding to rCan f 1 (IV, Fig. 4A). Reciprocally, rCan f 1 was able to inhibit IgE binding of the rTL-positive serum pool to rTL (IV, Fig. 4B). Interestingly, in both groups, rCan f 1 was a more efficient inhibitor of specific IgE binding than rTL. The concentration of rCan f 1 needed for 50% inhibition (IC₅₀) of Can f 1-specific IgE binding was about 400-fold lower than that of rTL. Furthermore, the IC₅₀ of rCan f 1 for the inhibition of rTL-specific IgE was 100-fold lower than IC₅₀ of rTL.

The IgE cross-reactivities between rCan f 2 and rCan f 1 and between rMus m 1 and rEqu c 1 were partial. The preincubation of the rCan f 2-specific serum pool with rCan f 1 inhibited IgE binding to rCan f 2 very weakly (IC₅₀=200µg/ml, Fig. 4C), whereas rCan f 2 could not prevent the binding of specific IgE to rCan f 1 at all. Furthermore, the preincubation of rMus m 1-specific serum pool with rEqu c 1 inhibited the binding of IgE to rMus m 1 (Fig. 4D). However, IC₅₀ was over 400-fold higher with rEqu c 1 than that of rMus m 1. In contrast, rMus m 1 could not inhibit the specific IgE binding to rEqu c 1. No IgE cross-reactivities were seen between Bos d 2 and other lipocalin proteins (data not shown).

5.6 Modelling of the IgE-cross-reactive site (IV)

The sequence identities between lipocalin proteins are shown in Table 6 (IV, Fig.5). The sequence alignments of the lipocalins with IgE cross-reactivity showed that identical amino acids form short continuous segments which were located quite evenly in the sequence and consequently in the structure (IV, Fig. 6). Equally located, similar segments in secondary
structural elements and in loops pointed to several potential areas for IgE cross-reactivity, for example, in Equ c 1 and rMus m 1.

Since the crystallographic three-dimensional structure of Can f 1 has not been solved, it is not possible to compare the identical residues on the surface of Can f 1 and TL. Nevertheless, the mapping of identical residues on the surfaces of Equ c 1 and Mus m 1 (Fig. 6) showed quite an even distribution. The largest similar area (and putative epitope) on the surface can be found around the two-fold axis containing the N-terminus of strand C and the C-terminus of strand D of the monomers. It is noteworthy that according to the amino acid sequences (IV, Fig. 5), these segments in strands C and D also have similarities in the IgE cross-reacting pair TL-Can f 1.

Table 6. The sequence identities between lipocalin proteins. The cross-reactive pairs are indicated with grey boxes.

<table>
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<th>3-D structure</th>
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<th>Can f 2</th>
<th>TL</th>
<th>Equ c 1</th>
<th>Mus m 1</th>
<th>Bos d 2</th>
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* the SIB BLAST network service at the Swiss Institute of Bioinformatics, April 5, 2007)
6. DISCUSSION

6.1 Humoral immune responses to lipocalins (I, III-IV)

6.1.1 Murine humoral immune responses of mice to Bos d 2 (I)

Several studies have shown that despite the strong IgE-inducing capacity of lipocalin allergens (Zeiler et al., 1999; Smith et al., 2004), they generally induce weak Th2-dominated cellular responses (Zeiler et al., 1999; Immonen et al., 2003; Jeal et al., 2004; Immonen et al., 2007). Therefore, it has been hypothesized that strong IgE and weak cellular immune responses to lipocalins may be associated with their allergenicity (Virtanen et al., 1999). In study I, Bos d 2-specific cellular and antibody responses were determined with six mouse strains of different haplotypes, including the BALB/c mouse. Of the six mouse strains, only BALB/c mice showed high nBos d 2-specific IgG levels after i.p. immunization. Repeated i.p. immunizations with nBos d 2 resulted in an increased IgG1 response but failed to induce an IgG2a response. This finding is in contrast to observations with HEL and TT. The high IgG1 and low IgG2a response of BALB/c mice to rBos d 2 pointed to a Th2-deviation of response, since the synthesis of IgG1 is one of the markers of the Th2 response, and IgG2a the marker of the Th1 response (Rosenkrands et al., 2005; Gizzarelli et al., 2006; Gomez et al., 2007). Furthermore, rBos d 2 induced weak specific IgG1 and IgG2a responses compared to the control antigens. Since the proliferative responses (see below) were also weak, it appears that Bos d 2 is a weak immunogen for several mouse strains. It also seems that the murine responses against Bos d 2 resemble those observed in humans.

