From the Department of Medicine, Solna
Clinical Immunology and Allergy Unit
Karolinska Institutet, Stockholm, Sweden

RECOMBINANT CAT AND MITE ALLERGENS AS TOOLS IN THE STUDY OF DIAGNOSIS AND TREATMENT OF ALLERGY

Tiiu Saarne

Karolinska Institutet

Stockholm 2009

Published by Karolinska Institutet. Printed by Reproprint AB.

ISBN 978-91-7409-583-8

"You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face… do the thing you think you cannot do."

Eleanor Roosevelt

ABSTRACT

Allergic diseases, such as allergic asthma, allergic rhinitis and atopic eczema, are a major health problem world-wide, affecting up to 30% of the population. A large proportion of the patients are allergic to indoor allergens, such as mite and cat allergens, which are airborne and thus cause symptoms in the airways upon inhalation. Hundreds of single allergens from different sources have been identified, cloned and expressed as highly pure and well-defined recombinant allergens. They can be produced in large amounts and are gradually replacing allergen extracts in allergy diagnosis and allergen-specific immunotherapy (SIT), the only treatment that may affect the natural course of allergic diseases. SIT may be further improved by genetically modifying allergens into hypoallergens, which have a reduced allergenic capacity, but a retained T-cell reactivity.

The aims of this thesis were to clone and characterise new allergens from the storage mite *Lepidoglyphus destructor*, and to apply the major recombinant cat allergen Fel d 1 in allergy diagnosis and in the construction and evaluation of hypoallergens for use in therapy of cat allergy.

In **paper I**, PCR and screening with sera were used to identify and isolate new allergen clones from a phage display cDNA library that was constructed previously from *L. destructor*. Two new clones, showing homology to tropomyosin and α-tubulin, were obtained and subsequently expressed in *Escherichia coli*. The recombinant proteins bound to IgE antibodies in sera of mite-allergic patients and were characterised as putative minor allergens Lep d 10 and *L. destructor* α-tubulin. Lep d 10 was found to be cross-reactive with tropomyosins from other mite species and crustaceans. In **paper II**, the previously well-characterised major recombinant cat allergen rFel d 1 was compared to cat dander extract (CDE) in the diagnosis of cat allergy in children of a large prospective birth cohort. The rFel d 1 was at least as good as CDE in detecting allergen-specific IgE and may be a better marker for early cat sensitisation. The study showed that children with allergen-specific IgE, but without symptoms to cat at 4 years of age all developed allergic symptoms at 8 years of age.

In **paper III**, the three-dimensional structure of rFel d 1 was genetically altered in a rational approach comprising duplication of known T-cell epitopes and disruption of disulphide bonds through introduction of point mutations. Three out of seven Fel d 1 derivatives generated were identified as hypoallergens with a strongly reduced IgE-binding capacity, a reduced allergenicity and a retained T-cell reactivity. The most promising candidate for SIT, rFel d 1 (DTE III) was further evaluated *in vivo* in **paper IV**. Therapeutic treatment of cat-allergic mice with the hypoallergen resulted in decreased airway hyperreactivity and induction of allergen-specific IgG with blocking capacity. In contrast to unmodified rFel d 1, the hypoallergen was tolerated at a high treatment dose without any observed side effects. In addition, rFel d 1 (DTE III) induced less skin prick test-reactivity compared to rFel d 1 in cat-allergic patients.

In summary, the results of this thesis show the wide usage of recombinant allergens. They are important in the characterisation of single allergens of allergen sources, as shown for the mite *L. destructor*. Furthermore, they reveal sensitisation to a specific allergen component, e.g. the major cat allergen Fel d 1, which in respect to diagnosis of cat allergy seems to be at least as good as CDE. In addition, recombinant allergens can be used in the development of safer and more efficient tools for SIT, as demonstrated with Fel d 1 (DTE III).

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numerals:

I. Tiiu Saarne, Liselotte Kaiser, Omid Rasool, Sonia Huecas, Marianne van Hage-Hamsten, Guro Gafvelin.
Cloning and characterisation of two IgE-binding proteins, homologous to tropomyosin and α-tubulin, from the mite *Lepidoglyphus destructor*.
Int Arch Allergy Immunol 2003;130:258-265

II. Tiiu Saarne, Hans Grönlund, Inger Kull, Catarina Almqvist, Magnus Wickman, Marianne van Hage.
Cat sensitization identified by recombinant Fel d 1 several years before symptoms – results from the BAMSE cohort.
Accepted for publication in *Pediatr Allergy Immunol*

III. Tiiu Saarne, Liselotte Kaiser, Hans Grönlund, Omid Rasool, Guro Gafvelin, Marianne van Hage-Hamsten.
Rational design of hypoallergens applied to the major cat allergen Fel d 1.
Clin Exp Allergy 2005;35:657-663

IV. Tiiu Saarne, Theresa Neimert-Andersson, Hans Grönlund, Marek Jutel, Guro Gafvelin, Marianne van Hage.
Treatment with Fel d 1 hypoallergen reduces allergic responses in a mouse model for cat allergy.
Manuscript

Publication not included in the thesis:

The major cat allergen, Fel d 1, in diagnosis and therapy
Hans Grönlund, Tiiu Saarne, Guro Gafvelin, Marianne van Hage
Accepted for publication in *Int Arch Allergy Immunol*

CONTENTS

LIST OF ABBREVIATIONS

AHR	Airway hyperrreactivity
alum	Aluminium hydroxide
APC	Antigen-presenting cells
BAL	Bronchoalveolar lavage
BAMSE	Children, Allergy, Milieu, Stockholm, Epidemiological survey
CBP	Carbohydrate-based particles
CA4	Certain cat allergy at 4
CDE	Cat dander extract
Cys, c	Cysteine
DC	Dendritic cell
DTE	Duplication of T cell epitopes
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FcεRI	High-affinity receptor for IgE
Fel d 1	Major cat allergen from *Felis domesticus*
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
i.l.	Intralymphatic
IL	Interleukin
ILIT	Intralymphatic immunotherapy
i.n.	Intranasal
i.v.	Intravenous
L. destructor	*Lepidoglyphus destructor* (storage mite)
LNIT	Local nasal immunotherapy
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NA8	New cat allergy at 8
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
r	Recombinant
SA4	Suspected cat allergy at 4
s.c.	Subcutaneous
SIT	Allergen-specific immunotherapy
SLIT	Sublingual immunotherapy
SPT	Skin prick test
TCR	T-cell receptor
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptors
TNF	Tumor necrosis factor
T_{Reg} cell	Regulatory T cell

1 INTRODUCTION

Allergic diseases with symptoms spanning from mild rhinitis to anaphylaxis are a major health problem, affecting the quality of life for millions of people all over the world, with highest prevalences in developed countries. Allergy is caused by the immune system's reaction to foreign substances, called allergens, which are otherwise harmless substances found in our environment. Before going into the details of the main focus of this thesis, I will give a short introduction to the immune system in general and the allergic reaction and disease in particular.

1.1 THE IMMUNE SYSTEM

The mammalian body has evolved different mechanisms to control and usually eliminate harmful foreign substances. When common microorganisms, such as bacteria and viruses, pass the epithelial barriers and enter the body for the first time, they are immediately met by cells and molecules belonging to the innate immune system [1, 2]. The most important cells in this first line of defence are phagocytic cells, such as macrophages and neutrophils, as well as natural killer (NK) cells. An innate immune response is initiated when common pathogen-associated molecular patterns (PAMPs) that are shared by many microbes are bound to pattern-recognition receptors, e.g. different Toll-like receptors (TLR), on the surface of the cells. A given set of germline-encoded receptors is present on all cells of the same cell type. The innate immune system can rapidly detect and destroy pathogens, but recognises only certain molecular patterns and cannot form memory of specific pathogens. Therefore, not all infectious organisms can be eliminated by the innate immune system.

The adaptive immune system is characterised by specific recognition of antigens and by the development of immunological memory [1, 2]. This results in an increased protection against reinfection with the same pathogen. An adaptive immune response is initiated when mature dendritic cells (DC) carry antigen of e.g. a pathogen to the peripheral lymphoid organs (lymph nodes, spleen or mucosal lymphoid tissues), process and present them to circulating T lymphocytes (T cells). Antigens are displayed as peptides on major histocompatibility complex (MHC) molecules on the surface of DC or other antigen-presenting cells (APC), such as macrophages or B lymphocytes (B cells). Specific combinations of peptide and MHC are recognised by T-cell receptors (TCR) on the surface of T cells. Endogenously synthesised peptides presented on MHC class I (MHC I) are recognised by T cells carrying the co-receptor CD8, which differentiate into CD8+ cytotoxic T cells that kill the infected target cells. Similarly, CD4+ T cells recognise peptides from proteolytically processed exogenous proteins presented on MHC II, and are differentiated into e.g. CD4+ T helper type 1 (Th1) or T helper type 2 (Th2) cells. CD4+ T helper cells mainly activate macrophages and B cells. Upon antigen stimulation, B cells also undergo clonal expansion. The B cell receptors, immunoglobulins (Igs), are cell-surface bound until binding of an antigen for the first time. Binding of antigen together with T-cell help activates the B cells to differentiate into plasma cells that secrete Igs as soluble antibodies. There are five isotypes of Igs (IgM, IgD, IgG, IgA and IgE); each specialised to activate different effector mechanisms. Like TCR on T cells, Igs are produced by B cells in a vast range

of antigen specificities, each B cell producing Ig of a single specificity. The antigen-specific receptors on B and T cells are assembled through somatic rearrangement of a collection of a few hundred germline-encoded gene elements. This may result in the formation of millions of different antigen receptors, each with specificity for a unique antigen. Upon antigen stimulation, antigen-specific lymphocytes proliferate to increase in number and thereby more effectively fight the pathogen. This clonal expansion is followed by differentiation into effector cells and affinity maturation of antibodies.

Most antigen-specific lymphocytes undergo apoptosis when the antigen is removed, but some persist as long-lived memory cells, mediating the immunological memory [1, 2]. These cells are more sensitive to the antigen than naïve lymphocytes and respond rapidly and effectively upon reexposure to the antigen.

1.2 MECHANISMS OF ALLERGY

1.2.1 The allergic immune response

The allergic reaction can be antibody- or cell-mediated [3]. In the majority of cases, the allergic symptoms are initiated by IgE antibodies that are produced in response to otherwise harmless environmental antigens, i.e. allergens. A person that has a genetic predisposition to produce IgE to allergens, i.e. to become sensitised, is called atopic. A large proportion of atopic individuals are sensitised to indoor allergens [4], such as cat and mite allergens. Indoor allergens are commonly airborne and thus cause allergic symptoms in the upper and lower airways upon inhalation. The reactions causing these symptoms are dominated by allergen-specific lymphocytes.

1.2.1.1 Sensitisation

The sensitisation process is initiated when APC, such as DC, take up and process allergens from mucosal surfaces, e.g. in the airways (Fig. 1) [5]. The allergen-derived peptides are bound to MHC II molecules on the surface of APC and are in this way presented to TCR on T cells [5]. In healthy individuals, presentation of allergen-derived peptides to T cells results in the induction of tolerance [5]. In atopic individuals, the presentation of allergens can in the presence of the cytokine interleukin (IL)-4, induce differentiation of naïve Th cells into allergen-specific Th2 cells [6]. Some possible mechanisms explaining the difference between healthy and atopic individuals are presented in section 1.3.

Allergic diseases are considered to be Th2-mediated, as Th2 cytokines (mainly IL-4, IL-5, and IL-13) are directly or indirectly responsible for a majority of the events in an allergic reaction. For instance, IL-4 and IL-13 take part in the induction of immunoglobulin class switch in B cells from production of IgM to allergen-specific IgE [6, 7]. The sensitisation leads to the formation of a pool of long-lived allergen-specific memory T cells as well as memory B cells producing and secreting allergen-specific IgE.

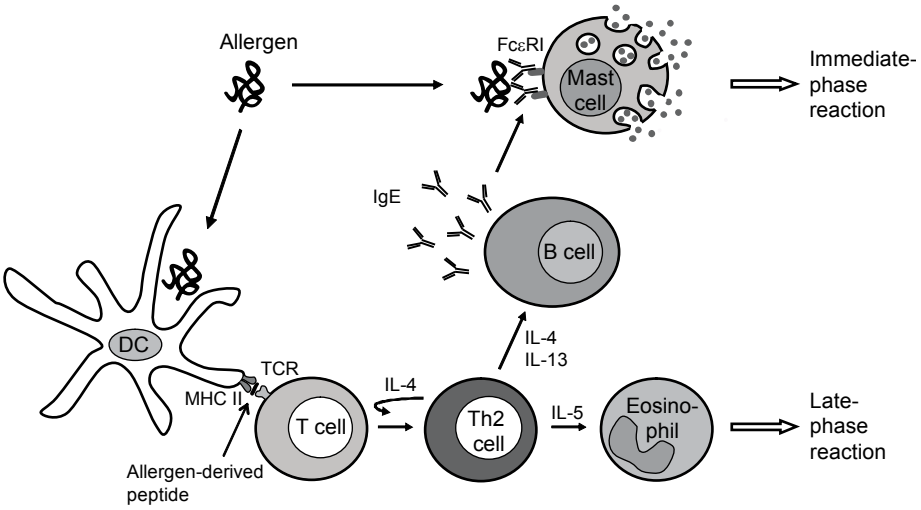

Figure 1. An overview of the allergic immune response. The allergen is taken up and processed by DCs that present allergen-derived peptides to T cells. T cells are differentiated into Th2 cells that induce class switching of B cells to production of allergen-specific IgE. Secreted IgE bind to FcεRI receptors on mast cells. Upon subsequent encounter with the same allergen, the receptors are cross-bound and cause degranulation of the mast cells, which results in the immediate-phase reaction. The Th2 cells can also cause late-phase reactions by recruiting other inflammatory cells, e.g. eosinophils.

1.2.1.2 The immediate-phase reaction

In sensitised individuals, subsequent exposure to a specific allergen results in the binding of allergen to preformed IgE molecules that are bound to high-affinity IgE receptors, FcεRI (Fig. 1). Cross-linking of adjacent receptors on tissue-based mast cells and circulating basophils by the allergen-bound IgE initiates a cascade of events, which eventually leads to mast cell or basophil activation [7]. This results in degranulation and release of preformed mediators, such as histamine and serine proteases, newly formed lipid mediators, such as prostaglandin and leukotrienes, as well as preformed cytokines, e.g. tumor necrosis factor (TNF)-α, IL-4 and IL-5 [7]. Within minutes after the allergen exposure, histamine and leukotrienes cause increased local blood flow and vascular permeability, leading to swelling and an increased cellular infiltration, while the proteases causes tissue damage. This results in immediate allergic symptoms, such as oedema and itching in the skin, watery and itchy eyes, sneezing, rhinorrhea and mucus secretion in the upper airways, and the acute asthma symptoms cough, wheezing, bronchoconstriction, oedema and mucus secretion in the lower airways [7].

1.2.1.3 Allergic anaphylaxis

The most severe case of allergic reactions is the systemic anaphylaxis, which has a rapid onset and may cause death [8, 9]. Allergic anaphylaxis is, like immediate-phase reactions, caused by the cross-linking of FcεRI on mast cells and basophils by allergen-bound IgE. Anaphylaxis is rarely caused by inhaled allergens, but more commonly by insect venoms, food or drug allergens. Mast cells all over the body are triggered to release large amounts of mediators, leading to a series of severe symptoms in multiple organ systems. Usually two or more body systems are concurrently involved in anaphylaxis. Common symptoms include urticaria, angioedema (swelling of lips, face, neck and throat), systemic vasodilation and

hypotension (eventually leading to shock), oedema of bronchial mucosa (leading to bronchoconstriction), and gastrointestinal symptoms such as abdominal pain, vomiting and diarrhoea.

1.2.1.4 The late-phase reaction

The release of pro-inflammatory mediators and cytokines from mast cells, and the increase in vascular permeability promotes subsequent recruitment of other effector cells [7]. Several hours after the allergen exposure, the activation of allergen-specific Th2 cells can in some patients induce a late-phase reaction that may persist for several days, e.g. sustained blockage of the nose or increased airway hyperrreactivity (AHR), which is IL-13-mediated [10]. Activation of allergen-specific Th2 cells might also be enhanced by FcεRI-IgE-dependent antigen presentation [11]. The late responses may lead to chronic inflammation, e.g. chronic asthma, which is characterised by the infiltration and activation of e.g. Th2 cells, eosinophils, neutrophils and basophils (Fig. 1) [10]. The maturation and activation of eosinophils is mediated through the Th2 cytokine IL-5 [7]. Furthermore, eosinophils secrete inflammatory mediators, including leukotrienes, and granule proteins, including major basic protein (MBP) and eosinophil cationic protein (ECP) that cause e.g. AHR and mucus secretion [7]. Neutrophils are also a source of many pro-inflammatory mediators, such as lipids, cytokines and proteases that contribute to epithelial damage and remodelling of the airways, and activate mast cells and eosinophils [12]. The airway remodelling, i.e. thickening of the airway wall, is caused by large repair processes after repeated airway injury [13].

1.2.2 Regulation of allergic T-cell responses

For a long time, Th1- and Th2-cell responses have been seen as antagonising, based on the cytokines these cells produce [14]. As mentioned earlier, Th2 cells are the dominating Th cell subset in allergic reactions. Via IL-4, the Th2 cells inhibit the development of Th1 cells, which produce the cytokines interferon (IFN)-γ, IL-2 and TNF-β. Conversely, IFN-γ from Th1 cells as well as from NK cells inhibits the development of Th2 cells. Th2 responses can also be shifted to Th1 or Th0 by IL-12 that is produced by DCs upon interaction of TLR on DCs with microbes, microbial products or synthetic adjuvants [15]. In addition, several chemokines have been shown to regulate the Th1/Th2 balance [16]. The regulation of Th1 and Th2 cytokines is also dependent on the lineage-specific transcription factors T-bet and GATA3, respectively [17]. Deficiency in the Th1 transcription factor T-bet has been suggested to predispose to a Th2 response [18].

More recently, new effector Th cell subsets and different regulatory T cells (T_{Reg} cells) have been discovered as important subsets of T cells that may contribute to the regulation of allergic responses. Th17 is a rather new subset of Th cells, while Th9 has only recently been suggested as yet another subset. Their roles in allergy are still unclear. The development of Th17 cells is induced by the combined action of IL-6 and transforming growth factor (TGF)-β [19, 20]. In addition, IL-23 seems to be involved in maturation and maintenance of Th17 cells [21]. The Th17 cells produce IL-17A, IL-17F and IL-22, which cooperate to induce tissue inflammation [21]. IL-17 is overexpressed in asthmatic airways and contributes to neutrophil recruitment [22].

Some data show that Th9 cells are reprogrammed Th2 cells that develop in the presence of TGF-β and IL-4 [23]. These cells produce IL-9 and IL-10 [20, 21], but lack suppressive function and promote tissue inflammation [24].

The T_{Reg} cells regulate or suppress effector T cells and are important in the maintenance of peripheral tolerance to e.g. allergens. So-called natural T_{Reg} cells are thymus-derived $CD4^+CD25^+$ T cells that express the transcription factor Foxp3 [25]. Other subsets of T_{Reg} cells include peripherally induced adaptive IL-10-secreting T_{Reg} type 1 (Tr1) cells [26] and TGF-β-secreting Th3 cells [27]. The Th3 cells are less studied and it is not clear to what extent they contribute to tolerance.

In healthy individuals, T_{Reg} cells have been shown to be responsible for active suppression against allergens [28, 29]. Also allergic individuals seem to have T_{Reg} cells, but the frequency of Th2 cells is higher [28], and Th2 cytokines inhibit the function and development of T_{Reg} cells [26]. It has been suggested that in allergic individuals, the suppressive effect of T_{Reg} cells could be deficient or overcome by strong activation signals [29, 30]. T_{Reg} cells may contribute to the regulation of allergen-specific immune responses in many ways, e.g. by suppression of DCs that support the generation of effector T cells, suppression of Th1, Th2 and Th17 effector cells, suppression of allergen-specific IgE and induction of IgG4, suppression of mast cells, basophils and eosinophils [31].

In addition, primarily mouse studies have proposed a role for natural killer T (NKT) cells in the development of asthma and allergen-induced airway hyperreactivity (AHR) [32, 33]. NKT cells share features of both classical T cells and NK cells and can rapidly produce large amounts of Th2 cytokines [34]. NKT cells have also been suggested to play a role in the pathogenesis of human asthma since a large proportion of these cells were detected in lungs of patients with moderate to severe asthma, in contrast to healthy subjects or subjects with sarcoidosis [35]. These data have however been questioned later [36].

1.3 THE DEVELOPMENT OF ALLERGIC DISEASE

The prevalence of allergic diseases, such as allergic asthma, allergic rhinitis and atopic eczema, has been increasing world-wide during the past 4-5 decades, particularly in western industrialised countries [3]. An increased prevalence of allergic diseases has been observed above all among children and young adults [37]. Up to 30% of the population in more developed countries is affected by allergy [37, 38]. Commonly, allergic diseases progress in a typical sequence of clinical signs. This “allergic march” often starts with atopic eczema and food allergies in early childhood before developing into asthma and allergic rhinitis [39]. For some patients, allergic rhinitis precedes asthma and acts as the starting point.

There is not a single gene or environmental factor explaining the development of allergic disease, but most probably it is regulated by an interaction between genes and environment. Multiple genes are thought to contribute and may influence the disease to variable degrees in different individuals. There is a cluster of cytokine genes on chromosome 5 that e.g. regulate the production of IgE and the proliferation and

maturation of effector cells of the allergic response [40, 41]. Many potential asthma susceptibility genes, such as *ADAM33*, *DPP10* and *NPSR1*, have been identified, but the impact of the genes are yet unclear [41, 42].