6.1.2 Human IgE responses to lipocalin allergens (III-IV)

The use of recombinant allergens for the diagnostics and immunotherapy of allergy has many advantages over allergen extracts (Chapman et al., 2000). The key advantage of recombinant allergens is their straightforward standardization. Importantly, yeasts such as Pichia pastoris, have been shown to produce bioactive, fully immunoreactive recombinant proteins in large quantities (Rouvinen et al., 1999; Daly et al., 2005; Macauley-Patrick et al., 2005). Therefore, all our recombinant proteins were chosen to be produced in Pichia pastoris yeast (Studies I, III-IV).
Mass-spectrometric analyses confirmed that the sequences of the HPLC-purified recombinant proteins were correct. Skin prick tests with the lipocalin allergens and lipocalin-specific IgE-reactivity in immunoblotting, immunoblot inhibition and in ELISA showed that the recombinant proteins were immunologically functional (III-IV).

In studies III-IV, the prevalence of Can f 1-specific IgE was found to be lower than that reported by Konieczny et al. (1997). Levels of detected Can f 1-specific IgE ranged from 44% (III) to 42% (IV) of the sera in dog-allergic patients, whereas 75% of the patients had Can f 1-specific IgE in the study of Konieczny et al. (1997). Detection by immunoblotting increased the prevalence of IgE reactivity to 52% (study III). The prevalence of Can f 2-specific IgE in study III was similar, and in study IV slightly lower than that detected by Konieczny et al. (Konieczny et al., 1997). One explanation for the difference between the studies in the prevalence of IgE reactivity to Can f 1 and Can f 2 may be the different methods used to define a positive reaction. In the study III, the cut-off value was the mean OD of the control subjects + 3 SD at a dilution 1:10. In contrast, the study of Konieczny et al. defined a positive sera as those that gave an OD of at least double the uncoated plate background at a dilution of 1:4 (Konieczny et al., 1997).

It can be speculated that this discrepancy can be attributed to differences in the selection and demographics of the patients. On the one hand, the prevalence of allergic diseases (Asher et al., 2006) and the HLA allele distribution (Partanen et al., 1997) in Finland do not differ from that in most Western European countries. On the other hand, 100% of birch-allergic patients in Finland and 98% in Sweden were found to be IgE positive to Bet v 1, whereas 62% and 65% of the patients in Italy and Switzerland, respectively, had Bet v 1-specific IgE (Moverare et al. 2002b). Moreover, in allergy to cherries, major differences have been observed across Europe in the sensitization pattern and prevalence of the systemic reaction (Reuter et al., 2006). Spanish patients were almost exclusively sensitized to Pru av 3 (91%), whereas 96% of the German patients were sensitized to Pru av 1 but only 11% to Pru av 3 (Reuter et al., 2006). Therefore, if recombinant allergens are used for allergy diagnostics or immununotherapy, it is important to take into consideration geographic and demographic factors.

Interestingly, IgE to human tear lipocalin (TL) was observed in the sera from seven out of 42 dog-allergic patients in study IV. It has been suggested that autoreactive IgE production could be induced by a cross-reactive exogenous allergen (Budde et al., 2002; Aichberger et al., 2005). This view is also in keeping with our results. TL-specific IgE levels of the patients were extremely low, with all but one of the patients with TL-specific IgE found to be Can f 1.
positive. It seems, therefore, reasonable to suppose that TL-specific IgE is Can f 1-specific with cross-reactivity to TL. This is also supported by the finding in inhibition experiments that rCan f 1 was a more potent inhibitor of both Can f 1 and TL-specific IgE binding than TL.

6.1.3 Use of recombinant Can f 1 and Can f 2 for the diagnostics of dog allergy (III)

Recombinant allergens have been shown to improve sensitivity over extract-based diagnostic tests. For example, the mix of cherry allergens rPru av 1, 3 and 4 had a sensitivity of 95% compared with 65% for cherry extract in ImmunoCAP tests (Reuter et al., 2006). One aim in study III was to evaluate the applicability of recombinant Can f 1 and Can f 2 for the diagnostics of dog allergy. Although, the recombinant dog allergens Can f 1 and Can f 2 were found to have high specificity (100%) for the diagnostics of dog allergy, additional allergens should be involved due to the poor sensitivity of the allergens. For example, the sensitivity of Can f 1 in ELISA was only 44%. Furthermore, it should be noted that the usage of Can f 2 does not increase the sensitivity of diagnostics for dog allergy, since all patients who recognized Can f 2 also recognized Can f 1.

Since the prevalence of Can f 1 and Can f 2-specific IgE reactivity among dog-allergic patients was relatively low, it seems that other dog allergens in addition to Can f 1 and Can f 2 are important in sensitization to dogs. One interesting candidate for a recombinant allergen mixture for the diagnostics of dog allergy would be the 18 kDa protein found in the dog SPT preparation. This previously unknown protein was recognized more frequently than Can f 1 by allergic patients. Furthermore, it is of special interest since the amino terminus of the protein contains a lipocalin-specific sequence motif (Flower et al., 2000), suggesting it as a new member in the lipocalin family. The protein was recently named Can f 4 by IUIS.

Serum albumin, Can f 3, is a protein with a molecular mass of approximately 70 kDa (Pandjaitan et al., 2000). Can f 3 would be useful for the diagnostics of dog allergy since it was also recognized by a considerable number of patients. The use of Can f 1, Can f 3, and Can f 4 in a mixture for diagnostics of dog allergy would increase the sensitivity to ~70%.

The results do not indicate which allergens are included in the commercial SPT test preparation that 28% of the allergic patients recognized in SPT but were not visualized in immunoblotting. It can be speculated that a natural SPT preparation contains some allergen determinants that could have deteriorated during the SDS-PAGE treatment and
immunoblotting and were therefore not recognized. On the other hand, the patients may exhibit low-level reactivity to more than one dog allergen, thus yielding positivity in SPT.

### 6.2 Murine cellular immune responses to lipocalins (I-II)

To elucidate murine cellular immune responses to the bovine lipocalin allergen Bos d 2, the proliferative and cytokine responses of six mouse strains were examined (I). The cellular responses with all six mouse strains against Bos d 2 immunized i.p. were weak. The results resemble those with human T cells against lipocalin allergens, including Bos d 2 (Zeiler et al., 1999; Immonen et al., 2003; Jeal et al., 2004; Immonen et al., 2007). The cytokine secretion by spleen cells of i.p. immunized BALB/c mice showed that Bos d 2 and the control proteins HEL and TT all induced a Th2-type response with moderate or high IL-4 and IL-5 levels and without detected IFN-γ. The result may be attributed to the characteristics of the BALB/c mouse which tends to favor Th2-type responses (Rosenkrands et al., 2005; Theiner et al., 2006). However, when the mice were immunized s.c. with Bos d 2 and HEL in CFA, the responses were more of the Th1 type. Therefore, a more probable explanation for the Th2-type cytokine profile with the Bos d 2 and the control antigen groups could be the influence of the immunization route (Yip et al., 1999) and type of adjuvant used (Comoy et al., 1998; Yip et al., 1999).

Zeiler et al. have previously shown that human T cells recognize one immunodominant epitope in Bos d 2 (Zeiler et al., 1999). The peptide p127-142 containing the immunodominant epitope was recognized by all cow dust-asthmatic patients (Zeiler et al., 1999). Furthermore, the peptide was also found to be immunodominant in the BALB/c mouse. For example, studies with allergens to house dust mite, Der p 1 (Jarman et al., 2005), and mugwort pollen, Art v 1 (Jahn-Schmid et al., 2002), suggest that the immunodominant epitopes of these allergens account for the Th2 dominance of the immune response. Furthermore, all the human T-cell clones to the immunodominant epitope of Bos d 2 were classified as Th0-like or Th2-like clones (Zeiler et al., 1999). Therefore, the role of p127-142 in determining the quality of immune response against Bos d 2 was studied in the BALB/c mouse in more detail (I-II). Although, it turned out that the quality of the response against p127-142 was connected to the immunization route, the type of adjuvant and the type of cell (spleen or lymph node cell), the response to the peptide tended to be more of the Th2 type. In addition, the proliferative response of the spleen cells of the BALB/c mouse to the immunodominant peptide seemed to be as weak as the response to the whole protein.
Interestingly, the other lipocalin peptide SP12 from horse also induced a low proliferative response with IL-4-dominant cytokine production (study II). In contrast, bacterial peptide SP7 from Spiroplasma citri elicited a strong proliferative response with a more balanced cytokine profile. These results are in keeping with those findings suggesting that a weak stimulation through the T-cell receptor (Janssen et al., 2000; Brogdon et al., 2002) favors the Th2-biased cellular response and may be one factor determining the allergenic capacity of a protein (Kinnunen et al., 2003).