Most probably, environmental factors increase the risk of allergic disease in genetically predisposed individuals. Among environmental factors that might explain the susceptibility to allergic disease are allergen exposure, maternal smoking during pregnancy, postnatal exposure to tobacco smoke, and indoor and outdoor air pollution [43]. The increased prevalence is, however, suggested to be explained above all by environmental factors related to the western lifestyle. This view is supported by studies from the early 1990s, which showed that the prevalence of allergic diseases was higher in Western than Eastern European countries [44]. During the past two decades, the countries of the former East block have undergone a rapid change in socio-economic standard and lifestyle, which seems to have resulted in an increased prevalence of sensitisation and allergy [45-47]. In 1989, a theory known as the "hygiene hypothesis" was formulated to explain the increased prevalence of allergic diseases in western societies over the last decades [48]. It proposed that a reduced exposure to infectious diseases in early childhood due to improved living conditions, higher personal hygiene and fewer siblings might result in an increased risk of developing allergic disease. Many epidemiological as well as animal studies support different aspects of the concept, as reviewed by Garn & Renz [49]. For example the use of antibiotics early in life might increase the risk of allergic disease and an anthroposophic life style seems to reduce the prevalence of sensitisation. However, a uniform underlying mechanism is still lacking, but there are two main mechanisms, or a combination of these, suggested to explain the hygiene hypothesis [49, 50]. First, a missing immune deviation from Th2 to Th1, which is a result of decreased stimulation of the innate immune cells through their TLRs by microbial components, and thereby reduced production of e.g. the Th1-driving cytokine IL-12. A second explanation is that a reduced stimulation of the innate immune system causes a reduced activation of T_{Reg} cells, which thereby fail to suppress Th2 activation. Thus, both these explanations connect innate and adaptive immune responses in the development of allergy.

1.4 ALLERGENS

1.4.1 Features of allergens

An allergen can be described as an antigen that has the ability to induce the production of IgE antibodies, to bind IgE and to elicit IgE-mediated immune responses [51]. Allergens originate from a large variety of sources, such as tree and grass pollen, mites, cockroaches, furred animals, venoms, foods, latex and medications, such as betalactam antibiotics. One source commonly contains many allergens, which are named according to the nomenclature maintained by the World Health Organization (WHO) and International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee [52, 53]. The first three letters of genus are followed by one letter for the species and an Arabic number for the order of discovery and purification of the allergen. Homologous allergens of related species are generally designated the same number. Thus, Der p 1 is a major group 1 allergen

from the house dust mite *Dermatophagoides pteronyssinus*. An allergen from a single source that reacts with IgE antibodies of more than 50% of the patients sensitised to that allergen source is considered a major allergen [52].

Most allergens are proteins, and many of these are glycosylated, which may contribute to their allergenicity [51]. It is only a limited number of proteins that induce allergic responses in susceptible individuals [54]. No single structural or functional feature has been identified for all allergens, but there are many common characteristics that may contribute toward the overall allergenicity [51]. Most allergens can be grouped into a small number of protein families with a limited range of biological functions, such as hydrolysis of proteins, polysaccharides and lipids, binding of metal ions and lipids, transport, storage, and cytoskeleton association [54]. It has been suggested that the enzymatic activity of some allergens, mainly proteolytic activity, might contribute to their allergenicity [51, 55]. Recently, it was shown that allergens have no or few homologues in bacteria, in contrast to randomly selected control proteins of other origin [56]. As early exposure to bacterial proteins could create tolerance to homologous proteins, it was suggested that non-homologous proteins may thus become allergens.

The majority of allergens are relatively small, negatively charged, highly soluble and stable proteins that are often carried on airborne particles such as pollen grains or animal dander [51]. Once inhaled, the soluble allergens can be readily eluted from the particle and diffuse into the mucosa of the airways. Other routes of exposure for allergens are ingestion, injection or bite, and skin contact. The allergen carriers might release or expose other factors together with the allergens, such as microbial molecules or lipid mediators, which may influence the immune response [51]. The allergenicity of the house dust mite allergen Der p 2 may at least partially be explained by its homology to the lipopolysaccharide (LPS)-binding protein MD-2 of the TLR4 signalling complex [57]. Der p 2 was recently shown to facilitate signalling through the TLR4 pathway in an adjuvant-like fashion [57]. This mechanism might underlie the allergenicity of similar lipid-binding allergens. TLR4 on pulmonary epithelial cells were shown to play an essential role in the activation of allergic immune responses [58]. Furthermore, environmental air pollutants might facilitate the release of allergens from their carriers or generate new allergenic epitopes, e.g. by nitration [43, 51].

The degree of allergenic activity is largely determined by the amino acid sequence and the protein fold of an allergen. In order to bind two or more IgE molecules that can cross-link FcεRI, an allergen must contain at least two IgE-binding epitopes. These specific structures, also called B-cell epitopes, are present on the surface of proteins (Fig. 2). The IgE-binding epitopes are represented either by a single continuous stretch of amino acids (linear/continuous epitopes), or by distantly separated amino acids or peptides that are brought together through folding of the protein (conformational/discontinuous epitopes) [59].

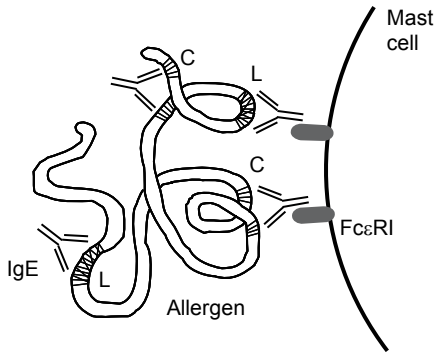

Figure 2. IgE-binding epitopes. An allergen contains linear (L) and conformational (C) epitopes that are recognised by different IgE anti-bodies.

IgE antibodies can thus recognise linear epitopes even in unfolded proteins, whereas IgE binding to conformational epitopes is disrupted when proteins unfold. It has been found that an allergen can reversibly take different conformations, which expose IgE epitopes to a varying degree [60]. It has also been suggested that the density and number of IgE epitopes together with the conformation determine the capacity of the allergen to activate effector cells, such as mast cells and basophils [60].

Furthermore, allergens contain multiple T-cell epitopes, i.e. the small peptide fragments that after processing of the protein by APC are displayed on MHC II molecules to TCR on T cells [59].

1.4.2 Allergen extracts

At present, the tools for both allergy diagnosis and immunotherapy are mainly based on crude allergen extracts prepared from natural sources, such as whole mites or cat dander. It is difficult to standardise these extracts, since batches of extracts from one allergen source may vary in allergen composition and content [61-63]. The desired allergens may be found in insufficient concentrations in some extracts, and in addition, the extracts contain variable amounts of other allergens or non-allergenic components [61, 62]. Furthermore, doses of allergen extracts are expressed in different ways by manufacturers, which complicates the use and comparison of extracts [61]. Standardisation protocols for major allergen content in allergen extracts are under development in Europe [64].

1.4.3 Recombinant allergens

Defined single allergens are preferable for both diagnosis and therapy, but it is relatively expensive and time-consuming to isolate and purify proteins for clinical use by biochemical and immunochemical procedures. The identification and analysis of allergens has been considerably improved since recombinant DNA technology was introduced to the field of allergen characterisation two decades ago [65]. This made it possible to clone the genes for specific allergens and produce them as highly pure recombinant proteins with high similarity to their natural counterparts. Up to day, more than 800 allergens from the most common allergen sources have been identified, isolated and produced as recombinant allergens [66]. Most of the recombinant allergens have been expressed in *Escherichia coli* (*E. coli*) and have structural and immunological properties comparable to those of the corresponding natural allergens [65, 66]. For each allergen, the most suitable expression system and purification procedure has to be established prior to the characterisation of the pure recombinant allergen.

The current knowledge about allergens is to a large extent based on the study of recombinant allergens. Recombinant allergens can be produced in large amounts for different applications in research as well as for clinical use. The identity and biological function of many allergens has been determined through sequence homology searches. Furthermore, homology searches and computer-based molecular modelling have been used to predict the 3D allergen structure, and now the definitive structures of many allergens are being determined by nuclear magnetic resonance spectroscopy or X-ray

crystallography using recombinant allergens [60]. By the use of recombinant allergens or fragments of allergens produced by recombinant technology, the location of amino acid residues in B-cell and T-cell epitopes can be determined [59]. The availability of recombinant allergens has been of major importance for the development of new tools for diagnosis and therapy of allergic diseases, which will be described in later sections of this thesis. In terms of clinical use, recombinant allergens have improved quality regarding purity, consistency, composition and dosage compared to crude allergen extracts.

1.4.4 Allergenic cross-reactivity

Most allergic patients have IgE antibodies to more than one single allergen or allergen source. This co-sensitisation can in some cases be due to IgE-reactivity against different epitopes on different allergens, but in other cases co-sensitisation can be due to cross-reactive allergens [67]. Two allergens are cross-reactive if they are recognised by the same IgE antibody or TCR [68]. A cross-reactive IgE antibody was originally raised against one allergen, the primary sensitising allergen, but is able to recognise a protein with similar structure and epitopes from another allergen source [68]. Due to cross-reactivity, allergic symptoms can arise in response to a source the patient has previously not been exposed to [69], i.e. the allergen does not necessarily have to induce IgE production [70].

The cross-reactivity between allergens is caused by similarity in the primary or 3D structure of proteins [70]. For example, sensitisation by the major birch pollen allergen Bet v 1 frequently leads to cross-reactions with homologous proteins in apple, hazelnut, carrot and other fruits and vegetables [67]. These allergens belong to the Bet v 1-like protein family and are very similar in 3D structure. About 30 other groups of cross-reactive proteins from various sources have been identified. Tropomyosin, for instance, is a cross-reactive protein found in both inhaled and ingested allergen sources [67]. It is a major food allergen that was first identified in shrimp [71, 72]. In invertebrates, it is a highly conserved protein that has subsequently been identified as an important cross-reactive allergen in many species of crustaceans, molluscs, nematodes, insects, as well as mites [73]. Such highly conserved proteins representing cross-reactive allergens from several sources are sometimes referred to as panallergens [67]. Proteins with a similar fold are, however, not necessarily cross-reactive [70], as exemplified by group 2 mite allergens (described section 1.5.2.).

1.5 MITE ALLERGY

1.5.1 Mites and sensitisation to mites

The main source of indoor allergens world-wide is mites [74], which are the most common cause of allergy around the world [75]. Sensitisation to house dust mites is a major independent risk factor for asthma in all areas where the climate is favourable for growth of mites [4, 74]. Mites can be found e.g. in house dust, furniture, mattresses, stored food and in farming environments [76]. More than 30 000 species of mites and the closely related ticks have been identified [74], and at present (August 2009), IgE

reactivities to at least 18 mite species are presented in the allergen database Allergome [66].

The house dust mite *Dermatophagoides pteronyssinus* is the most important inducer of allergic reactions in Europe [77]. One of the most abundant mite species causing allergic reactions in European farming environments is *Lepidoglyphus destructor* [78]. *L. destructor* and other related mites are mainly found in surroundings where hay, straw, cereals and food are stored [76], and are thus called storage mites. Consequently, *L. destructor* causes allergic disease in farmers [79, 80], grain handlers [81] and bakers [82]. In diverse climatic regions, it has been demonstrated that even urban populations without occupational exposure could have a high prevalence of sensitisation to *L. destructor* and other storage mites [77, 83-86]. Patients sensitised to *D. pteronyssinus* often have IgE reactivities against other mite species [77, 83, 87, 88]. It has been demonstrated that at least some of this co-sensitisation may be due to cross-reactivity [77, 83, 87, 88].

To date (August 2009), 58 mite allergens have been reported to the database Allergen Nomenclature from WHO/IUIS [53]. Mite allergens belonging to 24 different groups have been identified [53], of which many, but not all, have homologues in many different mite species. The majority of the mite allergens have been produced as recombinant proteins, although with varying success [53, 89].

1.5.2 Allergens from the mite *Lepidoglyphus destructor*

In the storage mite *L. destructor*, 21 IgE-binding components have been identified in blotted crude extract [88]. Four of them, Lep d 2, 5, 7 and 13, have subsequently been isolated, produced as recombinant proteins in *E. coli* and characterised as allergens [90, 91]. Lep d 2 (originally termed Lep d 1) was the first allergen from *L. destructor* to be cloned, sequenced [92, 93] and expressed as a recombinant protein [90]. Among *L. destructor*-sensitised patients, recombinant (r) Lep d 2 is detected by IgE from 60-70% [94, 95], and is therefore a major allergen of *L. destructor*. Lep d 5, 7 and 13 were isolated from a phage display cDNA library that was constructed from *L. destructor* [91]. The recombinant Lep d 5, 7 and 13 were detected by serum-IgE from 9%, 62% and 13% of *L. destructor*-sensitised patients, respectively. All of the characterised *L. destructor*-allergens have homologues in at least one other mite species [53]. Cross-inhibition studies demonstrated extensive cross-reactivity between group 2 allergens from the mites *L. destructor*, *Glycyphagus domesticus* (*G. domesticus*) and *Tyrophagus putrescentiae* (*T. putrescentiae*), whereas the cross-reactivity between these three allergens and Der p 2 from *D. pteronyssinus* was limited [96].

1.6 CAT ALLERGY

1.6.1 Cat allergens

Another important source of indoor allergens world-wide is the domestic cat *Felis domesticus*, which causes allergic disease in about 10-15% of the population [97-99]. Cat is one of the most important allergen sources associated with allergic asthma [100, 101]. Many studies have demonstrated that early sensitisation to cat increases the risk

of later asthma in childhood [101-103]. In Sweden, sensitisation to cat is more common than sensitisation to mites, both in children and adults [98, 104]. During the last decade, sensitisation to cat has increased in children from 13% to 19% [104].

Cat allergens originate from e.g. sebaceous glands and saliva, are transferred to the fur by licking, and are spread on airborne cat dander particles [105, 106]. Analysis of cat dander extract has demonstrated the presence of at least 10 IgE-binding components [107]. The dominant cat allergen, accounting for more than 60% of all IgE-binding reactivity to cat dander [108], is Fel d 1, which is detected by serum-IgE of about 95% of cat-allergic individuals [109-111]. Fel d 1 belongs to the secretoglobin family, which includes the human Clara cell protein CC16, but the biological function of Fel d 1 is unknown [112]. Four other allergens described in cat are Fel d 2 (cat serum albumin) [113], Fel d 3 (cystatin) [114], Fel d 4 (lipocalin) [115], and Fel d 5 (IgA) [116], which have sensitisation rates of approximately 20%,10%, 60% and 40%, respectively among cat-sensitised individuals. An additional cat immunoglobulin, IgM has been added to the WHO/IUIS allergen database [53], but is less well characterised.

1.6.2 The major cat allergen Fel d 1

Fel d 1 is a rather well characterised allergen, which was first identified and purified by Ohman et al. in 1974 [117]. Fel d 1 has a molecular weight of approximately 38 kDa [118] and consists of a tetramer that is formed by two non-covalently linked heterodimers [119]. Each heterodimer consists of two polypeptides, chain 1 and chain 2 [120], which are encoded by separate genes [121]. The two chains of 70 amino acid residues (chain 1) [120] and of 90 or 92 residues (chain 2) [120, 121] are linked by three interchain disulphide bonds (Fig. 3A). The bonds are formed between cysteine (Cys) 3 of chain 1 and Cys73 of chain 2, Cys44 of chain 1 and Cys48 of chain 2, and Cys70 of chain 1 and Cys7 of chain 2 [118, 122]. The T-cell epitopes of Fel d 1 have previously been determined by using overlapping synthetic peptides of both chains [123]. Another set of short overlapping peptides was used to define three important IgE epitopes on Fel d 1, two in chain 1 and one in chain 2, which were proposed to be conformational epitopes [124].

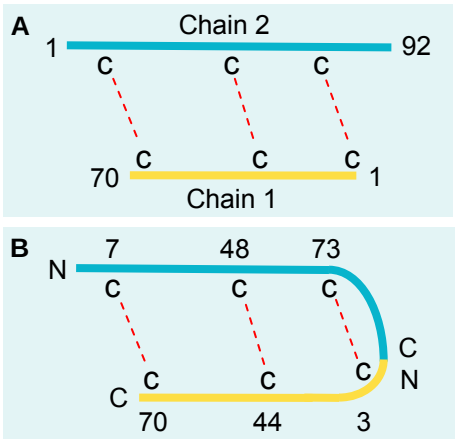

Figure 3. The major cat allergen Fel d 1. **A**, The two chains of the Fel d 1 heterodimer are linked by three disulphide bonds formed between pairs of cysteines. **B**, In the recombinant Fel d 1, chain 2 is directly fused to chain 1, and the same disulphide bonds are formed.

Fel d 1 has been expressed in *E. coli* as a direct fusion molecule of chain 1 and chain 2 [125]. The recombinant (r) Fel d 1 construct was termed rFel d 1 (2+1) as the C-terminal of chain 2 is directly fused to the N-terminal of chain 1 (Fig. 3B) [125]. The recombinant Fel d 1 displays the same disulphide bonds and fold as its natural counterpart, and has the same IgE-binding and allergenic activity as natural Fel d 1 *in vitro* [125]. Furthermore, the 3D structure of rFel d 1 was recently

solved by X-ray crystallography [126, 127]. The structure displays the three previously defined IgE epitopes on the surface of the Fel d 1 protein [126].

1.7 DIAGNOSIS OF ALLERGY

1.7.1 Methods

Once a doctor has diagnosed the symptoms of an allergic disease and identified the possible allergen exposure through a case history, there are several methods to identify the allergen that causes the symptoms. This is of great importance to be able to instruct the patient which allergens to avoid and to select the most appropriate treatment. Sensitisation can be diagnosed *in vivo* by different skin tests and *in vitro* by different serum tests.

In the most commonly used skin test, named skin prick test (SPT), the presence of mast-cell bound IgE antibodies is determined by eliciting an immediate reaction by local allergen provocation in the skin. A small amount of allergen is applied to the skin of e.g. the forearm before puncturing the skin to introduce the allergen into the epidermis [128]. Within 15-20 minutes, a wheal and flare reaction appears around the allergens the patient is sensitised to. A swollen wheal develops due to the release of histamine by mast cells in the skin, which increases the vascular permeability and leakage of mast cell contents into the tissue. Dilation of fine blood vessels around the area produces a diffuse red flare around the wheal. The area of the allergen-triggered wheal is compared to a histamine positive control.

Sensitisation can also be diagnosed *in vitro* by measuring circulating IgE antibodies in serum. Not only the presence, but also the level of IgE specific for an allergen or allergen source can be determined. There are many assays for these quantitative measurements, but the most common in clinical routine diagnostics worldwide is the ImmunoCAP™ system (formerly named Pharmacia CAP system™) from Phadia AB (Uppsala, Sweden) [129]. The ImmunoCAP is a capsule with a solid phase of a cellulose derivative to which allergens are bound. IgE antibodies that react with the sample are detected by enzyme-labelled anti-IgE. The assay is calibrated against a WHO reference standard for human IgE to a range of 0.35-100 kU_A/L [129, 130].

Other *in vitro* methods for identification of allergen-specific IgE or IgG are e.g. enzyme-linked immunosorbent assay (ELISA) and immunoblotting, which are widely used in allergen research. In research, the allergenicity is generally further evaluated by measuring the ability of an allergen to cross-link IgE-receptors and activate peripheral blood basophils [131]. The ability of peripheral blood basophils to be activated is related to the capability of tissue-bound mast cells to be activated by allergens. The allergen-dependent reactivity of peripheral blood basophils is most commonly measured by histamine release assays. In recent years, flow-cytometric methods, based on measurements of CD63 or CD203c, have been developed to analyse basophil activation. Various myeloid cells express CD63, and since it is a granulae protein, it is displayed on the plasma membrane of basophils upon degranulation [132]. The surface marker CD203c is a basophil-specific marker that is upregulated in response to allergen

cross-linking of FcεRI [133]. Most studies have used the two markers separately, but they can also be used in the same assay [131].

1.7.2 Allergen extracts versus recombinant allergens

The different methods for *in vivo* and *in vitro* diagnosis are still largely based on the use of natural allergen extracts, which are difficult to standardise. Diagnosis using allergen extracts can in most cases define the allergen source, but it does not allow the precise identification of the specific disease-eliciting allergens [134]. It has been demonstrated that allergen extracts can be contaminated with allergens from other sources that cause false-positive responses to the source tested for [135]. Fortunately, recombinant DNA technology now allows production of large amounts of highly pure and well-defined allergens. Thus, single recombinant allergens or cocktails of a few defined recombinant allergens can be used in allergy diagnosis to determine a patient's reactivity profile [134]. Recombinant allergens are also useful tools for evaluation of cross-reactivity, e.g. for identification of the initial sensitising allergen. A positive reaction to an allergen with cross-reactive potential may predict allergic reactions to other allergen sources that contain structurally related allergens [136].

Diagnostic multi-allergen tests based on microarray technology are currently being introduced into routine diagnostics [134, 137]. A panel of a wide variety of recombinant or highly purified native allergen molecules representing the most common allergen sources is immobilised on a chip, which allows IgE reactivity profiles (component-resolved diagnosis) to be determined by single measurements using only small amounts of serum [134]. According to a patient's sensitisation profile derived from the test results, the most appropriate form of treatment may be chosen for the individual patient.

1.8 TREATMENT OF ALLERGY

1.8.1 Methods

Treatments for allergies include allergen avoidance, use of anti-histamines, steroids or other symptom-relieving medications, as well as immunotherapy. The best way to prevent or reduce allergic symptoms in sensitised subjects is to avoid the sensitising allergen. Avoidance is only possible on the basis of a good allergy diagnosis and is depending on the nature of the allergen. It is for instance difficult to fully avoid pollen and other airborne allergens. For prevention of sensitisation, the situation is more complex, especially regarding pets. Some studies have shown that early-life exposure to cats decreases the risk of later sensitisation to cat [100, 138, 139]. It has been proposed that high exposure to cat is instead associated with modified Th2 response including a high levels of allergen-specific IgG4 [140]. Other studies have shown that early-life exposure to cats increases the risk of cat sensitisation [101, 141-143].

While the symptoms of different allergic diseases can be reduced by allergen avoidance and pharmacological treatment, allergen-specific immunotherapy (SIT) is the only treatment in use that may affect the natural course of allergic diseases [144]. The intention is to reduce the severity of allergic reactions, or cause unresponsiveness, to

the specific allergens that are recognised by the patient's IgE antibodies [144]. Allergen immunotherapy was first described by Noon in 1911 [145]. Grass pollen-allergic patients were subcutaneously injected with gradually increasing doses of timothy grass pollen extract, which resulted in decreased immune responses, as diagnosed by conjunctival provocation. The procedure for SIT remains essentially the same, but the approaches have been improved continuously.