The cellular immune responses of the spleen cells of the BALB/c mouse to p127-142 resembled those of a human T-cell clone (Kinnunen et al., 2003). The minimal core region of the peptide was close to that recognized by the clone (Kinnunen et al., 2003). Moreover, the critical amino acids for T-cell recognition by the murine spleen cells and the human T-cell clone (Kinnunen et al., 2003) colocalized closely. Since recognition of p127-142 by the BALB/c mouse resembles that with a human T-cell clone, the BALB/c mouse could provide a useful tool for peptide-based immunotherapy experiments.

Since human T cells also recognize an area corresponding to p127-142 in Rat n 1 (Jeal et al., 2004), Can f 1 (Immonen et al., 2003) and Equ c 1 (Immonen et al., 2007), the recognition of the areas corresponding to these in lipocalins was examined in the BALB/c mouse. Despite colocalization of human T-cell epitopes, it was found that of the 11 lipocalin peptides only p127-142 and the corresponding region in Equ c 1, SP12, were immunogenic for BALB/c mice. It has recently been shown by Immonen et al. that the immunodominant epitope of Equ c 1 localizes adjacent to p127-142 of Bos d 2 (Immonen et al., 2007).

6.3 Cross-reactivity among lipocalin proteins (II, IV)

6.3.1 T-cell cross-reactivity among lipocalin proteins (II)

The T-cell and IgE cross-reactivities of lipocalin allergens are poorly known. Immonen et al. have recently reported that specific T-cell lines induced with p107-122 of Can f 1 cross-reacted with a homologous peptide of human tear lipocalin (Immonen et al., 2007). To examine T-cell cross-reactivity among p127-142 and 20 database search peptides including 11 lipocalin peptides, BALB/c mice were primed with the peptides. The spleen cells of BALB/c mice primed with p127-142 did not exhibit T-cell cross-reactivity among the lipocalin peptides. This was likely due to low sequence identity among the peptides (Kinnunen et al., 2003). The p127-142 primed spleen cells, however, did cross-react with a
homologous bacterial peptide SP7 (*Spiroplasma citri*). Interestingly, the sequences of p127-142 and SP7 were almost identical in the core region of p127-142, but not in the N- or C-termini. Molecular mimicry has been suggested as a possible link between some autoimmune diseases and infections. For example, several type 1 diabetes-related HLA molecules have been shown to exhibit high binding affinity for the peptide of the Coxsackie virus B4 (Ellis et al., 2005). Moreover, T cells from MS patients recognizing peptide MBP(93-105) were observed to cross-react and be activated with a peptide identical to a segments of a protein from human herpervirus-6 (Tejada-Simon et al., 2003). Furthermore, T-cell cross-reactivity between an autoantigen and an exogenous allergen has been suggested to be involved in the induction and maintenance of severe forms of allergy (Bünder et al., 2004). The result that p127-142 and SP7 are T-cell cross-reactive supports the view that a T cell-response can be primed with a peptide from an unrelated protein.

In severe atopic dermatitis, it has been suggested that the molecular mimicry of a human homolog with a microbial protein (Crameri et al., 1996; Schmid-Grendelmeier et al., 2005) can exacerbate the disease shifting the Th2 response to become more of the Th1 type. However, deviation in the T-cell response can also be useful for allergen immunotherapy. For example, a Th1 skewing peptide analog of a dominant allergen epitope has been observed to modulate favourably the polarized Th2 response in *vitro* and in *vivo* in a murine asthma model (Janssen et al., 2000). Furthermore, it has been speculated that novel cross-reactive T-cell populations induced by peptide analogs can have therapeutic immunomodulatory effects *in vivo*, for example, through bystander suppression (Kinnunen et al., 2007). In human autoimmune diseases, trials with Th2-skewing altered peptide ligands have already been performed (Raz et al., 2001; Kim et al., 2002; Alleva et al., 2006).