1.8.2 Allergen extracts versus recombinant allergens

SIT is still mainly based on the use of crude allergen extracts, which, however, have major disadvantages. Due to the presence of many allergenic and non-allergenic components, there is a risk of local and systemic side effects, such as urticaria, asthma attacks and even anaphylactic shock [146-148]. When used in SIT, the crude extracts can in addition cause new sensitisations, in some cases even to other allergen sources due to the presence of cross-reactive allergens in the extract [149-151]. Furthermore, some allergens might not be present in the extract in sufficient amounts to treat the patient successfully [63]. Standardised extracts of cat hair and dander, containing different maintenance doses of the main cat allergen Fel d 1, have been used in immunotherapy studies [152-155]. All studies resulted in decreased sensitivity to cat, but many patients showed local or systemic reactions to the cat extract. In addition, many extracts contain endotoxins, which are derived from the outer membrane of Gram-negative bacteria, and are potent proinflammatory compounds [156]. The clinical effect of endotoxins in allergen extract preparations used in immunotherapy has so far not been evaluated.

The use of recombinant allergens enables a more precise performance of SIT with exactly those allergens to which a given patient is sensitised [157]. Accordingly, the allergic side effects can be reduced and the clinical efficacy of the treatment can be improved. For allergen sources where one major allergen dominates, SIT might be performed with one single recombinant allergen [158] whereas allergy to other sources might need treatment with a combination of allergens [159]. Recombinant allergens are not yet in use in SIT, but promising results have been obtained in clinical SIT trials with the recombinant major birch pollen allergen rBet v 1 [158], as well as with a cocktail of five recombinant grass pollen allergens [159]. The SIT study with rBet v 1 demonstrated that a single recombinant allergen is as effective as birch pollen extract in treating birch pollen allergy [158]. Both trials resulted in improved symptom-medication scores and increasing concentrations of allergen-specific IgG [158, 159]. In addition, the availability of recombinant allergens allows regular monitoring of IgE and IgG antibodies to individual allergens during SIT [134]. Both the success of SIT as well as possible new sensitisations can be detected.

1.9 MECHANISMS OF ALLERGEN-SPECIFIC IMMUNOTHERAPY

The immunological mechanisms behind SIT are complex and still not fully understood. The mechanisms may differ depending on the allergen (venoms or inhalant allergens) and the route of immunisation [160]. Many potential mechanisms have been identified for immunotherapy and it is probable that a combination of several different mechanisms is responsible for the observed effects.

During the initial phase of SIT, increased levels of allergen-specific IgE have been observed [150, 161, 162] but the levels decrease after long-term treatment [150]. Low allergen doses promote development of Th2 cells and thereby support IgE production, whereas high allergen doses suppress IgE production as Th1 cell development is induced [163]. SIT has been shown to be more efficient in allergic patients receiving high allergen doses compared to lower allergen doses [164]. Nevertheless, the risk of allergic side effects limits the possibility to use high therapeutic allergen doses.

The immunoglobulin response is, however, dominated by the induction of high levels of allergen-specific IgG (mainly IgG1 and IgG4) [150, 162]. IgG responses are induced by high doses of antigen, as shown both in a mouse model [165] and in a human study with grass pollen SIT [166]. These IgG antibodies are termed blocking antibodies, as they may directly compete with IgE antibodies for the same binding epitope on the allergen and thereby prevent allergen-induced activation of basophils and mast cells, as shown *in vitro* [150, 162, 166] and *in vivo* in a mouse model [167]. Another explanation for the inhibitory effect of IgG is that FcεRI is co-crosslinked with the FcγRIIb receptor, which is expressed by mast cells and basophils and inhibits the function of FcεRI [168]. Thereby the effector cell activation is downregulated. The blocking IgG antibodies may also inhibit IgE-mediated antigen uptake by APC [169-171], and subsequent presentation of allergens to T cells, which might reduce Th2 activation [169, 170]. The induction of blocking IgG antibodies may thus explain the reduction of both immediate and late-phase reactions, and possibly also the long-term effects observed after injection immunotherapy [172]. In healthy individuals, IgA takes part in the first-line immune response to allergens at mucosal surfaces [173, 174]. Increased levels of allergen-specific IgA have been observed in parallel to IgG4 in human SIT studies with inhalant allergens [166, 173, 174].

SIT has the ability to suppress allergen-induced late phase reactions in different organs [175, 176], which might be due to inhibited cell infiltration. Several findings indicate an important role for T cells in successful immunotherapy. As allergen-specific Th2 cells are thought to be the dominating T-cell subset in allergic diseases, it has been suggested that it would be desirable to shift the allergen-specific immune response from a Th2 to a Th1 response. This immune deviation has been supported as a potential mechanism of SIT by some immunotherapy studies in which a decrease of IL-4 and IL-5 production was accompanied by an increase of IFN-γ [177-179]. The shift from Th2 to Th1 has been suggested to partially be due to induction of apoptosis in allergen-specific Th2 cells [180-182]. Recently, it was found that SIT reduces the number of basophils in blood, which results in reduced production of IL-4 and IL-13 by basophils [183]. It has, however, been questioned whether it is desirable to shift Th2 responses to a strong Th1 response in allergic patients [172]. In mouse studies, Th1 responses have been associated with enhanced susceptibility to allergen sensitisation and aggravated allergic inflammation [184, 185].

During the last decade, much focus has been on the allergen-specific peripheral T-cell tolerance as a key mechanism for a successful outcome of SIT. Subsequently, T_{Reg} cells have been suggested as possible targets for therapy [31]. A suppression of allergen-specific Th1 and Th2 responses has been shown during SIT with allergen extract [173]. The T-cell unresponsiveness was accompanied by a slight increase in the levels of IgG4

[173]. Increased IL-10 production has been suggested to skew the specific antibody response from an IgE to an IgG4-dominated phenotype [171, 186]. IL-10 can also reduce the release of inflammatory cytokines from mast cells and downregulate the function of eosinophils [31]. Furthermore, IL-10 is thought to cooperate with TGF-β in inducing suppression of T-cell responses [173]. SIT has also been associated with enhanced numbers of $CD4^+CD25^+$ T_{Reg} cells, which are thought to be responsible for the increased production of IL-10 [173, 187, 188]. Increased numbers of $Foxp3^+CD4^+CD25^+$ T cells, correlating with clinical efficacy and suppression of allergic inflammation, have been demonstrated in the nasal mucosa after SIT [189]. This further supports the role of T_{Reg} cells in the induction of allergen-specific tolerance. The long-term mechanisms of SIT remain to be elucidated.

1.10 NEW STRATEGIES FOR ALLERGEN-SPECIFIC IMMUNOTHERAPY

The use of pure recombinant allergens in SIT has advantages over the use of crude allergen extracts, but since the recombinant allergens exhibit the allergenic activity of the naturally occurring allergens, they may induce allergic side effects similar to those induced by the allergen extracts [190]. Further improvements of the safety and efficacy as well as the convenience of SIT are crucial. In recent years, several strategies have been developed, of which, however, the majority are so far not in clinical use.

1.10.1 Routes of administration

SIT is traditionally administered by repeated subcutaneous injections of gradually increasing doses of the allergen, which has some limitations. Besides the inconvenience of getting a high number of injections, the treatment period is long (3-5 years), which implies frequent visits to the physician. The efficacy is usually partial and there is a risk of adverse reactions, both of which require continued use of symptom-relieving drugs [191]. This has led to the development of alternative routes of administration. Sublingual immunotherapy (SLIT), in which the allergen is given in high doses as soluble tablets or drops, is to some extent already used in clinical practice [191]. The allergen can be administered at home by the patient and systemic side effects are very rare, but local irritation in the mouth and other mild side effects are common. The costs for the high allergen doses might be limiting for the use of SLIT and only a few long-term follow-up studies have been published to date [192]. Also the nasal mucosa has been utilised in local nasal immunotherapy (LNIT), but the clinical use is declining, mainly due to technical limitations [193]. Recently, a new route of administration, the intralymphatic immunotherapy (ILIT), has been introduced. The allergen is directly injected into the lymph nodes, whereby considerably fewer (3 injections within 4 to 8 weeks) and lower doses of allergen than in traditional SIT are needed to induce tolerance [194, 195].

1.10.2 Adjuvants

An adjuvant is needed in SIT as well as other vaccines to enhance the specific immune response raised by an antigen. The antigen is most commonly adsorbed to aluminium hydroxide (alum), which has a depot effect, resulting in slow release of the allergen at the site of injection, and targets phagocytes [196]. Alum is rather safe and induces

relatively modest tissue reactions. However, it can cause granuloma at the site of injection, and, at least in mice, stimulate Th2 responses and IgE production [196, 197]. There is thus a need of other adjuvants in allergy treatment. As microbial agents have the capacity to activate DC, they have potential of being good adjuvants. Some Th1-inducing microbial agents that have been suggested to improve the efficacy of SIT are CpG-containing oligonucleotides [198], LPS-derived monophosphoryl lipid A [199], the bacterial surface layer proteins SbsC and SbpA [200], and the rhinovirus-derived VP1 surface protein [201]. Most recently, the coupling of bacteriophage Qβ-derived virus-like particles to major cat allergen Fel d 1 (Qβ-Fel d 1) resulted in a highly immunogenic derivative [202]. The coupling strongly reduced the allergenic reactivity of Fel d 1 in mice and in basophils from cat-allergic patients. Treatment of Fel d 1-sensitised mice with a single injection of Qβ-Fel d 1 prevented local and systemic allergic reactions, which was suggested to be mediated by allergen-specific IgG.

Another strategy is to use particulate adjuvants, as they structurally mimic pathogen size and shape and thus enable effective phagocytosis and antigen presentation [203]. A suggested adjuvant for use in SIT is the carbohydrate-based particles (CBP), which are spherical particles of 2 μm in diameter that can be covalently coupled to allergens [204]. CBP coupled to the grass pollen allergen rPhl p 5 induced a mixed Th1/Th2 response, as well as a stronger antibody and cytokine response than unbound rPhl p 5 after subcutaneous (s.c.) injection in mice [204]. CBP coupled to the cat allergen rFel d 1 (CBP-rFel d 1) was taken up by and stimulated a semi-mature state in human monocyte-derived DCs [205]. In mice s.c. injected with radioactively labelled CBP-rFel d 1, the allergen remained at the site of injection for a long time, prolonging the allergen exposure and antigen presentation [206]. Prophylactic and therapeutic treatment with CBP-rFel d 1 prevented and reduced allergic immune responses in mice sensitised to rFel d 1 [206, 207]. Furthermore, CBP did not induce any granuloma formation at the site of injection and no other side effects were observed in treated mice [204, 207].

1.10.3 Hypoallergens

Several approaches for the improvement of SIT intend to genetically modify allergens into hypoallergens, which have a reduced allergenic capacity, but a retained T-cell reactivity [208, 209]. Many allergens occur naturally in various isoforms that differ in only a few amino acid residues. Several isoforms of the major birch pollen allergen Bet v 1 have been identified with varying degrees of IgE reactivity and produced as recombinant proteins [210]. Two of the studied isoforms, Bet v 1.0104 and Bet v 1.1001, showed significantly lower IgE reactivity than the most allergenic isoform, Bet v 1.0101, and the highest levels of IgG4 were detected against Bet v 1.0104 [211]. The hypoallergenic isoforms had poor capability of inducing IgE production and were recognised by cross-reacting IgE induced by Bet v 1.0101. The varying immunogenicity of the isoforms was explained by the amino acid changes between the isoforms being located to the surface of the proteins.

Naturally occurring hypoallergens have only been identified for a few allergen families. Instead, a wide range of approaches are being used to genetically modify recombinant allergens into hypoallergens. Knowledge about the B- and T-cell epitopes as well as the

3D structure is useful when subjecting allergens for genetic engineering. In order to reduce the allergenic activity of allergens, their IgE-binding epitopes have to be changed in such a way that their ability to bind IgE is reduced. Since many IgE-binding epitopes are conformational, most strategies focus on the alteration of the 3D structure of allergens. Simultaneously, the structures inducing allergen-specific IgG should be preserved [208, 209]. The T-cell epitopes should also be retained in order to preserve the immunogenicity of the molecule, which is required for the induction of a beneficial immune response. These properties of hypoallergens may offer a possibility to administer higher allergen doses, thereby permitting fewer injections and enabling induction of allergen-specific unresponsiveness [208]. The use of hypoallergenic allergen derivatives has been suggested not only for treatment of allergic disease but also for prophylactic vaccination before initial sensitisation has taken place [212].

The main approaches used for genetic engineering of allergens into hypoallergens will be described below and are listed in Table 1. Most of the strategies have been used on more than one allergen, but will be exemplified by one allergen. So far, only three genetically modified hypoallergens, all derived from Bet v 1 (Bet v 1 fragments, Bet v 1 trimer and Bet v 1 folding variant) have been evaluated in clinical trials.

Conformational IgE-binding epitopes on allergens can be disrupted by fragmentation. Two large fragments of Bet v 1 showed a reduced *in vitro* IgE reactivity compared to wildtype rBet v 1 and induced proliferation of specific T-cell clones [213]. Oligomerisation of three covalently linked copies of Bet v 1 resulted in a hypoallergen with preserved T-cell as well as IgE-binding epitopes [214]. The allergenic activity was, however, strongly reduced, as shown in basophil degranulation experiments [214]. This was speculated to be due to reorientation or steric hindrance of the IgE epitopes. Both the Bet v 1 fragments and the Bet v 1 trimer showed a largely reduced reactivity in SPT [215] as well as in nasal provocation tests [216] when compared to rBet v 1. In mice and rabbits, the Bet v 1 hypoallergens induced IgG antibodies that blocked human IgE binding to rBet v 1 [214, 217]. In the first clinical immunotherapy trial ever testing hypoallergenic recombinant allergen derivatives, birch pollen-allergic patients were treated with a single course of preseasonal injections of rBet v 1 fragments, rBet v 1 trimer or placebo [218]. Trends towards improvement in the subjects' well-being and clinical symptoms (nasal scores) were obtained, although comparisons with the placebo group did not show significant differences in the main end-point, the combined symptom-medication scores [219]. Active treatment with either one of the

Table 1. Examples of approaches for construction of recombinant hypoallergens

Approach	Allergen	Allergen source	References
Fragmentation	Bet v 1	Birch pollen	[213, 215-220]
Oligomerisation	Bet v 1	Birch pollen	[214, 215-220]
Folding variant	Bet v 1	Birch pollen	[222, 223]
Mosaic	Phl p 2	Timothy grass pollen	[224]
Deletion	Phl p 6	Timothy grass pollen	[225]
Hybridisation	Phl p 1, 2, 5, 6	Timothy grass pollen	[226]
Point mutations	Lep d 2	Storage mite	[228-230]
DNA shuffling	Lep d 2, Gly d 2	Storage mite	[231]

hypoallergens reduced the immediate skin reactions to Bet v 1, and induced high levels of Bet v 1-specific IgG that inhibited *in vitro* basophil degranulation [218, 220]. Both treatments induced IgG4 and IgE directed against new epitopes on Bet v 1, not recognised by untreated birch-allergic patients [221]. These immunoglobulins are nevertheless considered to compete with allergic patient's IgE binding to the natural Bet v 1. In addition, a reduced Th2 cytokine response was observed after treatment in peripheral blood mononuclear cells (PBMC) from the trimer group [220].

In an attempt to disrupt the conformation of the allergen, but to leave the primary sequence and thereby T cell epitopes intact, a folding variant of rBet v 1 (rBet v 1-FV) was recently made [222]. The rBet v 1-FV showed reduced allergenic activity and preserved T cell-stimulating capacity. Furthermore, this hypoallergen induced high titres of Bet v 1-specific IgG in mice, but it remains to be investigated if the IgG can act as blocking antibodies. In a similar clinical approach as for the Bet v 1 fragments and trimer, the rBet v 1-FV was given to birch pollen-allergic patients as s.c. injections in pre-seasonal SIT [223]. In the first year, the hypoallergen treatment was well tolerated and clinically efficacious compared to the control treatment with birch pollen extract. After a second pre-seasonal course of treatment, the effect remained, but was the same for the extract-treated group. Both treatment groups got comparably high levels of Bet v 1-specific IgG1 and IgG4.

Another allergen that has been subjected to fragmentation is the major timothy grass pollen allergen Phl p 2. Three hypoallergenic peptides covering the entire Phl p 2 were obtained by fragmentation, but these were reassembled in a new order into a single rPhl p 2-derived mosaic protein [224]. The conformation of IgE epitopes was disrupted, which resulted in a reduced IgE reactivity and allergenic activity of the mosaic protein compared to Phl p 2. IgG antibodies from mice induced by the Phl p 2 mosaic protein cross-reacted with other grass species and inhibited binding of Phl p 2-specific IgE from grass-pollen allergic patients. The T-cell reactivity remains to be investigated.

Deletion mutants of yet another major timothy grass pollen allergen, Phl p 6, were produced by *E. coli* expression or chemical synthesis of fragments comprising amino acids (aa) 1-33, 1-57 and 31-110 [225]. The fragment aa 31-110, which lacked two α-helices, showed a strong reduction in IgE reactivity and allergenic activity compared to Phl p 6. It was the only mutant that induced Phl p 6-specific IgG upon immunisation of mice and rabbits, and these IgG inhibited binding of patients' IgE to Phl p 6.

The four major timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5 and Phl p 6 have been produced as recombinant hybrid molecules rP2-P6, rP6-P2 and rP5-P1 [226]. The hybrid molecules bound allergic patients' IgE equally well and induced stronger lymphoproliferation than the respective single allergen. Furthermore, hybrid immunisation in mice induced IgG antibodies that inhibited patients' IgE to the specific allergens and to grass pollen extract. As the hybrid molecules contain most of the B- and T-cell epitopes in grass pollen extract, it was suggested that a combination vaccine of these hybrids could suit treatment of the majority of grass pollen-allergic patients.

An additional hybrid molecule, P6-2 [227] was constructed by fusing two hypoallergens, the Phl p 2 mosaic protein [224] and the deletion mutant Phl p 6 (aa 31-

110) [225]. The IgE reactivity to the hP6-2 hybrid was strongly reduced [227] compared to the P6-P2 hybrid made previously [226]. Like the single components of hP6-2 [224, 225], the hybrid lacked allergenic activity and induced T-cell proliferation [227]. Immunisation of rabbits with hP6-2 induced higher levels of IgG than the hypoallergenic components alone, and these IgG antibodies recognised rPhl p 2 and rPhl p 6. The rabbit anti-hP6-2 IgG inhibited patients' IgE binding to rPhl p 2 and rPhl p 6.

So far, the most widely used strategy to make hypoallergens has been to introduce point mutations to recombinant allergens by site-directed mutagenesis. Site-directed mutagenesis has been used to change single amino acids within the IgE-binding epitopes of at least ten allergens, producing derivatives with reduced IgE-binding capacities. In addition, the protein folding and most probably the conformation of IgE-binding epitopes may be changed e.g. by disruption of disulphide bridges, which is achieved by mutations in cysteine codons. For this reason, site-directed mutagenesis has been applied on major mite allergens, e.g. Lep d 2, which resulted in derivatives with various degrees of reduction in IgE-binding capacity [228]. The derivative with all six cysteines changed to serines, Lep d 2.6Cys, resulted in lowest IgE reactivity [228], and the majority of patients were negative for Lep d 2.6Cys in SPT [229]. The same patients' T-cell reactivities to the hypoallergen were comparable to the unmodified Lep d 2 [230].

The knowledge about protein structure and B- and T-cell epitopes is limited for many allergens. In those cases, DNA shuffling has been suggested as a useful tool to introduce mutations into allergens and then screen a large number of genes for desired hypoallergenic properties. DNA shuffling was applied on genes encoding two isoforms of Lep d 2 (Lep d 2.01 and Lep d 2.02) and the cross-reacting mite allergen Gly d 2 from *G. domesticus* [231]. The mite group 2 library was subjected to selection for full-length, high-expressing clones and screening for low IgE-binding in sera from mite-allergic patients [231]. Two clones with reduced IgE reactivity and the most frequent T-cell proliferation (L4 and R2) were further characterised. These hypoallergens induced IgG antibodies in immunised mice with capacity to block binding of IgE specific for both Lep d 2 and Gly d 2.

1.10.4 Allergen-derived peptides

Allergen-derived peptides that contain either T-cell or B-cell epitopes can be produced by peptide synthesis. T-cell epitope-containing peptides are thought to induce unresponsiveness in allergen-specific T cells without causing immediate type reactions, as short peptides lack the ability to cross-link FcεRI-bound IgE. Due to the diversity of MHC II molecules that recognise different T-cell epitopes, a large number of T-cell peptides may be needed for SIT. SIT with peptides containing T-cell epitopes of the major bee venom allergen Api m 1 (phospholipase A2) has so far proven safe in small clinical trials, which resulted in reduced PBMC proliferation and Th1/Th2 cytokine production, and increased levels of IL-10 and allergen-specific IgG4 [232-234]. More extensive studies have been performed with T cell peptides of Fel d 1, with somewhat contradictory results. SIT with two synthetic peptides (27 amino acids in length), containing several dominant T-cell epitopes of Fel d 1,

reduced the allergic responses in many cat-sensitive patients receiving high doses of peptides [235-237]. The treatment was, however, associated with a relatively high frequency of adverse events [235-237]. The peptides used in these studies were thought to be large enough to elicit IgE responses, and were thus followed by trials with a mixture of 12 shorter overlapping Fel d 1 T-cell peptides (16-17 amino acids in length) [238]. Intradermal injections initially provoked late-phase reactions in cat-allergic asthmatics, but subsequent injections induced tolerance to cat dander [238, 239]. The treatment also showed promising results on clinical outcomes in the upper and lower airways [239]. When repeatedly administered by inhalation, the same peptides failed to induce tolerance [240]. Immunotherapy with the Fel d 1 peptides in both man and mouse was shown to reduce T cell responses to other Fel d 1 epitopes not covered by these peptides by so-called linked epitope suppression [241].

Synthetic peptides derived from B-cell epitopes of allergens contain solvent-exposed amino acids, but lack secondary structure and are aimed at the induction of blocking IgG antibodies. B-cell peptides of the major allergens of timothy grass pollen (Phl p 1) as well as birch pollen (Bet v 1) lacked IgE-binding capacities and failed to elicit allergic reactions in humans [167, 242]. Phl p 1 peptide immunisation in mice induced IgG antibodies that reacted with the complete Phl p 1 and cross-reacted with group 1 allergens from other grass species [242]. Prophylactic vaccination of mice with Bet v 1 peptides induced Bet v 1-specific blocking IgG and prevented sensitisation to Bet v 1 [167].