It was also found that p127-142 was able to induce the proliferation of spleen cells in naïve BALB/c and C57BL/6 mice *in vitro* (II). This is interesting, since the C57BL/6 mouse was found to be a nonresponder to Bos d 2 in study I. The influence of endotoxins can be excluded since the purity of the peptide was confirmed by mass spectrometry. As the phenomenon was observed repeatedly with the cells of the BALB/c mouse in two independently synthesized peptide lots but not with other peptides, it can be assumed that the phenomenon is related to the immunodominance of p127-142. This view is supported by several autoimmune studies. In EAE, the immunodominant epitope of myelin proteolipid protein (PLP) is recognized by the unprimed T cells of SJL mice (Anderson et al., 2000). Moreover, the immunodominant epitope of myelin basic protein (MBP) (Tejada-Simon et al., 2001) and several peptides of myelin oligodendrocyte glycoprotein (MOG) have been found to be recognized by human T
cells from healthy individuals (Van der Aa et al., 2003). In SJL mice, escape from central tolerance, combined with peripheral expansion by cross-reactive antigen(s) was thought to be responsible for the high frequency of PLP 139-151-reactive T cells (Anderson et al., 2000). On the other hand, it can be speculated that the mice in study IV might have been primed with cross-reactive environmental lipocalins, such as lipocalins of rat and rabbit, in the laboratory animal facility. However, on the basis of the results of study IV it is not possible to conclude whether the response of spleen cells of naïve BALB/c mice upon stimulation with p127-142 resulted from the proliferation of naïve or primed T cells.

6.3.2 IgE cross-reactivity among lipocalin proteins (IV)

The IgE cross-reactivities of animal allergens are poorly known, the exception being serum albumins. Taking into account the importance of animal-derived proteins as respiratory sensitizers, it is surprising that IgE cross-reactivity among these allergens has only been studied quite recently. In study IV, the aim was to characterize the cross-reactivity among five lipocalin allergens and one human lipocalin. Four out of the six studied lipocalins showed human IgE cross-reactivity, including human tear lipocalin.

T-cell cross-reactivity is known to be due to a high sequential similarity. However, the situation with B-cell cross-reactivity is different. IgE-binding epitopes, in contrast to T-cell epitopes, are mainly conformational (Limacher et al., 2007). In general, it seems that IgE cross-reactivity, for example, with pollen allergens mainly takes place when the sequence similarity approaches at least 50% (Radauer et al., 2006a). However, recent studies with phylogenetically conserved allergenic proteins suggest that high structural similarity may be more important than high similarity at the sequence level (Fluckiger et al., 2002; Jenkins et al., 2005; Sankian et al., 2005). Our results (study IV) support this view since cross-reactivity was also seen between Can f 1 and Can f 2, the sequence identity of which was only 23%.

In addition to sequence similarity, the so-called cross-reactive carbohydrate determinants (CCD) are known to be involved in the IgE cross-reactivity of plant and insect-derived glycoproteins (van Ree et al., 2000). However, since Mus m 1 and TL are nonglycosylated, it is reasonable to assume that the cross-reactivity between Mus m 1 and Equ c 1 as well as between TL and Can f 1 is due to a sequential or structural similarity. Furthermore, the sequence alignment and 3-D modeling of Equ c 1 and Mus m 1 pointed to one large similar area on the surface of these proteins. Therefore, it is conceivable that the cross-reactive epitope could localize there. The IgE cross-reactivity between glycosylated Can f 2 and
putatively glycosylated Can f 1 is also likely caused by a sequence or structural similarity since it has been observed with the allergens produced in *E. coli*, as well (Kamata et al., 2007).

The cross-reactivity between Mus m 1 and Equ c 1 was found to be partial because Equ c 1 inhibited Mus m 1-specific IgE binding, whereas Mus m 1 could not inhibit the binding of Equ c 1-specific IgE. The respective phenomenon was also observed in the inhibition of Can f 1 and Can f 2-specific IgE-binding. When partially inhibited, allergen-specific IgE points to common and unique epitopes between the allergens. Moreover, the cross-reactivity between Can f 1 and TL was also partial. Although both of the allergens were able to inhibit each others IgE binding, the inhibitory capacity of Can f 1 was clearly stronger. The situation is similar with the birch allergen Bet v 1, which is capable of inhibiting IgE binding to an apple allergen, Mal d 1, in a similar or even better way than Mal d 1 itself (Vanek-Krebitz et al., 1995; Kinaciyan et al., 2007). Mal d 1 can only partially inhibit IgE binding to Bet v 1.

The cross-reactivity between Can f 1 and TL is of special interest since TL is an endogenous lipocalin. Cross-reactivities by autoreactive IgE among environmental allergens have especially been described in severe atopies (Aichberger et al., 2005; Limacher et al., 2007). It can be speculated that endogenous proteins that are IgE cross-reactive with exogenous proteins may have a role in the regulation or tolerization of an allergic immune response, e.g., through indoleamine 2,3-dioxygenase (IDO). The activation of IDO via the high affinity IgE receptor (FceRI) may be part of the mechanism that suppresses unwanted T-cell responses (von Bubnoff et al., 2003). FceRI has been reported to be, for example, highly expressed on the surface of different subsets of dendritic cells in atopic (Allam et al., 2003; Foster et al., 2003) and nonatopic patients (Allam et al., 2003). Several reports have shown that dendritic cells can inhibit T-cell proliferation (Mellor et al., 2004) and tolerize the Th2 responses both *in vitro* and *in vivo* through the induction of IDO (von Bubnoff et al., 2003; Gordon et al., 2005).
7. CONCLUSIONS

The current study had three different aims. The first aim was to elucidate the allergenic properties of Bos d 2 and the impact of MHC haplotype on the response against Bos d 2 in a mouse model. Second, since the IgE cross-reactivity among lipocalin allergens is poorly known, cross-reactivity was examined with five animal-derived lipocalin allergens and one endogenous lipocalin. The third aim was to study the suitability of recombinant dog allergens, Can f 1 and Can f 2, for the diagnostics of dog allergy.

The major respiratory bovine allergen Bos d 2 was found to be a weak immunogen in six different mouse strains. Only the BALB/c mouse mounted a distinct humoral response to Bos d 2. Consistent with recognition by humans, BALB/c mice recognized one immunodominant epitope in Bos d 2. Recognition of the immunodominant epitope peptide p127-142 closely resembled that with human T cells. The p127-142 induced a Th2-type response in the BALB/c mouse. Interestingly, p127-142 was also recognized by naïve spleen cells of this mouse strain. It can be speculated that the phenomenon is related to the immunodominance of p127-142. On the other hand, reciprocal induction of the cytokine responses by p127-142 and a bacterial peptide from Spiroplasma citri (SP7) implies that modified allergen peptides can skew the phenotype of primed T cells. Furthermore, the finding points to the possibility that the Th2-type response can be primed with an unrelated protein or with a homolog of an allergen. This phenomenon may open prospects for allergen immunotherapy.

Four of the five lipocalin allergens and one human endogenous lipocalin were shown to be IgE cross-reactive. Since the sequence identities among the cross-reactive proteins were comparatively low (23-61%), it can be assumed that the phenomenon is partially caused by the similarity of the three-dimensional structures within the proteins. Whether the cross-reactivity among lipocalin allergens has clinical implications needs to be further elucidated. The cross-reactivity between the major dog allergen Can f 1 and human tear lipocalin, on the other hand, raises the question as to whether cross-reactivity between endogenous proteins and environmental allergens could be implicated in the regulation or tolerization of an allergic response.

Finally, the immunologic activity of recombinant dog allergens Can f 1 and Can f 2 were shown to be comparable to those with natural allergens. However, the sensitivity of the allergens was insufficient for dog-allergy diagnostics. It seems that other dog allergens in addition to Can f 1 and Can f 2 are important for sensitization to dogs. A new 18 kDa
allergen, Can f 4, was preliminarily characterized in the dog epithelial preparation. Can f 4 was recognized by 60% of the dog-allergic patients, thus more frequently than their recognition of Can f 1. Amino-terminal sequencing suggested that Can f 4 is a new member in the lipocalin family. Highly sensitive diagnostics of dog allergy will require other dog allergens in addition to Can f 1. These allergens could include, for example, dog albumin, Can f 3, and the new lipocalin allergen Can f 4.
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