1.10.5 Fusion proteins targeting immunoglobulin receptors

Saxon and co-workers have developed an approach for SIT by targeting the receptor FcγRIIb, which is expressed by mast cells and basophils and inhibits the function of FcεRI. A chimeric human Fcγ-allergen protein, GFD, was designed by joining the Fcγ1 part of human IgG with Fel d 1 through a flexible linker (Fig. 4) [243]. Fel d 1 binds to specific IgE on FcεRI, which contains immunoreceptor tyrosine-based activation motifs (ITAMs). The Fcγ part of GFD coaggregates FcγRIIB, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that inhibits the FcεRI signalling. This was demonstrated by an inhibition of mediator release from Fel d 1-sensitised human basophils and cord blood-derived mast cells by incubation with GFD [243]. Furthermore, therapeutic treatment with GFD inhibited Fel d 1-induced allergic asthmatic symptoms in Fel d 1-sensitised mice [244]. It remains to be investigated how GFD affects T cells and whether tolerance can be induced by GFD treatment.

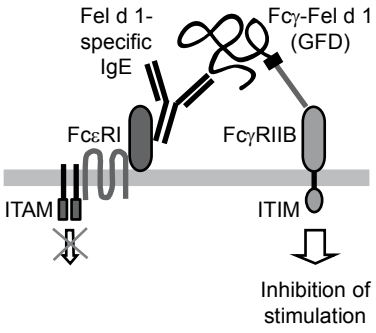

Figure 4. The Fcγ-Fel d 1 fusion protein (GFD). Fel d 1 is joined to the Fcγ part of human IgG through a linker. Fel d 1 binds specific IgE on FcεRI, but the activating signalling is inhibited as the Fcγ part binds to FcγRIIB, which contains an inhibitory ITIM motif. ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif

Fel d 1 has furthermore been linked to a fragment of the variable region of the humanised anti-CD64 monoclonal antibody, H22, which binds to the high affinity IgG receptor FcγRI (CD64) on APC [245]. One of the functions of FcγRI is to facilitate antigen presentation. It was shown i*n vitro* that H22-Fel d 1 stimulated DC to a tolerogenic phenotype and induced different IL-10-producing T cell subtypes [246].

1.10.6 Fusion proteins targeting MHC class II pathway

Recently, it was suggested that the MHC class II pathway of antigen presentation can be targeted to increase the allergen presentation to T cells [247]. The T cell responses could thereby be changed more efficiently during SIT without increasing the dose of allergen. By the so-called modular antigen translocation (MAT) technology, His-tagged allergens (Bet v 1, Api m 1, Der p 1 or Fel d 1) were fused to a transactivator of transcription (TAT) peptide, which converts extracellular into cytoplasmic protein, and a truncated invariant chain (Ii) peptide that targets the protein to endosomal/lysosomal compartments (Fig. 5) [247]. The association between MHC II and antigenic peptides can occur in endosomes after proteolytic degradation of Ii that prevented peptide binding to MHC II. A 10-100 fold lower dose of MAT-fusion protein than control allergen was needed for induction of T-cell proliferation from PBMC of allergic patients. The cytokine response shifted from Th2- to Th1-type and to production of IL-10. Immunisation of mice by intralymphatic (i.l.) injections of MAT-Fel d 1 induced higher levels of IgG2a, as well as IL-2 and IFN-γ from T cells, compared to rFel d 1 [248]. Cat-sensitised mice treated i.l. by MAT-Fel d 1 showed stronger protection against anaphylaxis than rFel d 1-treated mice. Furthermore, MAT-Fel d 1 reduced human basophil degranulation to a larger degree than rFel d 1. Recently, the first clinical trials with MAT-Fel d 1 were performed by using ILIT, and the results are under evaluation [249].

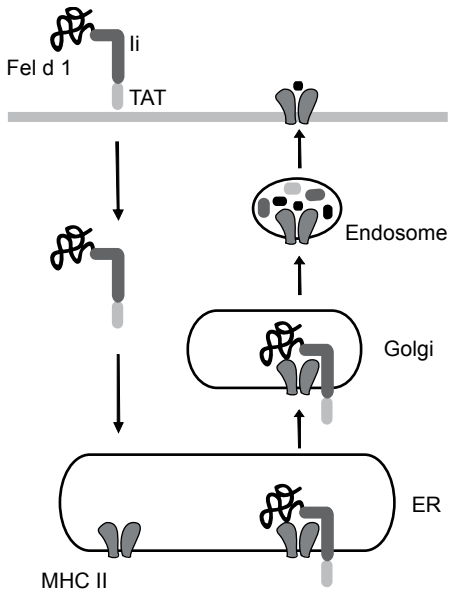

Figure 5. The MAT-Fel d 1 fusion protein. Fel d 1 is fused to a transactivator of transcription (TAT) peptide via a truncated invariant chain (Ii) peptide. TAT converts extracellular proteins into cytoplasmic. Ii prevents peptide binding to MHC II and targets endosomes. When the fusion reaches endosomes, Ii is degraded and MHC II can associate with allergen-derived peptides. (Modified from [247]).

2 AIMS OF THE THESIS

The general aim of the thesis was to obtain new tools for diagnosis and treatment of allergic disease. Molecular techniques were used for cloning and genetic modification of inhalant allergens, which were produced as recombinant proteins. The outcome was evaluated by different *in vitro* methods and *in vivo* in a mouse model for allergy.

The specific aims were:

Paper I: to clone and characterise new allergens from the storage mite *L. destructor* in order to increase the repertoire of recombinant allergens aimed for a more precise mite allergy diagnosis

Paper II: to investigate if the single major cat allergen rFel d 1 could replace CDE in the diagnosis of cat allergy and if IgE to rFel d 1 is a better marker for early cat sensitisation than IgE to CDE

Paper III: to apply a rational approach for genetic construction of hypoallergens of Fel d 1, and to evaluate these *in vitro* for use in allergen-specific immunotherapy

Paper IV: to evaluate the therapeutic potential of one of the rFel d 1 hypoallergens from paper III in a mouse model for cat allergy

3 MATERIALS AND METHODS

This section summarises the materials and methods used in this thesis with reference to the papers where they are applied. A more detailed description is found in the materials and methods section of each paper. In addition, the diagnostic tools used in this thesis are described in section 1.7.1.

3.1 SUBJECTS

The subjects in this thesis were patients allergic to mites [**I**], crustaceans [**I**] or cat [**II-IV**], as well as non-allergic healthy controls [**I-IV**]. In **papers I-IV**, serum samples were used to measure allergen-specific IgE. In **paper III**, heparinised blood was used for proliferation of PBMC and activation of basophils. In **paper IV**, subjects were tested for SPT reactivity. The studies were approved by local Ethics Committees in Stockholm, Sweden [**I-IV**] and Wroclaw, Poland (SPT) [**IV**].

In **paper II**, the subjects were selected from the ongoing BAMSE study (Children, Allergy, Milieu, Stockholm, Epidemiological survey), which has a prospective birth cohort design to assess risk factors for asthma and other allergic diseases in early childhood [250]. The study base constitutes 4089 unselected newborns recruited in Stockholm, Sweden, between 1994 and 1996. On the basis of parental questionnaire data, children with certain or suspected symptoms of asthma or rhinoconjunctivitis due to cat at 4 and/or 8 years of age were selected for **paper II**. Blood samples were drawn from these children at both 4 and 8 years of age.

3.2 MICE

In **paper IV**, female BALB/c mice were used in immunisation and treatment protocols. The mice (6-8 weeks of age) were obtained from Charles River (Sulzfeld, Germany) and housed with food and water ad libitum. The experiments were approved by the Swedish local ethics committee for animal welfare.

3.3 METHODOLOGY

Airway hyperreactivity (AHR) measurements [**IV**]	AHR was induced by intravenous (i.v.) injection of methacholine and respiratory resistance was measured in a small animal ventilator (FlexiVent®).
Bronchoalveolar lavage (BAL) [**IV**]	Lungs of mice were lavaged with PBS to collect pulmonary cells and BAL fluid.
Cytometric bead array (CBA) [**IV**]	Cytokines from cell culture supernatants were captured on a set of beads, which were analysed by flow cytometry.
Cloning of recombinant allergens [**I, III**]	The full-length allergen-coding sequences were amplified from cDNA by PCR, subcloned into plasmids for verification by DNA sequencing, and further cloned into high-level expression vector pET17b [**I**] or pET20b [**III**].
Differentiated cell count [**IV**]	BAL cells were cytospun and stained with May-Grünwald-Giemsa for differentiated counting of eosinophils, neutrophils, lymphocytes and macrophages.
ELISA	
Blocking [**IV**]	To allow IgG to block binding of IgE to Fel d 1, Fel d 1-bound wells were preincubated with sera from hypoallergen-treated mice before incubation with sera from cat-allergic patients.
Competition [**III**]	To allow two antigens to compete for binding to IgE, serum was preincubated with serial dilutions of a competitor prior to incubation in the well that was coated with Fel d 1.
Quantitative [**II**]	Serial dilutions of plasma from a cat-allergic patient were used in Fel d 1-coated wells to set calibration points for measurement of Fel d 1-specific IgE.
Immunisation, sensitisation and treatment of mice [**IV**]	Antigen was s.c. injected in the neck.
Immunoblot inhibition [**I**]	To compare the binding affinity to IgE, sera were preincubated with an antigen before incubation with another antigen immobilised on a membrane.

Intranasal allergen challenge of mice [**IV**]	Mice were anaesthetised with 3.5% isoflurane before intranasal (i.n.) application of CDE.
PBMC or splenocyte proliferation assays [**III, IV**]	PBMC and splenocytes were prepared from human peripheral blood and mouse spleens, respectively. T-cell proliferation was measured in antigen-stimulated cell cultures by incorporation of [^{3}H]-thymidine.
Phage display library and biopanning [**I**]	A phage display cDNA library was constructed from *L. destructor* mRNA by using the pJuFo vector. IgE-binding proteins displayed on phages were screened with sera from *L. destructor*-sensitised individuals.
Preparation of allergen extract and native allergen [**I**]	Whole *L. destructor* culture was extracted in 0.15 M NaCl, and the supernatant was desalted and lyophilised. Native tropomyosin was enriched from the extract by repeated rounds of precipitation at its isoelectric point.
Production of recombinant allergens [**I-IV**]	Expression vectors containing allergen-encoding DNA were transformed into BL21 *E. coli* expression strains. Recombinant allergens were expressed as His-tagged proteins.
Protein analysis [**I-IV**]	Protein concentration was measured by spectrophotometry at 280 nm [**I**], by BCA™ protein assay [**II-IV**] or total amino acid composition analysis [**III**]. The molecular masses of proteins were determined by SDS-PAGE [**I, III**] or mass spectrometry [**I**].
Protein purification [**I-IV**]	Proteins were mostly found in inclusion bodies that were solubilised in Guanidine-HCl. Proteins were purified by immobilised metal chelate affinity chromatography (IMAC) [**I-IV**], ion exchange chromatography [**II, IV**] and size exclusion chromatography [**II-IV**].
Site-directed mutagenesis [**III**]	Point mutations were introduced via primers to change cysteine codons to serine codons.

4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION AND PRODUCTION OF TWO NEW PUTATIVE RECOMBINANT ALLERGENS FROM *LEPIDOGLYPHUS* DESTRUCTOR [I]

Tropomyosin is an important cross-reactive allergen found in many species of e.g. crustaceans and mites [73]. It has earlier been identified and produced as a recombinant allergen from the mites *Dermatophagoides farinae* (*D. farinae*) [251], *D. pteronyssinus* [252] and *Blomia tropicalis* (*B. tropicalis*) [253], and recently from the storage mite *T. putrescentiae* [254]. However, no tropomyosin-homologous clone was identified during the biopanning for IgE-binding proteins in a phage display *L. destructor* cDNA library, in which three allergens (Lep d 5, 7 and 13) were previously identified [91].

In an attempt to directly search for tropomyosin in *L. destructor*, we designed primers corresponding to the conserved 5'- and 3'-regions of the house dust mite tropomyosins Der f 10 and Der p 10. PCR amplification from the phage display *L. destructor* cDNA library, using the tropomyosin primers, resulted in a sequence encoding 284 amino acids with a calculated molecular mass of 32 930 Da. The amino acid sequence contained two tropomyosin-like motifs and two regions that are suggested to be IgE-binding regions in the shrimp tropomyosin Pen a 1 [255]. Based on a high amino acid sequence homology (96% identity) to both Der f 10 and Der p 10, and in accordance with the WHO/IUIS Allergen Nomenclature Sub-Committee, the *L. destructor* tropomyosin was named Lep d 10 [53].

Biopanning of the phage display *L. destructor* cDNA library revealed yet another clone encoding an IgE-binding protein. The clone was a partial nucleotide sequence of 370 bp, showing homology (up to 86% identity) to α-tubulin of many organisms. The full-length cDNA sequence spanning 1350 bp was obtained by RACE-PCR. It resulted in an open reading frame of 450 amino acids, corresponding to a calculated molecular mass of 50 007 Da. The amino acid sequence showed up to 94% identity to α-tubulins from other organisms, and contained a tubulin pattern as well as one N-glycosylation site.

The cDNAs encoding Lep d 10 and *L. destructor* α-tubulin were expressed as His_6-tagged recombinant proteins in *E. coli* and purified by IMAC. The calculated molecular masses of approximately 34 kDa (rLep d 10) and 51 kDa (recombinant α-tubulin) were confirmed by mass spectrometry. SDS-PAGE showed somewhat higher molecular weights (Fig. 6), which possibly could be explained by the repetitive, non-globular structure of the proteins.

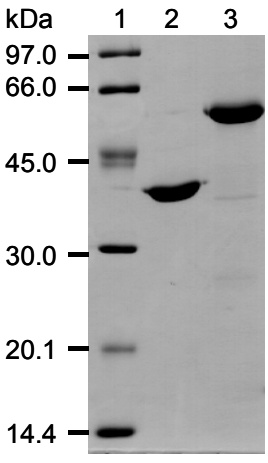

Figure 6. SDS-PAGE analysis of purified recombinant mite allergens. Lane 1: molecular weight marker; lane 2: rLep d 10; lane 3: recombinant *L. destructor* α-tubulin.

In immunoblotting experiments, rLep d 10 was recognised by serum-IgE of 13% (18/136) of subjects ImmunoCAP-positive to mites and/or crustaceans. As expected from the high sequence homology between Lep d 10 and Der p 10, a monoclonal anti-Der p 10 antibody, 1A6 [256], could recognise rLep d 10. The mAb 1A6 also recognises tropomyosins from shrimp [256], snail [257], and cockroach [258]. All of the 18 sera that detected rLep d 10 were ImmunoCAP-positive to shrimp (*P. borealis*), 13 were ImmunoCAP-positive to *D. pteronyssinus* and 7 were ImmunoCAP-positive to *L. destructor*. The characteristics of the sera detecting rLep d 10 and the detection by the anti-Der p 10 mAb demonstrate a cross-reactivity of Lep d 10 to other invertebrate tropomyosins. The importance of taking the cross-reactivity of tropomyosins into consideration has been highlighted in immunotherapy studies using whole mite extract. During immunotherapy with *D. pteronyssinus* and *D. farinae* extracts, IgE-reactivity was induced or increased against snail and shrimp [149, 259, 260]. In one of the studies, tropomyosin was identified as one of the cross-reactive allergens involved [149]. Therefore, it is of importance to diagnose IgE-reactivities to tropomyosins before using crude allergen extracts from tropomyosin-containing species in SIT. It is possible that all tropomyosin allergens are cross-reactive and therefore a positive IgE-reaction to any tropomyosin could predict cross-sensitivities to a variety of allergen sources. This is important to know, not only when considering treatment with SIT, but also when advising the patient of which allergen sources should be avoided. The fact that all rLep d 10-positive sera were ImmunoCAP-positive to shrimp, does not necessarily mean that all the patients are sensitised to shrimp. The IgE antibodies in some of the sera might originate from sensitisation to a tropomyosin from another allergen source, such as a mite species.

The results of the immunoblot inhibitions where rLep d 10 was compared to nLep d 10 suggest that rLep d 10 is correctly folded in the bacterial expression system used in this study. The fact that a tropomyosin clone was not found during the biopanning is therefore probably not due to incorrect folding of Lep d 10 on the phage. Instead, it is likely that the sera used for biopanning lacked sufficient amount of allergen-specific IgE to detect Lep d 10 displayed on the phage in the library.

In immunoblotting analysis, the recombinant α-tubulin was recognised by the anti-α-tubulin mAb DM 1A (Sigma-Aldrich Inc., Saint Louis, Mo., USA). Immunoblotting with sera from subjects ImmunoCAP-positive to mites and/or crustaceans showed an IgE-binding frequency of 12% (11/95) to recombinant α-tubulin. Several of these *L. destructor* α-tubulin-positive sera were ImmunoCAP-negative to *L. destructor*, but ImmunoCAP-positive to *D. pteronyssinus* and/or crustaceans, implying that α-tubulin is a cross-reactive allergen. Recently, α-tubulin was identified as a potential allergen in *T. putrescentiae*, with 97.3% sequence identity to *L. destructor* α-tubulin and an IgE-binding frequency of 29.3% in sera from storage mite-allergic subjects [261]. Furthermore, α-tubulin was one of 140 IgE-binding self-antigens found in a phage display human cDNA library, and showed IgE-binding in 21.7% of patients with atopic eczema [262].

Our results from immunoblot analysis, clearly indicating α-tubulin as an IgE-binding protein, were not supported by the immunoblot inhibition experiments. The IgE-binding to recombinant α-tubulin blotted on the membrane could not be inhibited by

recombinant α-tubulin or by *L. destructor* whole extract. Alpha-tubulin was very weakly detected in the *L. destructor* whole extract blotted on membrane, probably due to a very low concentration of this protein. Thus, it was not possible to interpret the results from the inhibition experiment with membrane-bound extract. These results could be due to different IgE-binding epitopes being exposed or masked when the protein is in solution compared to when it is immobilised on membrane. IgE-binding epitopes might also be hidden in the native α-tubulin in the whole extract, as it forms dimers with β-tubulin [263]. Furthermore, sequence analysis of the full-length α-tubulin revealed the presence of one N-glycosylation site. Since no post-translational modifications are made in the *E. coli* expression system, the lack of sugar moieties might influence the protein folding, and hence the IgE-reactivity, of the recombinant α-tubulin.

4.2 RECOMBINANT FEL D 1 IN THE DIAGNOSIS OF CAT SENSITISATION [II]

The major cat allergen Fel d 1 has earlier been constructed as a recombinant protein with biochemical, immunological and biological properties mimicking those of natural Fel d 1 [125, 126]. Today, sensitisation to cat is still most commonly diagnosed by the use of cat dander extract (CDE). In this study, rFel d 1 was compared to CDE in the diagnosis of cat allergy and in the use as an early marker of sensitisation. The IgE reactivity to rFel d 1 and CDE was analysed in blood samples from children belonging to the large prospective birth cohort BAMSE [250]. Based on parental questionnaires, children with symptoms of asthma or rhinoconjunctivitis due to cat at 4 and/or 8 years of age, or where such symptoms were suspected, were selected for this study. Of these children, 144 had blood samples available at both time points and comprised the three study groups *Certain cat allergy at 4* (CA4), *Suspected cat allergy at 4* (SA4), and *New cat allergy at 8* (NA8) including 33, 42 and 69 children, respectively. For control purposes, 104 children at age 4 and 135 separate children at age 8 years, who were reported to not have any certain or suspected symptoms in contact with cat were randomly selected from the BAMSE cohort. Of the 4 and 8 year controls, seven (6.7%) and sixteen (12.0%), respectively, were scoring positive in Phadiatop®, which is an ImmunoCAP containing a mix of common inhalant allergens (cat, dog, horse, birch, timothy, mugwort, house dust mite (*D. pteronyssinus)* and mold.

IgE antibodies to CDE were analysed by the ImmunoCAP system by using the cut-off limit 0.35 kU_A/L. A quantitative ELISA was developed to measure IgE to rFel d 1 with the cut-off limit for positive samples set to 0.037 kU_A/L. Among the 4 and 8 year controls, 5/104 (4.8%) and 6/135 (4.4%) children, respectively, had detectable IgE levels to rFel d 1.

A high correlation between the IgE levels to rFel d 1 and CDE among all subjects was observed (r_s=0.94, $p<0.001$). Therefore, comparisons of IgE levels within and between the different groups could be focused on rFel d 1-specific IgE. Children in the CA4 group had the earliest establishment of both rFel d 1-specific IgE and allergic symptoms. In most children, the IgE levels did not change between 4 and 8 years of age

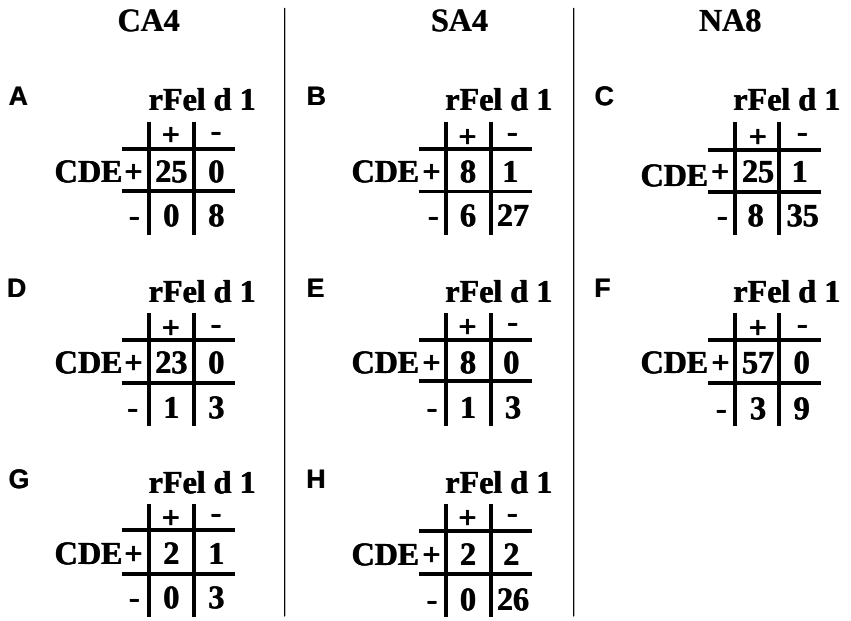

Figure 7. Number of children in the study groups with and without IgE to rFel d 1 and cat dander extract (CDE). A-C, 4 year samples from all children in the respective study group. 8 year samples from **D-F**, children with certain symptoms related to asthma or rhinoconjunctivitis in contact with cat at 8 years and from **G-H**, children with no reported symptoms related to asthma or rhinoconjunctivitis in contact with cat at 8 years. CA4, Certain cat allergy at 4 (*N* = 33); SA4, Suspected cat allergy at 4 (*N* = 42); NA8, New cat allergy at 8 (*N* = 69)

and the symptoms persisted in 27 out of 33 children at age 8 years. All children in the CA4 group that had IgE to CDE at age 4 years (25/33) also had IgE to rFel d 1 (Fig. 7A), and similar results were seen at age 8 years (26/33) (Fig. 7D, G).

Compared to the CA4 group, children in the SA4 group were less frequently sensitised to rFel d 1 and/or CDE both at 4 and 8 years of age. However, more children had IgE to rFel d 1 (14/42) than to CDE (9/42) at age 4 years (Fig. 7B). At age 8 years, 11 and 12 out of the 42 children had IgE to rFel d 1 and CDE, respectively (Fig. 7E, H). Neither in this group did the IgE levels to rFel d 1 change between 4 and 8 years of age. In the entire SA4 group, it was much less common than in the CA4 group to have symptoms at age 8 years. However, nearly 30% were certain of symptoms at age 8 years. Thus, IgE to rFel d 1 could be used as a marker to aid parents and clinicians to resolve suspicion of cat allergy and true sensitisation. Detection of early sensitisation may be useful since the development of further disease may be prevented e.g. by avoidance of the allergen source or premedication before being exposed to the allergen source. In some cases the possibility of SIT might be taken into consideration.

In the NA8 group, in which no children had reported suspicion or certainty of symptoms in contact with cat until between 4 and 8 years of age, nearly 50% were already sensitized to cat at 4 years of age. Like in the SA4 group, the children in the NA8 group were more frequently sensitized to rFel d 1 (33/69) than to CDE (26/69) at this age (Fig. 7C). All children with IgE to rFel d 1 or CDE at age 4 years were also IgE positive at age 8 years. At 8 years of age, when all children in the NA8 group reported symptoms, almost 90% had IgE to rFel d 1 (60/69) and a few less to CDE (57/69) (Fig. 7F). In the entire NA8 group, the IgE levels to rFel d 1 were high already before the

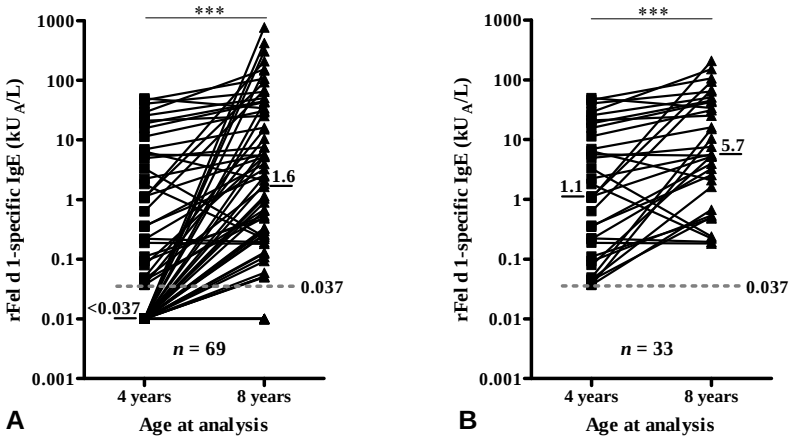

Figure 8. IgE levels to rFel d 1 in the NA8 group. Levels of IgE to rFel d 1 were compared between 4 and 8 years **A**, among all children in the NA8 group and **B**, among children in the NA8 group with IgE to rFel d 1 at 4 years. (*** p<0.001). Dotted line indicates the cut-off limit and horizontal lines show median values. NA8, New cat allergy at 8

symptoms were observed, and still a significant increase in the IgE levels was observed from 4 to 8 years of age (Fig. 8A). Interestingly, when only children in the NA8 group with IgE antibodies to rFel d 1 already at age 4 years were included, a significant increase from age 4 to 8 years remained (Fig. 8B). Children with IgE but without symptoms to cat at 4 years of age all developed allergic symptoms at 8 years of age, suggesting that early detection of cat sensitisation is important. Why symptoms were not suspected or observed at 4 years of age in the NA8 group despite the high levels of IgE antibodies to rFel d 1 and CDE could depend on the fact that cat allergens are found all over public places and it may hence be difficult to recognize the connection between cat exposure and possible symptoms to cat [264-266]. We observed that children within the NA8 group who were sensitised to cat already at age 4 years were more often sensitised to other inhalant allergens than those who were not sensitised to cat (data not shown). However, not all of them were reported to have symptoms to the other inhalant allergens either. These children may be in the beginning of their "allergic march" and may develop symptoms later on. Previous studies have shown that IgE antibodies to individual allergens often appear before the symptoms to the allergens [267]. The presence of sensitivities and symptoms to other allergen sources should be a strong indicator for evaluating cat allergy at an early age.

At 4 years of age, rFel d 1 and CDE were equally good at predicting allergic symptoms to cat among the 144 subjects at 8 years of age, while at 8 years of age, rFel d 1 was better than CDE in the classification of symptoms at the same age.

The cut-off limit of our quantitative ELISA was about ten times lower (0.037 kU_A/L) than the globally used limit of the ImmunoCAP system (0.35 kU_A/L), the most common system worldwide for measurement of IgE antibodies. In our study, the low cut-off limit for IgE to rFel d 1 resulted in a higher sensitivity for rFel d 1 compared to CDE. With a few exceptions, these children had IgE levels to rFel d 1 below 0.35 kU_A/L, and most often just above 0.037 kU_A/L. This highlights the usefulness of rFel d 1 and a sensitive ELISA in early diagnosis of sensitisation to cat. One explanation for the high sensitivity is that more IgE antibodies may be detected to the single Fel d 1

allergen than to CDE, since Fel d 1 represents only about 1% of the protein content in the extract, as determined by ELISA [Dr T. Neimert-Andersson, personal communication]. Because of the dominance of IgE antibodies to Fel d 1, it may be argued that addition of minor allergens, such as cat cystatin, albumin or lipocalin will not improve the diagnostic efficacy. On the other hand, it can not be ruled out that addition of some allergens may improve the diagnostics of cat allergy in occasional cases. Our results also indicate that although most children with symptoms to cat have IgE to CDE at 8 years of age, even more children can be classified with symptoms by measuring IgE to rFel d 1. Already at 4 years of age, IgE to rFel d 1 was equally good as IgE to CDE in predicting symptoms to cat at 8 years of age. These results further emphasize the usefulness of the single Fel d 1 allergen in the diagnosis of cat allergy.

4.3 CONSTRUCTION AND *IN VITRO* CHARACTERISATION OF FEL D 1 HYPOALLERGENS [III]

In order to make SIT more safe and efficient, we used the major cat allergen Fel d 1 as a model for a rational design of hypoallergens. In total, seven hypoallergen candidates were created by systematic engineering of rFel d 1 (Table 2). Genetic modifications were made by introducing duplications of T-cell epitopes (DTE) and point mutations. First, the new concept of DTE was applied in order to preserve the T-cell reactivity and to simultaneously alter the 3D structure of Fel d 1, thereby reducing the allergenic activity. We chose to duplicate the sequences encoding amino acid residues 23-30 on chain 1 and 46-53 on chain 2 (Fig. 9A), which are parts of epitopes that have earlier been shown to induce frequent T-cell responses in a panel of Fel d 1-reactive T-cell lines [123]. The two sequences selected for DTE are parts of solvent-exposed loops between helix 2 and 3 of chain 2, and helix 6 and 7 of chain 1 (Fig. 9C) [126], known to harbour IgE-binding epitopes [124]. By PCR, we applied DTE separately on synthetic genes of Fel d 1 chain 1 and chain 2, which were subsequently joined to each other to form rFel d 1 (DTE) (Fig. 9A). In a

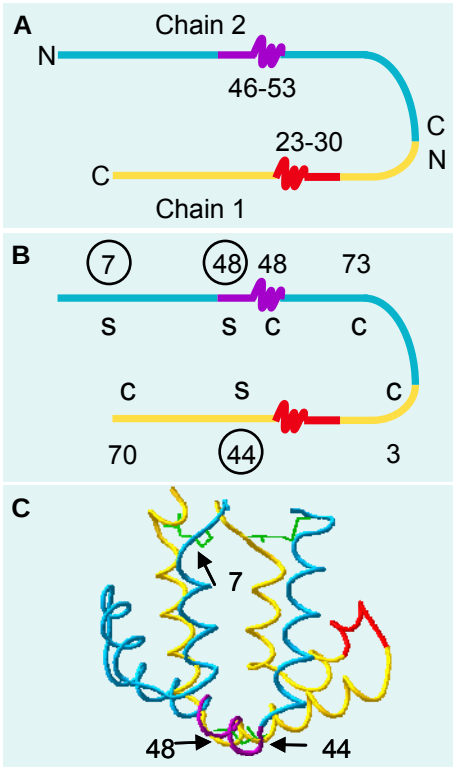

Figure 9. Rational design of hypoallergens by the use of DTE and point mutations. A, The amino acids 46-53 on chain 2 and 23-30 on chain 1 were duplicated, and the two chains were joined to each other. **B**, Cys44 on chain 1, and Cys7 and Cys48 on chain 2 (only Cys48 in the parental sequence) were changed to serines. The construct with three cysteine skin to introduce the allergen into the epidermis ADDIN EN.CITE <EndNote><Cite><Author>Douglass</Author><Year>2006</Year><RecNum>47</RecNum><record><rec-number>47</rec-number><foreign-keys><own in red and purple, respectively. The three disulphide bonds that link chain 1 and 2 are displayed in green, and cysteine residues subjected to mutagenesis are indicated by their respective numbers. DTE, duplication of T cell epitopes

Table 2. Fel d 1 derivatives

Name	Modification
rFel d 1 (I)	C44S chain 1
rFel d 1 (II)	C44S chain 1, C48S chain 2
rFel d 1 (III)	C44S chain 1, C48S chain 2, C7S chain 2
rFel d 1 (DTE)	DTE
rFel d 1 (DTE I)	DTE, C44S chain 1
rFel d 1 (DTE II)	DTE, C44S chain 1, C48S chain 2
rFel d 1 (DTE III)	DTE, C44S chain 1, C48S chain 2, C7S chain 2

second step, disulphide bonds in rFel d 1 and rFel d 1 (DTE) were disrupted. This was achieved by changing cysteine residues to serines. Point mutations were made by site-directed mutagenesis in the cysteine codons at position 44 on chain 1, and at positions 7 and 48 on chain 2 (Fig. 9B). Hence, molecules with one (Fel d 1 (I)), two (Fel d 1 (II)) or three (Fel d 1 (III)) cysteines replaced by serines were created, generating six point-mutated derivatives in total (Table 2). In three of the derivatives point-mutations were combined with DTE and one additional was modified by DTE only.

The IgE-binding reactivities of the seven Fel d 1 derivatives were compared to rFel d 1 in competition-ELISA. During this screening process, three of the Fel d 1 derivatives were selected for further investigations on the basis of reduced IgE-binding capacity in combination with recovery of readily soluble recombinant protein. The selected derivatives were DTE-modified rFel d 1 molecules with one (rFel d 1 (DTE I)), two (rFel d 1 (DTE II)) or three (rFel d 1 (DTE III)) cysteines replaced by serines. They exhibited 400 to 900 times reduced IgE-binding capacity compared to rFel d 1 (Fig. 10).

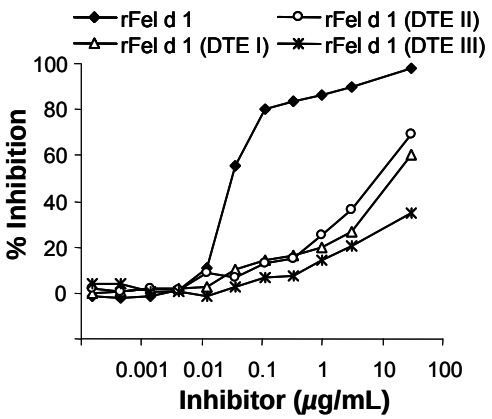

Figure 10. Reduced IgE-binding of Fel d 1 derivatives in competition-ELISA. Micro-titre plates were coated with rFel d 1. A serum pool from cat-sensitised patients was preincubated with either rFel d 1 or the rFel d 1 derivatives. 0.034 µg/mL of rFel d 1, 21.8 µg/mL of rFel d 1 (DTE I) and 14.3 µg/mL of rFel d 1 (DTE II) were needed to reach 50% inhibition. For rFel d 1 (DTE III), the highest concentration tested (30 µg/mL) resulted in 35% inhibition.

The derivative with only DTE (rFel d 1 (DTE)) displayed a marked reduction in IgE-binding (data not shown), but a more extensive investigation was not possible, since this construct was expressed in limited amounts and was mainly found in an aggregated form. A reason for unsuccessful folding might be that a cysteine residue is present in the duplicated sequence of chain 2 (Fig. 9). The derivatives with only cysteine mutations (rFel d 1 (I), rFel d 1 (II) and rFel d 1 (III)) were expressed and refolded in adequate amounts, but they exhibited only moderate reduction in IgE-binding (data not shown). Thus, disruption of the disulphide bonds of Fel d 1 had only

minor effects on the overall fold, but improved the solubility of DTE-modified rFel d 1.

The allergenic activities of the three Fel d 1 derivatives, rFel d 1 (DTE I), rFel d 1 (DTE II) and rFel d 1 (DTE III), were further compared to rFel d 1 by evaluating the activation of peripheral blood basophils from four cat-allergic patients. A dose-dependent activation of basophils was seen in all four patients after stimulation with all three derivatives as well as with rFel d 1. Generally, a lower concentration of rFel d 1 was needed to reach the same level of activation achieved by the derivatives. The ability to induce degranulation of basophils was decreased with an increased number of cysteine mutations. Accordingly, rFel d 1 (DTE III) showed the lowest capacity to activate basophils.

The aim of DTE was not only to alter the 3D structure of the allergen, but also to preserve or enhance the T-cell reactivity. Therefore, the lymphoproliferative activity of the three selected rFel d 1 derivatives was assayed in PBMC from nine cat-allergic patients. PBMC from seven patients were stimulated to proliferate (SI > 2.0) in response to the different Fel d 1 proteins, while no proliferation was detected in PBMC from two of the cat-allergic patients. In six of the seven responding patients, the rFel d 1 derivatives had a stronger stimulatory effect than rFel d 1. Only a weak response to rFel d 1 (DTE I) (SI 2.3), but not to the other Fel d 1 molecules, was observed in PBMC from a non-atopic control.

4.4 *IN VIVO* EVALUATION OF FEL D 1 HYPOALLERGEN IN A MOUSE MODEL AND BY SKIN PRICK TEST [IV]

Recently a mouse model for cat allergy was established by using rFel d 1 [206, 207]. The model showed characteristics of experimental allergic inflammation with elevated serum levels of allergen-specific IgE and IgG1, increased infiltration of eosinophils in the lungs and enhanced airway hyperreactivity to methacholine after allergen challenge. Based on the *in vitro* evaluation of the Fel d 1 hypoallergens in **paper III**, we selected the variant with the lowest IgE-binding capacity, rFel d 1 (DTE III), for further evaluation *in vivo*. The mouse model for cat allergy was used to investigate the potential of the hypoallergen in treatment.

At first, humoral responses against rFel d 1 (DTE III) and rFel d 1 were studied. BALB/c mice were s.c. immunised with the respective antigens adsorbed to alum five times every second week and blood was collected three days after the last immunisation. Both antigens raised IgG1 responses to the respective antigen, with variations in titres. Sera from rFel d 1 (DTE III)-immunised mice were further analysed for rFel d 1-specific IgG1. IgG1 antibodies specific for both rFel d 1 and rFel d 1 (DTE III) were detected in serum from the rFel d 1 (DTE III)-immunised mice. This indicates that the hypoallergen and the wild-type allergen possess some common IgG epitopes, which is a prerequisite for the induction of allergen-specific blocking antibodies by hypoallergen treatment.

Thus, as expected, the rFel d 1 (DTE III) is a good immunogen and its effect was further evaluated in a treatment protocol. After sensitisation with rFel d 1/alum, groups

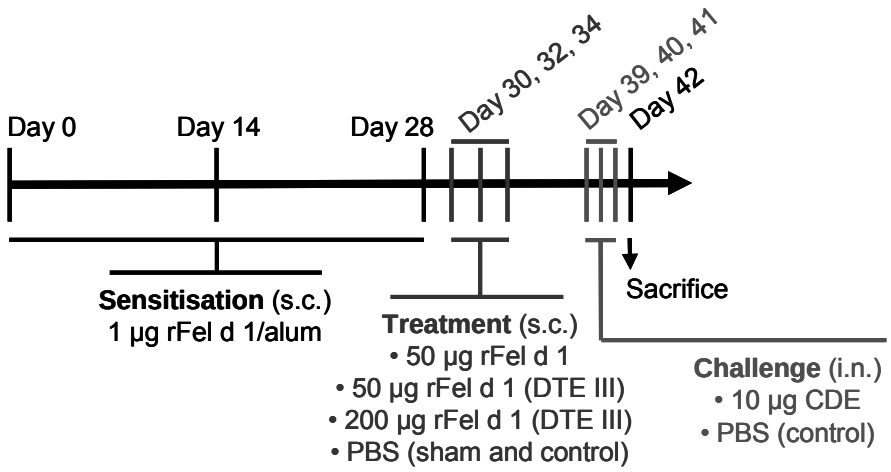

Figure 11. Treatment protocol for cat-allergic mouse model.

of mice (n=8-11) were therapeutically treated with two doses (50 µg or 200 µg) of rFel d 1 (DTE III), or with rFel d 1 (50 µg) at three occasions, followed by i.n. challenge with CDE to provoke airway inflammation (Fig. 11). Sham and control mice were sensitised with rFel d 1, treated with PBS, and challenged with CDE or PBS, respectively. All mice from the groups treated with rFel d 1 (DTE III) tolerated the treatments, even with the high dose, without any observed complications, while only four out of ten mice tolerated the three treatment injections with rFel d 1. Thus, solely four mice from this group could be characterised after treatment and challenge.

AHR was measured one day after the last CDE challenge. Treatment with the higher dose of hypoallergen decreased the maximal AHR, compared to the sham treatment, while treatment with unmodified rFel d 1 or the lower dose of hypoallergen did not have any impact on AHR (Fig. 12). As only mice treated with the higher dose of rFel d 1 (DTE III) showed a tendency of decreased AHR, this indicates that high treatment doses are required to reduce the AHR. The high treatment doses of the hypoallergen were indeed tolerated without any observed side effects, in contrast to wild-type allergen that was administered in a lower dose.

The i.n. challenge with CDE established an inflammation in the airways of rFel d 1-sensitised mice, which was marked by infiltration of macrophages, neutrophils and eosinophils in BAL fluid. Total cell numbers and differentiated cell counts in BAL fluid showed that all groups of mice had an ongoing inflammation in the airways. The inflammation was apparently so strong that it could not be decreased, neither by treatment with rFel d 1 or rFel d 1 (DTE III).

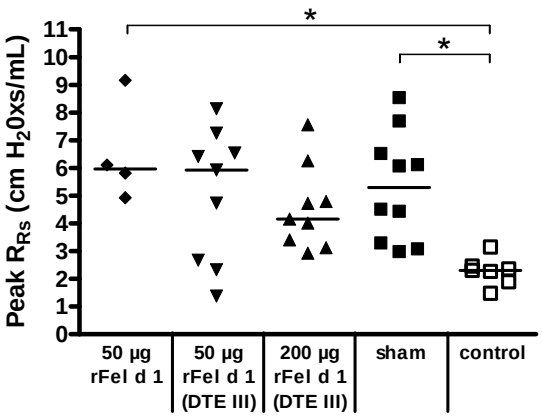

Figure 12. Airway hyperreactivity (AHR) in treated mice. AHR expressed as peak respiratory resistance (R_{Rs}) after challenge with 1 mg/kg methacholine at day 42 of the treatment protocol. *n* = 4-10, median.
* p<0.05

However, the cytokine levels in the supernatants of BAL fluid indicated a somewhat increased degree of inflammation in the sham-treated compared to the rFel d 1- or rFel d 1 (DTE III)-treated mice. IL-13 was only increased in the sham-treated mice, and although IL-5 was also significantly increased in all groups of treated mice, the sham-treated mice had the highest increase compared to control mice.

Cellular responses and cytokines were further analysed in cell cultures prepared from spleen. Splenocytes from all groups of mice had a strong ability to proliferate in response to rFel d 1. The proliferation was highest in the control mice, while a significantly lower proliferation was seen in the groups treated with 50 μg rFel d 1 ($p<0.05$) and 50 μg rFel d 1 (DTE III) ($p<0.01$). The cytokine profiles in the splenocyte culture supernatants showed a somewhat decreased Th2 response with lower levels of IL-5 and IL-13 in hypoallergen-treated compared to sham-treated mice. We could however not detect any differences between treated and sham-treated mice in the levels of e.g. the Th1-driving cytokine IFN-γ or other pro-inflammatory cytokines, like IL-6. Furthermore, allergen-stimulated splenocytes from treated mice produced less IL-10 than sham-treated mice. Despite the presence of duplicated T-cell epitopes, no clear change to T-cell tolerance or anergy could be detected. Thịs apparent lack of induction of a counter-acting immune response, Th1 or a regulatory response, may explain the inability to reduce the Th2-driven airway inflammation. Our mouse model reflects an acute allergic disease and it is possible that repeated application of the Fel d 1 hypoallergen in a chronic allergy model (or in human allergic disease) would have a more beneficial effect on the T-cell driven inflammation.

The most important result in this *in vivo* evaluation was that treatment with both doses of rFel d 1 (DTE III) and with rFel d 1 had a beneficial effect on the antibody responses. Only baseline levels of rFel d 1-specific IgE were found in all treated mice, which were significantly lower than the IgE levels in sham-treated mice. The levels of rFel d 1-specific IgG2a were significantly higher in the mice treated with the high dose of hypoallergen compared to sham ($p<0.05$). In addition, the levels of rFel d 1-specific IgG1 were significantly higher in all treated mice compared to sham-treated mice. The finding from the immunization experiments indicating some common IgG epitopes in the hypoallergen and the wild-type allergen was further supported by the fact that the IgG1 antibodies from both hypoallergen- and rFel d 1-treated mice in addition to rFel d 1 recognised rFel d 1 (DTE III). We could also show that IgG in sera from all groups of treated mice inhibited binding of IgE from cat-allergic patients to rFel d 1, supporting the theory of blocking antibodies produced during SIT. Importantly, IgG from the hypoallergen-treated mice blocked the IgE-binding to the same extent as IgG from rFel d 1-treated mice.

To further evaluate the IgE reactivity of rFel d 1 (DTE III) *in vivo*, we performed skin prick testing of seven cat-allergic patients and ten healthy controls. SPT in all the cat-allergic patients revealed less reactivity to rFel d 1 (DTE III) than to rFel d 1, while none of the healthy controls displayed any positive SPT to the allergens. These data indicate that rFel d 1 (DTE III) acts as a hypoallergen also *in vivo* in humans.

5 CONCLUSIONS

Paper I: Two new putative minor allergens, Lep d 10 and *L. destructor* α-tubulin were isolated from a phage display cDNA library of the storage mite *L. destructor*. Full-length clones were obtained and recombinant proteins were subsequently expressed in *E. coli* and tested for IgE reactivity. Evidence was found for cross-reactivity of Lep d 10 with other tropomyosins from different mite species and crustaceans, while the cross-reactive potential of α-tubulin remains to be investigated.

Paper II: We showed that serum-IgE to the single recombinant major cat allergen rFel d 1 is at least as good as IgE to CDE in the diagnosis of cat allergy in children, and may be a better marker for early cat sensitisation than IgE to CDE. By using a new sensitive rFel d 1 assay, low levels of IgE to rFel d 1 were found in children that were not detected with IgE to CDE. Furthermore, our results confirm that sensitisation at an early age is associated with later appearance of symptoms and suggest that early detection of cat sensitisation could be important in order to prevent disease development to asthma.

Paper III: Recombinant Fel d 1 hypoallergens were constructed by a rational approach. We altered the structure of Fel d 1 by applying the new strategy of DTE in combination with the established method of introducing point mutations, whereby disulphide bonds were disrupted. Three out of seven Fel d 1 derivatives generated were identified as hypoallergens with a strongly reduced IgE-binding capacity, a reduced allergenicity and similar or stronger T-cell reactivity. The hypoallergen rFel d 1 (DTE III) had the lowest IgE-binding capacity and appeared as the most promising candidate for SIT.

Paper IV: The first *in vivo* evaluation of rFel d 1 (DTE III) support the earlier *in vitro* results showing that this hypoallergen is a promising candidate for SIT of cat allergy. Therapeutic treatment of cat-allergic mice with the hypoallergen rFel d 1 (DTE III) resulted in decreased AHR and induction of rFel d 1-specific IgG with blocking capacity. In contrast to unmodified rFel d 1, the hypoallergen was tolerated at a high treatment dose without any observed side effects. The results indicated that high treatment doses are required to reduce the AHR. In addition, it was shown that rFel d 1 (DTE III) can act as a hypoallergen in humans, which was demonstrated by less SPT reactivity induced by rFel d 1 (DTE III) compared to rFel d 1 in cat-allergic patients.

In summary, the results of this thesis show the wide usage of recombinant allergens. They are important in the characterisation of single allergens of an allergen source, as shown for the mite *L. destructor*. Furthermore, they provide diagnosis of sensitisation to a specific allergen component, and can be used in the development of safer and more efficient tools for SIT, as demonstrated with the major cat allergen Fel d 1.

6 FUTURE PERSPECTIVES

This thesis focuses on the use of recombinant allergens for allergy diagnosis and immunotherapy, as exemplified by recombinant Lep d 10 and α-tubulin from the storage mite *L. destructor*, and recombinant Fel d 1 from domestic cat. From a Swedish point of view, it is of particular importance to improve the diagnosis and treatment of cat allergy, since it is a major cause of allergic disease. Especially children sensitised to cat have an increased risk of developing asthma [101-103].

As mentioned earlier, more than 800 allergens from the most common allergen sources are today available as recombinant allergens [66]. Many identified allergens still remain to be cloned and expressed as correctly folded recombinant allergens. The most important major allergens are already commercially available as diagnostic tools, but can in many cases be supplemented by minor allergens. In **paper I**, the cloning, expression and *in vitro* IgE-binding demonstrated the *L. destructor* allergens Lep d 10 and α-tubulin as minor allergens. The clinical relevance of these recombinant allergens remains to be evaluated *in vivo* by e.g. SPT. Furthermore, the natural *L. destructor* α-tubulin should be purified in order to allow a comparison between the recombinant and the naturally occurring *L. destructor* α-tubulin. The cross-reactive capacity of Lep d 10 and *L. destructor* α-tubulin could be further analysed by cross-inhibition studies with homologous proteins from other allergen sources. Alpha-tubulin has so far only been identified as an allergen in one other mite species, *T. putrescentiae* [261]. Preliminary studies have furthermore indicated that *L. destructor* α-tubulin cross-reacts with human α-tubulin that binds autoreactive IgE [Dr S. Zeller, personal communication].

The availability of Lep d 10 and *L. destructor* α-tubulin will open new opportunities for refined mite allergy diagnosis. Minor allergens may aid in the diagnosis of patients that are not sensitised to any major allergen. In addition, minor allergens give useful information in the characterisation of an allergen source regarding the composition of the disease-eliciting molecules. In case of *L. destructor* allergens, particularly rLep d 10 may be included in a cocktail of allergens for diagnosis. This allergen might be a useful tool in the evaluation of cross-reactivities. Tropomyosin-specific IgE may be important to diagnose, for at least two reasons. Firstly, Lep d 10-sensitised patients can be informed about the risk of cross-reactivity to tropomyosin in other allergenic sources. Secondly, the IgE reactivity to Lep d 10 could be followed before or during the application of SIT with crude allergen extracts from tropomyosin-containing allergenic sources to avoid development of side effects due to cross-reactivity.

In **papers II-IV**, the well-characterised recombinant major cat allergen Fel d 1 was used. A sensitive ELISA for the measurement of rFel d 1-specific IgE in kU/L was established for **paper II**. A similar assay could be established for any allergen, at least for small-scale research purposes. Our results, showing that sensitisation to rFel d 1 may be detected in children many years before the appearance of symptoms to cat, support the importance of measuring low levels of allergen-specific IgE. An assay with a low cut-off level for measurement of Fel d 1-specific IgE could be used to screen selected groups of high-risk children before the possible appearance of symptoms to cat. This could e.g. include children whose both parents are atopic or cat-allergic,

children with other sensitivities and possibly families who are planning to buy a cat. Awareness of early sensitisation could be used to prevent the progression of a mild allergic disease into asthma. The results furthermore support the use of rFel d 1 instead of CDE in the diagnosis of cat allergy. In fact, Fel d 1-based diagnostic tests have recently been introduced to the clinic.

Regarding the children in the BAMSE birth cohort in **paper II**, it would be interesting to do follow-up measurements of rFel d 1-specific IgE when the children are older. Unfortunately, no blood samples were taken at the 12 year follow-up of the BAMSE cohort. However, the 12 year questionnaires could be used to study which of the cat-sensitised children at 4 or 8 years do have symptoms to cat at 12 years. In the 16 year follow-up, starting in 2010, both questionnaires and clinical examinations, including blood sampling, will be carried out. This opens up for an opportunity for follow-up measurements of rFel d 1-specific IgE. The results from those measurements could further indicate how important the detection of very low allergen-specific IgE levels at 4 years could be.

Another issue that could be studied in the BAMSE cohort is the relation between cat sensitisation/symptoms and cat exposure. However, exposure to cat is a very complex issue, since contact to cat is not restricted to the home of the patient. It is difficult to compare the level of cat exposure at the patient's home compared to possible cat exposure outside the own household. Nevertheless, data on cat exposure at an early age could be connected to levels of rFel d 1-specific IgG4 in order to study if healthy children are tolerant to cat due to higher levels IgG4 compared to cat-allergic children.

In **paper III,** the structure of Fel d 1 was disrupted by applying DTE on T-cell epitopes that were shown to be parts of two out of three IgE-binding loops in Fel d 1. Thereby, the use of DTE most probably resulted in changed conformation of IgE epitopes as we indeed observed a reduction in IgE-binding. The disruption of disulphide bridges did not alter the IgE-binding as much as expected, but as these disruptions alter the conformation of the allergen, they probably also had some effect on conformational IgE epitopes. Results from circular dichroism spectrometry suggested that the structure of Fel d 1 (DTE III) is mostly unfolded (unpublished data). Furthermore, linear IgE epitopes in Fel d 1 have so far not been found, but if they exist, their disruption could result in different degrees of reduction in IgE-binding. It would be interesting to compare the treatment effect of hypoallergens with less reduced IgE-binding capacity with the highly hypoallergenic rFel d 1 (DTE III). An alternative strategy to derive Fel d 1 hypoallergens with different degrees of IgE-binding capacity is to apply DTE and disruption of disulphide bridges separately. This would also demonstrate which modification has the largest effect in SIT. As goes for DTE, the difficulties with protein expression remain to be solved. Furthermore, preliminary results show that the IgE-binding to Fel d 1 with only one cysteine changed to serine is around ten times reduced compared to unmodified Fel d 1. Hypoallergens with less reduced IgE-binding than rFel d 1 (DTE III) would nevertheless probably lead to side effects.

In **paper IV**, we were not able to see substantial effects of the hypoallergen treatment compared to unmodified Fel d 1 at the T cell level. An additional method to study possible induction of T cell tolerance could be by adoptive transfer of T cells from the

hypoallergen-treated mice into naïve mice, which are thereafter sensitised with Fel d 1. Furthermore, it remains to be analysed if the blocking IgG antibodies induced during treatment of the cat-allergic mouse can block basophil degranulation. This may be demonstrated by using rat basophil leukaemia cells.

Our mouse model reflects an acute allergic disease. To be able to study long-lasting effect of the hypoallergen treatment, a mouse model for chronic disease needs to be established. This could, however, result in a model with high degree of airway remodelling, which could be hard to reduce by hypoallergen treatment. Alternatively, the allergen challenge after treatment could be given during a longer period of time. Thereby the airway physiology, as well as cellular and immunoglobulin responses could be analysed later than in our present model.

In this first *in vivo* evaluation of the Fel d 1 (DTE III) hypoallergen, presented in this thesis, we studied the effect and safety of free hypoallergen alone. Next, the immune response to the hypoallergen could be further increased during treatment. For induction of tolerance it might be necessary to activate T_{Reg} cells, e.g. by coupling the hypoallergen to an adjuvant or to an immunomodulatory molecule, which could increase the antigen presentation.

Many approaches have been used for the construction of hypoallergens, but so far only three Bet v 1 hypoallergens have been evaluated in clinical immunotherapy trials [218-221, 223] and are yet not in clinical use. No strategy for design of hypoallergens will probably be applicable on all allergens, but many approaches will contribute to the construction of those hypoallergens that will reach the clinic in the future. Except for the Fel d 1 (DTE III) hypoallergen, we are currently working on several candidates for the treatment of cat-allergic patients. We need to carefully evaluate which of the hypoallergens should eventually be selected for clinical SIT trials. Finally, the recombinant allergens and hypoallergens in this thesis could be useful tools in the study of mechanisms behind the allergic disease as well as SIT.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Att vara allergiker kan innebära att man varje dag måste tänka på t ex vilka djur, växter eller födoämnen som måste undvikas för att slippa de symptom som dessa kan framkalla. Allergi är ett stort hälsoproblem som drabbar människor världen över, särskilt i den s.k. västvärlden och framförallt barn och unga vuxna. Uppemot 30% av befolkningen i vissa länder lider av allergiska sjukdomar, som allergisk astma, allergisk rinit eller atopiskt eksem. Allergiska besvär uppkommer vid en immunologisk reaktion mot ämnen som normalt är oskadliga, s.k. allergen. En allergenkälla, t ex kattmjäll, innehåller oftast flera olika allergen, vilka vanligen är proteiner. Denna avhandling fokuserar på luftburna allergen från kvalster och katt som många individer är sensibiliserade (överkänsliga) mot. När immunsystemet reagerar felaktigt mot ett allergen uppstår en allergisk reaktion. Denna reaktion kännetecknas av speciella antikroppar, IgE, som binder specifikt till allergenet och speciella T-celler av T-hjälpar typ 2 (Th2). Den allergiska reaktionen leder snabbt till symptom, såsom kliande ögon, nysningar och snuva, samt kan orsaka astma, vilket är en kronisk inflammation i luftvägarna.

I Sverige är kattallergi en av de vanligaste orsakerna till astma bland barn. Därför finns det ett stort behov av förbättrad diagnostik och nya terapier för att förhindra kattutlöst astma. Vid allergidiagnostik analyseras bl.a. hudreaktioner eller blodprov för förekomsten av IgE-antikroppar mot de vanligaste allergenen. Idag används mestadels allergener i form av extrakt från den naturliga allergenkällan, t.ex. kattmjäll eller hela kvalster, som kan variera mellan olika tillredningar i innehåll av både allergener och andra komponenter. Detta innebär bl.a. att man inte alltid kan identifiera det specifika allergenet. Allergenextrakt används även i allergen-specifik immunterapi (SIT), som är den enda behandlingen med vilken det är möjligt att ge bestående symptomfrihet vid allergi. Patienten får under en lång tidsperiod upprepade injektioner av gradvis ökande doser av det allergen som patienten är sensibiliserad mot. Vid SIT ändras T-cellssvaret från ett allergiskt Th2-dominerat immunsvar till ett icke-allergiskt immunsvar som i slutändan ger symptomfrihet. Med dagens behandling med extrakt finns det en risk för biverkningar och uppkomst av nya sensibiliseringar. För att få en mer specifik diagnostik samt en säkrare och effektivare immunterapi har man börjat ersätta extrakten med rena och väldefinierade allergen som är framställda med hybrid DNA-teknik. Dessa s.k. rekombinanta allergen har under de två senaste årtiondena tagits fram från ett stort antal allergenkällor, men täcker ännu inte hela spektrumet av allergen. Målsättningen i denna avhandling var att ta fram nya rekombinanta kvalsterallergen samt att utveckla förbättrad diagnostik och behandling av kattallergi m.h.a. ett rekombinant kattallergen.

En av de vanligaste orsakerna till allergier i jordbruksmiljöer är förrådskvalstret *Lepidoglyphus destructor*. I **delarbete I** hittade vi två nya IgE-bindande proteiner från ett s.k. *L. destructor* cDNA fagbibliotek. Proteinerna visade stor likhet till α-tubulin och tropomyosin från andra arter och framställdes som rekombinanta proteiner i bakterien *Escherichia coli*. Analyser av blodprover från kvalster- och skaldjursallergiker visade IgE-bindning till rekombinant α-tubulin och tropomyosin hos 12% respektive 13%. Tropomyosinet döptes till Lep d 10 då det är homologt till

kvalsterallergenen Der f 10 och Der p10. Våra resultat tyder på att Lep d 10 är ett korsreagerande allergen, vilket innebär att patienter som reagerar mot Lep d 10 även kan få symptom av tropomyosin från andra kvalster samt skaldjur utan att tidigare ha sensibiliserats mot dessa.

Vi har tidigare framställt kattallergenet Fel d 1 som ett rekombinant allergen (rFel d 1) med samma egenskaper som det naturligt förekommande Fel d 1. Fel d 1 känns igen av IgE-antikroppar i blodprover hos upp till 95% av alla kattallergiker och är därför ett mycket viktigt allergen. I **delarbete II** jämförde vi IgE-reaktiviteten mot rFel d 1 och kattmjällsextrakt i blodprover hos kattallergiska barn i den stora svenska barnkohorten BAMSE. En känslig detektionsmetod sattes upp för att kunna kvantifiera låga nivåer av IgE mot rFel d 1. Vi kunde visa att rFel d 1 är minst lika bra som kattmjällsextrakt för att diagnostisera kattallergi hos barn. Dessutom kunde kattsensibilisering, d.v.s. IgE mot rFel d 1, detekteras hos barn flera år innan symptom mot katt upptäcktes.

Risken för biverkningar vid SIT kan minskas ännu mer genom användandet av genetiskt modifierade rekombinanta allergen, s.k. hypoallergen. Dessa har en reducerad förmåga att binda IgE, men kan fortfarande stimulera ett T-cellssvar. I **delarbete III** använde vi oss av strukturell information om Fel d 1 för att ta fram hypoallergen. Den tredimensionella strukturen förändrades samtidigt som epitoper som stimulerar T-celler duplicerades på DNA-nivå. Vi lyckades framställa tre Fel d 1 hypoallergen, som jämfört med Fel d 1 hade sänkt IgE-bindande kapacitet, lägre förmåga att stimulera basofiler och bibehållen kapacitet att stimulera T-celler i perifert blod hos kattallergiska patienter. Hypoallergenet rFel d 1 (DTE III) sågs som den mest lovande kandidaten för SIT p.g.a. lägst förmåga att binda IgE.

I **delarbete IV** använde vi oss av en nyligen framtagen musmodell för kattallergi för att utvärdera rFel d 1 (DTE III) för immunterapibehandling. Tre grupper av möss som sensibiliserats med rFel d 1 genomgick behandlingar med två olika doser av hypoallergenet eller med rFel d 1. Därefter provocerades en allergisk reaktion i lungan med kattmjällsextrakt. De möss som behandlats med en hög dos av hypoallergenet hade en lägre luftvägsreaktivitet än övriga behandlade samt obehandlade möss. Behandlingen påverkade inte inflammationen i luftvägarna och var därmed inte tillräckligt effektiv för att ändra det cellulära svaret. Jämfört med obehandlade möss hade dock alla tre grupper av behandlade möss mycket lägre nivåer av rFel d 1-specifikt IgE och förhöjda nivåer av rFel d 1-specifikt IgG, som blockerade bindningen av IgE till rFel d 1. Till skillnad från behandlingen med rFel d 1, som endast tolererades av 4 av 10 möss, tolererade alla möss hypoallergenbehandlingarna. Dessutom visade hudpricktester på kattallergiska patienter att reaktiviteten var lägre mot hypoallergenet jämfört med rFel d 1. SIT med rFel d 1 (DTE III) kan därmed minska riskerna för biverkningar, men effekten av behandlingen behöver undersökas ytterligare.

Sammanfattningsvis visar resultaten i denna avhandling på många möjliga användningsområden för rekombinanta allergen. De är viktiga för att karakterisera specifika allergen från en allergenkälla, som visades för kvalstret *L. destructor*. Vidare kan de bidra till diagnostisering av sensibilisering mot ett specifikt allergen samt användas i utvecklingen av säkrare och effektivare SIT, vilket visades med huvudallergenet i katt, Fel d 1.

8 ACKNOWLEDGEMENTS

I am so grateful to all colleagues, friends and family for your continuous help and support during my PhD studies, for caring about my well-being and for reminding me about other important things in life than work. My warmest thanks go to my **supervisors** as well as **Jonas**, **Natalija**, **Patricia** and **Toomas** for proofreading the whole thesis or parts of it. In addition, I would more specifically like to thank:

My main supervisor, **Marianne van Hage**, for accepting me as a PhD student and providing such a nice working environment. I appreciate very much your interest and concern regarding my projects, for sharing your great knowledge in allergy and clinical immunology and for all support in scientific writing. I am also happy that you have given me the opportunity to go to congresses and allergy schools to present my data, and to interact with both experienced scientists and other PhD students.

My co-supervisors, **Guro Gafvelin** and **Hans Grönlund**, for sharing your scientific knowledge, ideas and enthusiasm. **Guro**, for being calm and having a realistic view of things, for always having time to explain things (especially concerning all cellular parts of my projects) and for very constructive advice in scientific writing. **Hans**, for always being optimistic and having rational ideas, for endless guidance and discussions about proteins and ELISAs, and for always finding time for pep talks when needed. I have appreciated that a lot.

My co-authors and co-workers; **Omid Rasool**, my first supervisor in molecular biology, for all enthusiasm. **Liselotte (Lotta) Kaiser**, my confident collaborator and support during my first two projects. Thank you also for friendship, great days in Boston and NYC, and for being my most equal and faithful badminton opponent (we've had a too long break now though…). **Theresa Neimert-Andersson**, my coach, rescuer and support in mouse experiments, whom I can never thank enough for all the tick-tacking FlexiVent events. Thank you also for putting up the cat-allergic mouse model, without that my last project would have been impossible.

My other co-authors **Sonia Huecas**, **Magnus Wickman**, **Catarina Almqvist**, **Inger Kull** and **Marek Jutel** for important contributions to my projects.

Eva Hallner and **Stina Gustavsson** for technical support with the BAMSE material, and **Nazanin Hashemi** and **Jakob Bergström** for statistical advice in that project.

Ann-Britt, **Pia**, **Carina** and all others at the Department of Clinical Immunology and Transfusion Medicine who have carried out numerous ImmunoCAP analyses for me.

Sven-Erik Dahlén and **Cfa** at KI for providing a nice lab and good instruments at the FyFa animal facility. **Linda S**, **Cecilia**, **Sus** and **Magnus** for good company and useful advice in the lab.

My mentor, **Outi Hovatta**, for being sincere about your own experiences in the world of research, and for inspiring me to positive thinking.

Everyone who has ever worked at **L2:04** during all my years, for creating such a lovely place to work at. There is always someone ready to solve any problem, or answer any stupid question. A lot of social events, like parties, dinners and pubs (in and outside the lab), the Filmclub (thanks to Le Groupe!), all sporting (especially spinning at Friskis), and crazy step-counting, have contributed very much to the friendly and relaxing atmosphere. The congresses that I have attended with people from the lab have been the funniest ones. Without doubt, the first congress trip to Naples is the most unforgettable one, with a lost bus driver in the middle of the night, a guided bus tour without guide, crazy taxi rides and a "nervoso" chef. Sunny Göteborg with Flumeride at Liseberg (guess who got most wet…) and Ulf driving loads of people to Saltholmen was pretty crazy too.

The **MvH-group** for good collaborations, inspiring discussions and the best company during labwork, fika and lunches. In addition to my supervisors, I would like to thank the other current members of the group for different contributions: **Gerd**, for all your smiles and for always being so helpful in administrative matters. **Neda**, the most faithful fika companion, for assistance in the lab and for concerning about the organisation in the lab at least as much as I. **Theresa**, the master of efficient lab work, for entertaining with practical jokes. **Ola**, for your trust in my BCA assays, your amusing moaning (sorry, but it has been fun), and your bizarre humour. **Jeanette**, for good collaboration in the Fel d 1 factory and nice excursions to the animal facility. **Jonas**, for opening my centrifuge flasks and for telling the funniest stories. **Natalija**, for showing pure enthusiasm for everything, even dry ice deliveries. **Erik**, for all interesting stories and for finding answers to almost everything on your iPhone. It has also been a pleasure to work in the same group as **Maria T-L**, **Eva J**, **Klas**, the previous PhD students **Tove**, **Sam**, **Lotta K**, **Linda J**, **Nariman**, **Sarah** and **Justus**, as well as **all the nice students** that we have had throughout all my years at L2:04.

All the kind and funny room mates I have shared offices with throughout the years, especially the members of crazy (and crowded) Grottan office: **Theresa**, **Fredrik**, **Mattias**, **Ulf**, **Patricia**, **Marisa**, **Mats** (who recently left) and the previous ones; **Ricardo**, **Sara J** and **Lotta A**, for creating such a cheerful and friendly working place to come to every day. You might have doubted from time to time, but I have really enjoyed most of the strange and funny ideas, thoughts and discussions in the main habitat of gripklon and fredagsstämning, that previously included weekly statistics on DN's nutidstest (what happened to those, was I too good?), and some rare themes like "handkrämsparty". Not to forget, it has been really convenient to have you around for more serious discussions and help in scientific matters.

My sweet and funny friends from the lab. Without your company and support (especially during the last months) everything would have been so much harder. In order of appearance to the lab, thank you:
Anna, the crazy lover of coldness**,** for your warm laughter and clever ideas, for showing me the wonderful Höga kusten, and for shared passion for watching sports, both on TV and live. I'm so happy you have been back and forth to the lab during all my years.
Natalija, our lovely Serbian diva**,** for compassion, encouragement and help in every matter at any time (will never forget the endless EndNote night), for sharing your

knowledge in biochemistry and allergy, for so much fun and for almost all your laughter…
Jeanette, the master of knitting, tea and Wiiiii, for kindness and care (also for our mice), for being patient with my advice and habits in the lab, and for organising or participating in most of the funny parties which I have had the pleasure to take part of the last years.
Patricia, the Spanish or Indian (?) bee-keeper, for your kindness and understanding, for samåkning, samcykling and samgång. Årsta-kontakten turned out to be a great contact, which will keep being plugged-in even if you move to anti-friends-in-acknowledgements **Vasco**, who also deserves to be mentioned here for friendship and funny discussions.
Jonas, my PT and walking encyclopaedia (had to check up this elsewhere), for putting so much time, effort and interest in reading, correcting and discussing my thesis. I have also valued our pep talks and nice discussions about the silliest and most serious matters, also outside science.
Cindy, the German pasta lady, who is finding French words when looking for English ones, for care and concern. Luckily the writing of my thesis and your big grant application took place the same summer. However, we have had too much bad luck and we shouldn't let Murphy's Law rule our lives anymore!

Other friends that I had the pleasure to get to know when you were at L2:04: **Ulrika**, **Linda J** and **Min** for sharing experiences as PhD students and for all the good times I have had with each of you and your families.

My oldest and one of my dearest friends from KI, **Maria**, for sharing both good and bad moments, for endless chats, mutual pep talks and so much fun. Our Estonian background connected us from start and has been of more importance to our friendship than we might have realised.

My dear multinational pen pal since ages, **Kirsi**, for being so present in my life although always living too far away. I'm looking so much forward to see you and **Vincent** again, this time in Sydney.

Last, but not least, my **relatives** all around the world that are so important to me. In particular, my big and lovely family in Sweden and Finland, including my grandmothers **Ira** and **Maija-Liisa**, all **aunts**, **uncles** and **cousins with families**, especially life-long friends **Liisa**, **Vilja-Maria** and **Pauliina**. You have contributed so much to my well-being through your love, care and support. I'm so happy Finland isn't as far away as it felt when we were kids. Kiitos kaikille ihanille sukulaisille!
Luckily, the closest family lives even closer. My dear brothers **Matti** and **Toomas**, thank you for all your care and ever-growing friendship, for teaching me new things about myself, and for sibling trips and get-togethers, often including Matti's **Ida**, whose positive attitude and friendliness I admire so much. Finally, my parents **Heli** and **Henno**, who have contributed the most to my Finnish-Estonian-Swedish identity, which I'm so proud of. Thank you for showing such interest in what I'm doing and for endless support. Simply for always being there for me, first in line, no matter what happens, even when a bedroom ceiling falls down in the most stressful time of my life.

9 REFERENCES

1. Janeway C, *et al.* Janeway's immunobiology. 7th ed. New York, NY: Garland Science; 2008.
2. Chaplin DD. 1. Overview of the human immune response. J Allergy Clin Immunol 2006;117(2 Suppl Mini-Primer):S430-435.
3. Johansson SG, *et al.* Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 2004;113(5):832-836.
4. Platts-Mills TAE, *et al.* Indoor allergens and asthma: report of the Third International Workshop. J Allergy Clin Immunol 1997;100(6 Pt 1):S2-24.
5. Novak N, *et al.* The role of antigen presenting cells at distinct anatomic sites: they accelerate and they slow down allergies. Allergy 2004;59(1):5-14.
6. Romagnani S. The role of lymphocytes in allergic disease. J Allergy Clin Immunol 2000;105(3):399-408.
7. Prussin C, Metcalfe DD. 5. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 2006;117(2 Suppl Mini-Primer):S450-456.
8. Sampson HA, *et al.* Second symposium on the definition and management of anaphylaxis: summary report--Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. J Allergy Clin Immunol 2006;117(2):391-397.
9. Simons FE. 9. Anaphylaxis. J Allergy Clin Immunol 2008;121(2 Suppl):S402-407; quiz S420.
10. Larché M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. J Allergy Clin Immunol 2003;111(3):450-463; quiz 464.
11. Stingl G, Maurer D. IgE-mediated allergen presentation via Fc epsilon RI on antigen-presenting cells. Int Arch Allergy Immunol 1997;113(1-3):24-29.
12. Sampson AP. The role of eosinophils and neutrophils in inflammation. Clin Exp Allergy 2000;30 Suppl 1:22-27.
13. Holgate ST, *et al.* Local genetic and environmental factors in asthma disease pathogenesis: chronicity and persistence mechanisms. Eur Respir J 2007;29(4):793-803.
14. Romagnani S. Regulation of the T cell response. Clin Exp Allergy 2006;36(11):1357-1366.
15. Hammad H, Lambrecht BN. Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation. J Allergy Clin Immunol 2006;118(2):331-336.
16. Bisset LR, Schmid-Grendelmeier P. Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective. Curr Opin Pulm Med 2005;11(1):35-42.
17. Kay AB. The role of T lymphocytes in asthma. Chem Immunol Allergy 2006;91:59-75.
18. Finotto S, *et al.* Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. Science 2002;295(5553):336-338.
19. Mangan PR, *et al.* Transforming growth factor-beta induces development of the TH17 lineage. Nature 2006;441(7090):231-234.
20. Bettelli E, *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441(7090):235-238.
21. Korn T, *et al.* IL-17 and Th17 Cells. Annu Rev Immunol 2009;27:485-517.
22. Oboki K, *et al.* Th17 and allergy. Allergol Int 2008;57(2):121-134.
23. Veldhoen M, *et al.* Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. Nat Immunol 2008;9(12):1341-1346.
24. Dardalhon V, *et al.* IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nat Immunol 2008;9(12):1347-1355.

25. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299(5609):1057-1061.
26. Roncarolo MG, *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006;212:28-50.
27. Carrier Y, *et al.* Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. J Immunol 2007;178(1):179-185.
28. Akdis M, *et al.* Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;199(11):1567-1575.
29. Ling EM, *et al.* Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 2004;363(9409):608-615.
30. Grindebacke H, *et al.* Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. Clin Exp Allergy 2004;34(9):1364-1372.
31. Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. J Allergy Clin Immunol 2009;123(4):735-746; quiz 747-738.
32. Akbari O, *et al.* Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat Med 2003;9(5):582-588.
33. Meyer EH, *et al.* Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells. Proc Natl Acad Sci U S A 2006;103(8):2782-2787.
34. Umetsu DT, Meyer EH, DeKruyff RH. Natural killer T cells regulate the development of asthma. Int Rev Immunol 2007;26(1-2):121-140.
35. Akbari O, *et al.* CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. N Engl J Med 2006;354(11):1117-1129.
36. Vijayanand P, *et al.* Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. New Engl J Med 2007;356(14):1410-1422.
37. Asher MI, *et al.* Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006;368(9537):733-743.
38. Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. Eur Respir J 2004;24(5):758-764.
39. Spergel JM. Atopic march: link to upper airways. Curr Opin Allergy Clin Immunol 2005;5(1):17-21.
40. Blumenthal MN. The role of genetics in the development of asthma and atopy. Curr Opin Allergy Clin Immunol 2005;5(2):141-145.
41. Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. Genes Immun 2006;7(2):95-100.
42. Bossé Y, Hudson TJ. Toward a comprehensive set of asthma susceptibility genes. Annu Rev Med 2007;58:171-184.
43. Kim H, Bernstein JA. Air pollution and allergic disease. Curr Allergy Asthma Rep 2009;9(2):128-133.
44. Matricardi PM. Prevalence of atopy and asthma in eastern versus western Europe: why the difference? Ann Allergy Asthma Immunol 2001;87(6 Suppl 3):24-27.
45. von Mutius E, *et al.* Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. Lancet 1998;351(9106):862-866.
46. Heinrich J, *et al.* Trends in prevalence of atopic diseases and allergic sensitization in children in Eastern Germany. Eur Respir J 2002;19(6):1040-1046.
47. Annus T, *et al.* Modest increase in seasonal allergic rhinitis and eczema over 8 years among Estonian schoolchildren. Pediatr Allergy Immunol 2005;16(4):315-320.
48. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989;299(6710):1259-1260.

49. Garn H, Renz H. Epidemiological and immunological evidence for the hygiene hypothesis. Immunobiology 2007;212(6):441-452.
50. Romagnani S. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? Immunology 2004;112(3):352-363.
51. Traidl-Hoffmann C, Jakob T, Behrendt H. Determinants of allergenicity. J Allergy Clin Immunol 2009;123(3):558-566.
52. King TP, *et al.* Allergen nomenclature. Allergy 1995;50(9):765-774.
53. WHO/IUIS. WHO/IUIS Allergen Nomenclature Sub-Committee. Official list of allergens. In: www.allergen.org; 2009.
54. Radauer C, *et al.* Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol 2008;121(4):847-852 e847.
55. Chapman MD, *et al.* Nomenclature and structural biology of allergens. J Allergy Clin Immunol 2007;119(2):414-420.
56. Emanuelsson C, Spangfort MD. Allergens as eukaryotic proteins lacking bacterial homologues. Mol Immunol 2007;44(12):3256-3260.
57. Trompette A, *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature 2009;457(7229):585-588.
58. Hammad H, *et al.* House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med 2009;15(4):410-416.
59. Tanabe S. Epitope peptides and immunotherapy. Curr Protein Pept Sci 2007;8(1):109-118.
60. Valenta R, Kraft D. Recombinant allergen molecules: tools to study effector cell activation. Immunol Rev 2001;179:119-127.
61. Nelson HS. Allergen immunotherapy: where is it now? J Allergy Clin Immunol 2007;119(4):769-779.
62. Valenta R, Niederberger V. Recombinant allergens for immunotherapy. J Allergy Clin Immunol 2007;119(4):826-830.
63. Focke M, Marth K, Valenta R. Molecular composition and biological activity of commercial birch pollen allergen extracts. Eur J Clin Invest 2009;39(5):429-436.
64. van Ree R, *et al.* The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. Allergy 2008;63(3):310-326.
65. Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. Curr Opin Immunol 2002;14(6):718-727.
66. Allergome. Allergome - a Database of Allergenic Molecules. In: www.allergome.org; 2009.
67. Ferreira F, *et al.* Allergic cross-reactivity: from gene to the clinic. Allergy 2004;59(3):243-267.
68. Aalberse RC, Akkerdaas JH, van Ree R. Cross-reactivity of IgE antibodies to allergens. Allergy 2001;56(6):478-490.
69. Fernandes J, *et al.* Immunoglobulin E antibody reactivity to the major shrimp allergen, tropomyosin, in unexposed Orthodox Jews. Clin Exp Allergy 2003;33(7):956-961.
70. Aalberse RC. Structural features of allergenic molecules. Chem Immunol Allergy 2006;91:134-146.
71. Nagpal S, *et al.* Isolation and characterization of heat-stable allergens from shrimp (Penaeus indicus). J Allergy Clin Immunol 1989;83(1):26-36.
72. Shanti KN, *et al.* Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. J Immunol 1993;151(10):5354-5363.
73. Jeong KY, Hong CS, Yong TS. Allergenic tropomyosins and their cross-reactivities. Protein Pept Lett 2006;13(8):835-845.
74. Fernández-Caldas E. Dust mite allergens: mitigation and control. Curr Allergy Asthma Rep 2002;2(5):424-431.
75. Chapman MD, *et al.* Recombinant mite allergens. New technologies for the management of patients with asthma. Allergy 1997;52(4):374-379.

76. Fernández-Caldas E, *et al.* Mite allergens. Clin Allergy Immunol 2008;21:161-182.
77. van der Heide S, *et al.* Prevalence of sensitization to the storage mites *Acarus siro*, *Tyrophagus putrescentiae*, and *Lepidoglyphus destructor* in allergic patients with different degrees of sensitization to the house-dust mite *Dermatophagoides pteronyssinus*. Allergy 1998;53(4):426-430.
78. Boström S, *et al.* Characterization of the mite fauna (acari) in Swedish barn dust. Internat J Acarol 1997;23(2):127-132.
79. van Hage-Hamsten M, *et al.* Storage mite allergy is common in a farming population. Clin Allergy 1985;15(6):555-564.
80. Kronqvist M, *et al.* Increasing prevalence of asthma over 12 years among dairy farmers on Gotland, Sweden: storage mites remain dominant allergens. Clin Exp Allergy 1999;29(1):35-41.
81. Blainey AD, *et al.* Allergic respiratory disease in grain workers: the role of storage mites. J Allergy Clin Immunol 1989;84(3):296-303.
82. Storaas T, *et al.* Occupational rhinitis: diagnostic criteria, relation to lower airway symptoms and IgE sensitization in bakery workers. Acta Otolaryngol 2005;125(11):1211-1217.
83. Ebner C, *et al.* Sensitization to storage mites in house dust mite (*Dermatophagoides pteronyssinus*) allergic patients. Comparison of a rural and an urban population. Clin Exp Allergy 1994;24(4):347-352.
84. Bernd LAG, Ambrozio LC, Baggio D. Storage mite allergy in perennial rhinitis patients not sensitized to house dust mites. J Investig Allergol Clin Immunol 1996;6(2):94-97.
85. Warner A, *et al.* Mite fauna in the home and sensitivity to house-dust and storage mites. Allergy 1999;54(7):681-690.
86. Vidal C, *et al.* High prevalence of storage mite sensitization in a general adult population. Allergy 2004;59(4):401-405.
87. Luczynska CM, *et al.* Prevalence of specific IgE to storage mites (*A. siro*, *L. destructor* and *T. longior*) in an urban population and crossreactivity with the house dust mite (*D. pteronyssinus*). Clin Exp Allergy 1990;20(4):403-406.
88. Johansson E, *et al.* Immunoblot multi-allergen inhibition studies of allergenic cross-reactivity of the dust mites *Lepidoglyphus destructor* and *Dermatophagoides pteronyssinus*. Clin Exp Allergy 1991;21(4):511-518.
89. Thomas WR, *et al.* Characterization and immunobiology of house dust mite allergens. Int Arch Allergy Immunol 2002;129(1):1-18.
90. Olsson S, *et al.* Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems. Clin Exp Allergy 1998;28(8):984-991.
91. Eriksson TLJ, *et al.* Cloning of three new allergens from the dust mite *Lepidoglyphus destructor* using phage surface display technology. Eur J Biochem 2001;268(2):287-294.
92. Varela J, *et al.* Primary structure of Lep d I, the main *Lepidoglyphus destructor* allergen. Eur J Biochem 1994;225(1):93-98.
93. Schmidt M, *et al.* cDNA analysis of the mite allergen Lep d 1 identifies two different isoallergens and variants. FEBS Lett 1995;370(1-2):11-14.
94. Kronqvist M, *et al.* Skin prick test and serological analysis with recombinant group 2 allergens of the dust mites *L. destructor* and *T. putrescentiae*. Clin Exp Allergy 2000;30(5):670-676.
95. Johansson E, *et al.* Evaluation of specific IgE to the recombinant group 2 mite allergens Lep d 2 and Tyr p 2 in the Pharmacia CAP system. Int Arch Allergy Immunol 1999;120(1):43-49.
96. Gafvelin G, *et al.* Cross-reactivity studies of a new group 2 allergen from the dust mite *Glycyphagus domesticus*, Gly d 2, and group 2 allergens from *Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae* with recombinant allergens. J Allergy Clin Immunol 2001;107(3):511-518.
97. Freidhoff LR, Meyers DA, Marsh DG. A genetic-epidemiologic study of human immune responsiveness to allergens in an industrial population. II. The associations among skin sensitivity, total serum IgE, age, sex, and the reporting

of allergies in a stratified random sample. J Allergy Clin Immunol 1984;73(4):490-499.
98. Plaschke P, *et al.* Association between atopic sensitization and asthma and bronchial hyperresponsiveness in Swedish adults: pets, and not mites, are the most important allergens. J Allergy Clin Immunol 1999;104(1):58-65.
99. Roost HP, *et al.* Role of current and childhood exposure to cat and atopic sensitization. European Community Respiratory Health Survey. J Allergy Clin Immunol 1999;104(5):941-947.
100. Perzanowski MS, *et al.* Effect of cat and dog ownership on sensitization and development of asthma among preteenage children. Am J Respir Crit Care Med 2002;166(5):696-702.
101. Lau S, *et al.* Longitudinal study on the relationship between cat allergen and endotoxin exposure, sensitization, cat-specific IgG and development of asthma in childhood--report of the German Multicentre Allergy Study (MAS 90). Allergy 2005;60(6):766-773.
102. Lévesque B, *et al.* Total and specific immunoglobulin E and their relationship to respiratory symptoms in Quebec children and adolescents. Can Respir J 2005;12(8):426-432.
103. Korppi M, *et al.* Early exposure and sensitization to cat and dog: different effects on asthma risk after wheezing in infancy. Pediatr Allergy Immunol 2008;19(8):696-701.
104. Rönmark E, *et al.* Major increase in allergic sensitization in school children from 1996 to 2006 in northern Sweden. J Allergy Clin Immunol 2009.
105. Dabrowski AJ, *et al.* Cat skin as an important source of Fel d I allergen. J Allergy Clin Immunol 1990;86(4 Pt 1):462-465.
106. Charpin C, *et al.* Fel d I allergen distribution in cat fur and skin. J Allergy Clin Immunol 1991;88(1):77-82.
107. Løwenstein H, Lind P, Weeke B. Identification and clinical significance of allergenic molecules of cat origin. Part of the DAS 76 Study. Allergy 1985;40(6):430-441.
108. Kleine-Tebbe J, *et al.* Role of the major allergen (Fel d I) in patients sensitized to cat allergens. Int Arch Allergy Immunol 1993;100(3):256-262.
109. van Ree R, *et al.* Purified natural and recombinant Fel d 1 and cat albumin in in vitro diagnostics for cat allergy. J Allergy Clin Immunol 1999;104(6):1223-1230.
110. Ichikawa K, *et al.* High prevalence of sensitization to cat allergen among Japanese children with asthma, living without cats. Clin Exp Allergy 1999;29(6):754-761.
111. Grönlund H, *et al.* Higher immunoglobulin E antibody levels to recombinant Fel d 1 in cat-allergic children with asthma compared with rhinoconjunctivitis. Clin Exp Allergy 2008;38(8):1275-1281.
112. Klug J, *et al.* Uteroglobin/Clara cell 10-kDa family of proteins: nomenclature committee report. Ann N Y Acad Sci 2000;923:348-354.
113. Hilger C, Grigioni F, Hentges F. Sequence of the gene encoding cat (*Felis domesticus*) serum albumin. Gene 1996;169(2):295-296.
114. Ichikawa K, *et al.* Molecular cloning, expression and modelling of cat allergen, cystatin (Fel d 3), a cysteine protease inhibitor. Clin Exp Allergy 2001;31(8):1279-1286.
115. Smith W, *et al.* Fel d 4, a cat lipocalin allergen. Clin Exp Allergy 2004;34(11):1732-1738.
116. Adedoyin J, *et al.* Cat IgA, representative of new carbohydrate cross-reactive allergens. J Allergy Clin Immunol 2007;119(3):640-645.
117. Ohman JL, Jr., Lowell FC, Bloch KJ. Allergens of mammalian origin. III. Properties of a major feline allergen. J Immunol 1974;113(6):1668-1677.
118. Kristensen AK, Schou C, Roepstorff P. Determination of isoforms, N-linked glycan structure and disulfide bond linkages of the major cat allergen Fel d1 by a mass spectrometric approach. Biol Chem 1997;378(8):899-908.
119. Duffort OA, *et al.* Studies on the biochemical structure of the major cat allergen *Felis domesticus* I. Mol Immunol 1991;28(4-5):301-309.

120. Morgenstern JP, *et al.* Amino acid sequence of Fel dI, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. Proc Natl Acad Sci U S A 1991;88(21):9690-9694.
121. Griffith IJ, *et al.* Expression and genomic structure of the genes encoding FdI, the major allergen from the domestic cat. Gene 1992;113(2):263-268.
122. Bond JF, *et al.* Native and recombinant Fel dI as probes into the relationship of allergen structure to human IgE immunoreactivity. Mol Immunol 1993;30(16):1529-1541.
123. Counsell CM, *et al.* Definition of the human T-cell epitopes of Fel d 1, the major allergen of the domestic cat. J Allergy Clin Immunol 1996;98(5 Pt 1):884-894.
124. van Milligen FJ, *et al.* IgE epitopes on the cat (*Felis domesticus*) major allergen Fel d I: a study with overlapping synthetic peptides. J Allergy Clin Immunol 1994;93(1 Pt 1):34-43.
125. Grönlund H, *et al.* Formation of disulfide bonds and homodimers of the major cat allergen Fel d 1 equivalent to the natural allergen by expression in *Escherichia coli*. J Biol Chem 2003;278(41):40144-40151.
126. Kaiser L, *et al.* The crystal structure of the major cat allergen Fel d 1, a member of the secretoglobin family. J Biol Chem 2003;278:37730-37735.
127. Kaiser L, *et al.* Production, crystallization and preliminary crystallographic study of the major cat allergen Fel d 1. Acta Crystallogr D Biol Crystallogr 2003;59(Pt 6):1103-1105.
128. Douglass JA, O'Hehir RE. 1. Diagnosis, treatment and prevention of allergic disease: the basics. Med J Aust 2006;185(4):228-233.
129. Yman L. Standardization of in vitro methods. Allergy 2001;56 Suppl 67:70-74.
130. Seagroatt V, Anderson SG. The second international reference preparation for human serum immunoglobulin E and the first British standard for human serum immunoglobulin E. J Biological Standardization 1981;9(4):431-437.
131. Valent P, *et al.* Assays for measuring in vitro basophil activation induced by recombinant allergens. Methods 2004;32(3):265-270.
132. Knol EF, *et al.* Monitoring human basophil activation via CD63 monoclonal antibody 435. J Allergy Clin Immunol 1991;88(3 Pt 1):328-338.
133. Hauswirth AW, *et al.* Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. J Allergy Clin Immunol 2002;110(1):102-109.
134. Mothes N, Valenta R, Spitzauer S. Allergy testing: the role of recombinant allergens. Clin Chem Lab Med 2006;44(2):125-132.
135. van der Veen MJ, *et al.* False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (Dermatophagoides pteronyssinus) allergens. J Allergy Clin Immunol 1996;98(6 Pt 1):1028-1034.
136. Kazemi-Shirazi L, *et al.* Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. Int Arch Allergy Immunol 2002;127(4):259-268.
137. Deinhofer K, *et al.* Microarrayed allergens for IgE profiling. Methods 2004;32(3):249-254.
138. Hesselmar B, *et al.* Does early exposure to cat or dog protect against later allergy development? Clin Exp Allergy 1999;29(5):611-617.
139. Ownby DR, Johnson CC, Peterson EL. Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. JAMA 2002;288(8):963-972.
140. Platts-Mills T, *et al.* Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet 2001;357(9258):752-756.
141. Almqvist C, *et al.* Direct and indirect exposure to pets - risk of sensitization and asthma at 4 years in a birth cohort. Clin Exp Allergy 2003;33(9):1190-1197.
142. Schäfer T, *et al.* Associations between cat keeping, allergen exposure, allergic sensitization and atopic diseases: results from the Children of Lubeck Allergy and Environment Study (KLAUS). Pediatr Allergy Immunol 2009;20(4):353-357.

143. Chen CM, *et al.* Longitudinal study on cat allergen exposure and the development of allergy in young children. J Allergy Clin Immunol 2007;119(5):1148-1155.
144. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;102(4 Pt 1):558-562.
145. Noon L. Prophylactic inoculation against hay fever. Lancet 1911;177:1572-1573.
146. Lockey RF, *et al.* Systemic reactions and fatalities associated with allergen immunotherapy. Ann Allergy Asthma Immunol 2001;87(1 Suppl 1):47-55.
147. Nettis E, *et al.* Safety of inhalant allergen immunotherapy with mass units-standardized extracts. Clin Exp Allergy 2002;32(12):1745-1749.
148. Winther L, *et al.* Side-effects of allergen-specific immunotherapy: a prospective multi-centre study. Clin Exp Allergy 2006;36(3):254-260.
149. van Ree R, *et al.* Possible induction of food allergy during mite immunotherapy. Allergy 1996;51(2):108-113.
150. Ball T, *et al.* Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. Eur J Immunol 1999;29(6):2026-2036.
151. Movérare R, *et al.* Development of new IgE specificities to allergenic components in birch pollen extract during specific immunotherapy studied with immunoblotting and Pharmacia CAP System. Allergy 2002;57(5):423-430.
152. Van Metre TE, Jr., *et al.* Immunotherapy for cat asthma. J Allergy Clin Immunol 1988;82(6):1055-1068.
153. Hedlin G, *et al.* Immunotherapy with cat- and dog-dander extracts. V. Effects of 3 years of treatment. J Allergy Clin Immunol 1991;87(5):955-964.
154. Alvarez-Cuesta E, *et al.* Monoclonal antibody-standardized cat extract immunotherapy: risk-benefit effects from a double-blind placebo study. J Allergy Clin Immunol 1994;93(3):556-566.
155. Varney VA, *et al.* Clinical efficacy of specific immunotherapy to cat dander: a double-blind placebo-controlled trial. Clin Exp Allergy 1997;27(8):860-867.
156. Trivedi B, Valerio C, Slater JE. Endotoxin content of standardized allergen vaccines. J Allergy Clin Immunol 2003;111(4):777-783.
157. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002;2(6):446-453.
158. Pauli G, *et al.* Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. J Allergy Clin Immunol 2008;122(5):951-960.
159. Jutel M, *et al.* Allergen-specific immunotherapy with recombinant grass pollen allergens. J Allergy Clin Immunol 2005;116(3):608-613.
160. Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006;6(10):761-771.
161. Ebner C, *et al.* Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. Clin Exp Allergy 1997;27(9):1007-1015.
162. Mothes N, *et al.* Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clin Exp Allergy 2003;33(9):1198-1208.
163. Ruedl C, Bachmann MF, Kopf M. The antigen dose determines T helper subset development by regulation of CD40 ligand. Eur J Immunol 2000;30(7):2056-2064.
164. Powell RJ, *et al.* Effect of grass pollen immunotherapy with Alutard SQ on quality of life in seasonal allergic rhinoconjunctivitis. Allergy 2007;62(11):1335-1338.
165. Kolbe L, Heusser CH, Kölsch E. Isotype-associated recognition of allergen epitopes and its modulation by antigen dose. Immunology 1995;84(2):285-289.

166. Francis JN, *et al.* Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. J Allergy Clin Immunol 2008;121(5):1120-1125 e1122.
167. Focke M, *et al.* Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. Clin Exp Allergy 2004;34(10):1525-1533.
168. Daeron M, *et al.* Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. J Clin Invest 1995;95(2):577-585.
169. van Neerven RJ, *et al.* Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. J Immunol 1999;163(5):2944-2952.
170. Wachholz PA, *et al.* Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. J Allergy Clin Immunol 2003;112(5):915-922.
171. Nouri-Aria KT, *et al.* Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. J Immunol 2004;172(5):3252-3259.
172. Valenta R, *et al.* Immunotherapy of allergic disease. Adv Immunol 2004;82:105-153.
173. Jutel M, *et al.* IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33(5):1205-1214.
174. Pilette C, *et al.* Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF-beta expression. J Immunol 2007;178(7):4658-4666.
175. Varney VA, *et al.* Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. J Clin Invest 1993;92(2):644-651.
176. Durham SR, *et al.* Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. J Allergy Clin Immunol 1996;97(6):1356-1365.
177. Jutel M, *et al.* Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. J Immunol 1995;154(8):4187-4194.
178. Bellinghausen I, *et al.* Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. Eur J Immunol 1997;27(5):1131-1139.
179. Benjaponpitak S, *et al.* The kinetics of change in cytokine production by CD4 T cells during conventional allergen immunotherapy. J Allergy Clin Immunol 1999;103(3 Pt 1):468-475.
180. Guerra F, *et al.* T_H2 lymphocytes from atopic patients treated with immunotherapy undergo rapid apoptosis after culture with specific allergens. J Allergy Clin Immunol 2001;107(4):647-653.
181. Gardner LM, O'Hehir RE, Rolland JM. High dose allergen stimulation of T cells from house dust mite-allergic subjects induces expansion of IFN-γ+ T Cells, apoptosis of CD4+IL-4+ T cells and T cell anergy. Int Arch Allergy Immunol 2004;133(1):1-13.
182. Tsai YG, *et al.* Induced apoptosis of TH2 lymphocytes in asthmatic children treated with Dermatophagoides pteronyssinus immunotherapy. Pediatr Allergy Immunol 2005;16(7):602-608.
183. Plewako H, *et al.* Basophil interleukin 4 and interleukin 13 production is suppressed during the early phase of rush immunotherapy. Int Arch Allergy Immunol 2006;141(4):346-353.
184. Hansen G, *et al.* Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. J Clin Invest 1999;103(2):175-183.
185. Dahl ME, *et al.* Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. Nat Immunol 2004;5(3):337-343.

186. Akdis CA, *et al.* Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102(1):98-106.
187. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 2003;111(6):1255-1261.
188. Bohle B, *et al.* Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J Allergy Clin Immunol 2007;120(3):707-713.
189. Radulovic S, *et al.* Grass pollen immunotherapy induces Foxp3-expressing CD4+ CD25+ cells in the nasal mucosa. J Allergy Clin Immunol 2008;121(6):1467-1472, 1472 e1461.
190. Vrtala S. From allergen genes to new forms of allergy diagnosis and treatment. Allergy 2008;63(3):299-309.
191. Frew AJ. Sublingual immunotherapy. N Engl J Med 2008;358(21):2259-2264.
192. Durham SR. Sublingual immunotherapy: what have we learnt from the 'big trials'? Curr Opin Allergy Clin Immunol 2008;8(6):577-584.
193. Canonica GW, Passalacqua G. Noninjection routes for immunotherapy. J Allergy Clin Immunol 2003;111(3):437-448; quiz 449.
194. Martinez-Gómez JM, *et al.* Intralymphatic Injections as a New Administration Route for Allergen-Specific Immunotherapy. Int Arch Allergy Immunol 2009;150(1):59-65.
195. Senti G, *et al.* Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. Proc Natl Acad Sci USA 2008;105(46):17908-17912.
196. Brewer JM. (How) do aluminium adjuvants work? Immunol Lett 2006;102(1):10-15.
197. Gupta RK, Siber GR. Adjuvants for human vaccines--current status, problems and future prospects. Vaccine 1995;13(14):1263-1276.
198. Broide DH. Immunostimulatory sequences of DNA and conjugates in the treatment of allergic rhinitis. Curr Allergy Asthma Rep 2005;5(3):182-185.
199. Puggioni F, Durham SR, Francis JN. Monophosphoryl lipid A (MPL) promotes allergen-induced immune deviation in favour of Th1 responses. Allergy 2005;60(5):678-684.
200. Gerstmayr M, *et al.* Natural self-assembly of allergen-S-layer fusion proteins is no prerequisite for reduced allergenicity and T cell stimulatory capacity. Int Arch Allergy Immunol 2009;149(3):231-238.
201. Edlmayr J, *et al.* A combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived surface protein VP1 and a nonallergenic peptide of the major timothy grass pollen allergen Phl p 1. J Immunol 2009;182(10):6298-6306.
202. Schmitz N, *et al.* Displaying Fel d1 on virus-like particles prevents reactogenicity despite greatly enhanced immunogenicity: a novel therapy for cat allergy. J Exp Med 2009.
203. Storni T, *et al.* Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev 2005;57(3):333-355.
204. Grönlund H, *et al.* Carbohydrate-based particles: a new adjuvant for allergen-specific immunotherapy. Immunology 2002;107(4):523-529.
205. Andersson TN, *et al.* A novel adjuvant-allergen complex, CBP-rFel d 1, induces up-regulation of CD86 expression and enhances cytokine release by human dendritic cells in vitro. Immunology 2004;113(2):253-259.
206. Thunberg S, *et al.* Prolonged antigen-exposure with carbohydrate particle based vaccination prevents allergic immune responses in sensitized mice. Allergy 2009;64(6):919-926.
207. Neimert-Andersson T, *et al.* Carbohydrate-based particles reduce allergic inflammation in a mouse model for cat allergy. Allergy 2008;63(5):518-526.
208. Ferreira F, *et al.* Modified recombinant allergens for safer immunotherapy. Inflamm Allergy Drug Targets 2006;5(1):5-14.
209. Velickovic TC, Jankov RM. Design and modifications of allergens for improving specific immunotherapy. Inflamm Allergy Drug Targets 2008;7(4):270-278.

210. Ferreira F, *et al.* Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996;183(2):599-609.
211. Wagner S, *et al.* Naturally occurring hypoallergenic Bet v 1 isoforms fail to induce IgE responses in individuals with birch pollen allergy. J Allergy Clin Immunol 2008;121(1):246-252.
212. Niederberger V, Valenta R. Molecular approaches for new vaccines against allergy. Expert Rev Vaccines 2006;5(1):103-110.
213. Vrtala S, *et al.* Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. J Clin Invest 1997;99(7):1673-1681.
214. Vrtala S, *et al.* Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. Faseb J 2001;15(11):2045-2047.
215. van Hage-Hamsten M, *et al.* Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. J Allergy Clin Immunol 1999;104(5):969-977.
216. van Hage-Hamsten M, *et al.* Nasal challenges with recombinant derivatives of the major birch pollen allergen Bet v 1 induce fewer symptoms and lower mediator release than rBet v 1 wild-type in patients with allergic rhinitis. Clin Exp Allergy 2002;32(10):1448-1453.
217. Vrtala S, *et al.* T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. J Immunol 2000;165(11):6653-6659.
218. Niederberger V, *et al.* Vaccination with genetically engineered allergens prevents progression of allergic disease. Proc Natl Acad Sci U S A 2004;101 Suppl 2:14677-14682.
219. Purohit A, *et al.* Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. Clin Exp Allergy 2008;38(9):1514-1525.
220. Gafvelin G, *et al.* Cytokine and antibody responses in birch-pollen-allergic patients treated with genetically modified derivatives of the major birch pollen allergen Bet v 1. Int Arch Allergy Immunol 2005;138(1):59-66.
221. Pree I, *et al.* Analysis of epitope-specific immune responses induced by vaccination with structurally folded and unfolded recombinant Bet v 1 allergen derivatives in man. J Immunol 2007;179(8):5309-5316.
222. Kahlert H, *et al.* Characterization of a hypoallergenic recombinant Bet v 1 variant as a candidate for allergen-specific immunotherapy. Int Arch Allergy Immunol 2008;145(3):193-206.
223. Kettner J, *et al.* Specific immunotherapy with recombinant birch pollen allergen rBet v 1-FV results of 2 years of treatment (Phase II trial). Allergy 2007;62(Suppl. 83):724.
224. Mothes-Luksch N, *et al.* Disruption of allergenic activity of the major grass pollen allergen Phl p 2 by reassembly as a mosaic protein. J Immunol 2008;181(7):4864-4873.
225. Vrtala S, *et al.* Genetic engineering of the major timothy grass pollen allergen, Phl p 6, to reduce allergenic activity and preserve immunogenicity. J Immunol 2007;179(3):1730-1739.
226. Linhart B, *et al.* Combination vaccines for the treatment of grass pollen allergy consisting of genetically engineered hybrid molecules with increased immunogenicity. Faseb J 2002;16(10):1301-1303.
227. Linhart B, *et al.* A hypoallergenic hybrid molecule with increased immunogenicity consisting of derivatives of the major grass pollen allergens, Phl p 2 and Phl p 6. Biol Chem 2008;389(7):925-933.
228. Olsson S, van Hage-Hamsten M, Whitley P. Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite *Lepidoglyphus destructor*. Mol Immunol 1998;35(16):1017-1023.
229. Kronqvist M, *et al.* A hypoallergenic derivative of the major allergen of the dust mite *Lepidoglyphus destructor*, Lep d 2.6Cys, induces less IgE reactivity and

cellular response in the skin than recombinant Lep d 2. Int Arch Allergy Immunol 2001;126(1):41-49.
230. Eriksson TLJ, *et al.* T cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*. Clin Exp Allergy 2001;31(12):1881-1890.
231. Gafvelin G, *et al.* Hypoallergens for allergen-specific immunotherapy by directed molecular evolution of mite group 2 allergens. J Biol Chem 2007;282(6):3778-3787.
232. Müller U, *et al.* Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 1998;101(6 Pt 1):747-754.
233. Fellrath JM, *et al.* Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. J Allergy Clin Immunol 2003;111(4):854-861.
234. Tarzi M, *et al.* Induction of interleukin-10 and suppressor of cytokine signalling-3 gene expression following peptide immunotherapy. Clin Exp Allergy 2006;36(4):465-474.
235. Norman PS, *et al.* Treatment of cat allergy with T-cell reactive peptides. Am J Respir Crit Care Med 1996;154(6 Pt 1):1623-1628.
236. Simons FE, *et al.* Fel d 1 peptides: effect on skin tests and cytokine synthesis in cat-allergic human subjects. Int Immunol 1996;8(12):1937-1945.
237. Maguire P, *et al.* The safety and efficacy of ALLERVAX CAT in cat allergic patients. Clin Immunol 1999;93(3):222-231.
238. Oldfield WLG, Larché M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. Lancet 2002;360(9326):47-53.
239. Alexander C, *et al.* The effect of Fel d 1-derived T-cell peptides on upper and lower airway outcome measurements in cat-allergic subjects. Allergy 2005;60(10):1269-1274.
240. Ali FR, *et al.* Late asthmatic reactions induced by inhalation of allergen-derived T cell peptides. Am J Respir Crit Care Med 2004;169(1):20-26.
241. Campbell JD, *et al.* Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. J Exp Med 2009.
242. Focke M, *et al.* Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. Faseb J 2001;15(11):2042-2044.
243. Zhu D, *et al.* A chimeric human-cat fusion protein blocks cat-induced allergy. Nat Med 2005;11(4):446-449.
244. Terada T, *et al.* A chimeric human-cat Fcgamma-Fel d1 fusion protein inhibits systemic, pulmonary, and cutaneous allergic reactivity to intratracheal challenge in mice sensitized to Fel d1, the major cat allergen. Clin Immunol 2006;120(1):45-56.
245. Vailes LD, *et al.* High-level expression of immunoreactive recombinant cat allergen (Fel d 1): Targeting to antigen-presenting cells. J Allergy Clin Immunol 2002;110(5):757-762.
246. Hulse KE, *et al.* Targeting Fel d 1 to FcgammaRI induces a novel variation of the T(H)2 response in subjects with cat allergy. J Allergy Clin Immunol 2008;121(3):756-762 e754.
247. Crameri R, *et al.* Design, engineering and in vitro evaluation of MHC class-II targeting allergy vaccines. Allergy 2007;62(2):197-206.
248. Martinez-Gómez JM, *et al.* Targeting the MHC class II pathway of antigen presentation enhances immunogenicity and safety of allergen immunotherapy. Allergy 2009;64(1):172-178.
249. ImVisioN Therapeutics AG M, Switzerland. In: www.imvision-therapeutics.com.
250. Wickman M, *et al.* The BAMSE project: presentation of a prospective longitudinal birth cohort study. Pediatr Allergy Immunol 2002;13 Suppl 15:11-13.

251. Aki T, *et al.* Immunochemical characterization of recombinant and native tropomyosins as a new allergen from the house dust mite, *Dermatophagoides farinae*. J Allergy Clin Immunol 1995;96(1):74-83.
252. Asturias JA, *et al.* Sequencing and high level expression in *Escherichia coli* of the tropomyosin allergen (Der p 10) from *Dermatophagoides pteronyssinus*. Biochim Biophys Acta 1998;1397(1):27-30.
253. Yi FC, *et al.* Identification of shared and unique immunoglobulin E epitopes of the highly conserved tropomyosins in *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. Clin Exp Allergy 2002;32(8):1203-1210.
254. Jeong KY, *et al.* Molecular cloning and the allergenic characterization of tropomyosin from *Tyrophagus putrescentiae*. Protein Pept Lett 2007;14(5):431-436.
255. Ayuso R, Lehrer SB, Reese G. Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). Int Arch Allergy Immunol 2002;127(1):27-37.
256. Witteman AM, *et al.* Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. Int Arch Allergy Immunol 1994;105(1):56-61.
257. van Ree R, *et al.* Asthma after consumption of snails in house-dust-mite-allergic patients: a case of IgE cross-reactivity. Allergy 1996;51(6):387-393.
258. Santos ABR, *et al.* Cockroach allergens and asthma in Brazil: identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. J Allergy Clin Immunol 1999;104(2 Pt 1):329-337.
259. Peroni DG, *et al.* Snail anaphylaxis during house dust mite immunotherapy. Pediatr Allergy Immunol 2000;11(4):260-261.
260. Pajno GB, *et al.* Harmful effect of immunotherapy in children with combined snail and mite allergy. J Allergy Clin Immunol 2002;109(4):627-629.
261. Jeong KY, *et al.* Immunoglobulin E binding reactivity of a recombinant allergen homologous to alpha-Tubulin from *Tyrophagus putrescentiae*. Clinical Diag Lab Immunol 2005;12(12):1451-1454.
262. Zeller S, *et al.* Exploring the repertoire of IgE-binding self-antigens associated with atopic eczema. J Allergy Clin Immunol 2009;124(2):278-285, 285 e271-277.
263. Mandelkow E, Mandelkow E-M. Microtubule structure. Curr Opin Struct Biol 1994;4:171-179.
264. Berge M, Munir AK, Dreborg S. Concentrations of cat (Fel d1), dog (Can f1) and mite (Der f1 and Der p1) allergens in the clothing and school environment of Swedish schoolchildren with and without pets at home. Pediatr Allergy Immunol 1998;9(1):25-30.
265. Partti-Pellinen K, *et al.* Occurrence of dog, cat, and mite allergens in public transport vehicles. Allergy 2000;55(1):65-68.
266. Almqvist C, *et al.* Worsening of asthma in children allergic to cats, after indirect exposure to cat at school. Am J Respir Crit Care Med 2001;163(3 Pt 1):694-698.
267. Guttormsen AB, *et al.* No consumption of IgE antibody in serum during allergic drug anaphylaxis. Allergy 2007;62(11):1326-1330.