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ALLERGY TO PETS; MOLECULAR APPROACHES FOR IMPROVED DIAGNOSTICS AND VACCINATION

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Cover illustration by Tatyana Sandalova. Surface representation of the theoretical model of the linked construct (Can f 1-Can f 2-Can f 4-Can f 6, from left to right) from **paper IV**. The surface is coloured by electrostatic potential.

Illustrations on pages 5, 8 (Fig. 2a) and 19 by Anna Zoltowska

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Till mormor

Stop me if you think you've heard this one before

Steven Morrissey and Johnny Marr

ABSTRACT

Allergic diseases are a major health problem, affecting up to 30% of the population in affluent countries. Domestic pets such as cats, dogs and horses are among the most common agents of IgE-mediated allergies. Conventional diagnostics and allergen-specific immunotherapy (SIT) still rely on crude allergen extracts, even though several commercial extracts have demonstrated poor quality with regard to the included allergen content. Particularly in dog allergy, SIT may show a low clinical effectiveness upon treatment with allergenic extracts. As an alternative, recombinant DNA technologies allow for the construction of recombinant (r) allergens for improved allergy diagnostics and induction of a long-lasting protection by SIT. However, this therapy is still limited by unwanted side-effects such as IgE-mediated acute reactions, or late phase reactions (LPR). To combat these limitations, allergens have been modified for safer use in SIT, i.e. hypoallergens, or several allergenic molecules have been combined in one construct for improved diagnostics and treatment of allergic patients.

The aim of this thesis was to demonstrate how development in molecular allergology may increase the understanding of the allergenicity and cross-reactivity of dog allergens. We also set out to demonstrate how this knowledge can be used to engineer safer and more efficacious allergenic constructs for improved diagnostics and therapy.

In **paper I**, we described the crystal structure and the structural characteristics of the dog lipocalin allergen Can f 2. The recombinant protein was shown to mimick the IgE-binding characteristics of the natural allergen. Furthermore, we demonstrated an IgE-mediated cross-reactivity between Can f 2 and the cat lipocalin allergen Fel d 4, and suggested a potential conserved epitope as a cross-reactive site. In **paper II**, the primary structures for Fel d 4 and the major horse allergen Equ c 1 were used to identify a novel dog lipocalin allergen, Can f 6. The recombinant allergen elicited a positive IgE-reaction in ~40% of dog-sensitized subjects, and most individuals also had a positive reaction to the homologous allergens Fel d 4 and Equ c 1. The biological activity of rCan f 6 and the homologous allergens was demonstrated by basophil activation test (BAT). Finally, we identified IgE-mediated cross-reactivity between these three allergens, which may account for cross-species sensitization between dog, cat and horse.

In **paper III**, the method phage display was for the first time used to construct hypoallergenic mutants of the major cat allergen, Fel d 1. The four vaccine candidates were produced as folded proteins in *E. coli* and showed a lower IgE-binding than the wild type protein in ELISA. By T cell proliferation and BAT assays, we demonstrated that two of the four candidates had a significantly lower T cell activation capacity. Moreover all mutants had a lower allergenic activity than rFel d 1, which could indicate a safer profile for SIT of allergic patients. The effectiveness of the mutants was demonstrated in a mouse model for cat allergy, by the induction of blocking IgG antibodies. In **paper IV,** a different vaccine concept was described, where four dog lipocalin allergens were assembled in one molecule. The corresponding fusion protein comprised the biochemical and immunological properties of the original allergens, investigated by CD-spectra and ELISA. Importantly, the linked construct bound more IgE than the individual allergens, demonstrating the usefulness for diagnostics. Finally, the linked molecule induced comparable IgG levels to all included allergens compared with an equimolar mix, while showing a lower T cell reactivity which could implicate a lower risk of LPR.

To summarize, this thesis demonstrates how molecular approaches may be used to improve our understanding of allergenicity, to characterize new allergens and define their relationship with other allergens. This detailed knowledge was used to formulate modified allergen constructs for improved diagnosis and vaccination of allergic patients.

LIST OF PUBLICATIONS

I. Chaithanya Madhurantakam†, Ola B. Nilsson†, Hannes Uchtenhagen, Jon Konradsen, Tiiu Saarne, Erik Högbom, Tatyana Sandalova, Hans Grönlund†, Adnane Achour†
† Shared first and last authors
Crystal Structure of the Dog Lipocalin Allergen Can f 2: Implications for Cross-reactivity to the Cat Allergen Fel d 4.
The Journal of Molecular Biology 2010; Mar;21(2 Pt 1):277-283

II. Ola B. Nilsson, Jonas Binnmyr, Anna Zoltowska, Tiiu Saarne, Marianne van Hage, Hans Grönlund
Characterization of the dog lipocalin allergen Can f 6: the role in cross-reactivity with cat and horse.
Allergy, 2012*;* Jun;67(6):751-757.

III. Ola B. Nilsson, Justus Adedoyin, Claudio Rhyner, Theresa Neimert-Andersson, Jeanette Grundström, Kurt D. Berndt, Reto Crameri, Hans Grönlund
In Vitro Evolution of Allergy Vaccine Candidates, with Maintained Structure, but Reduced B Cell and T Cell Activation Capacity.
PLoS One 2011; 6(9):e24558

IV. Ola B. Nilsson, Mattias Bronge, Jeanette Grundström, Hannes Uchtenhagen, Adnane Achour, Erik Holmgren, Marianne van Hage, Hans Grönlund
Engineering and evaluation of a linked molecule comprising four dog lipocalin allergens for improved allergy diagnosis and vaccination
Manuscript

Publications not included in this thesis

Chaithanya Madhurantakam, Ola B. Nilsson, Klas Jönsson, Hans Grönlund, Adnane Achour
Production, crystallization and preliminary X-ray diffraction analysis of the allergen Can f 2 from Canis familiaris.
Acta Crystallogr Sect F Struct Biol Cryst Commun. 2009; May 1;65(Pt 5):467-71.

Jeanette Grundström, Theresa Neimert-Andersson, Cecilia Kemi, Ola B. Nilsson, Tiiu Saarne, Mats Andersson, Marianne van Hage, Guro Gafvelin
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Pediatr Allergy Immunol. 2012 Mar 21. DOI: 10.1111/j.1399-3038.2011.01260.x.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
APT	Atopy patch test
BAL	Bronchoalveolar lavage
BAT	Basophil activation test
CD	Cluster of differentiation
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
Ig	Immunoglobulin
IEC	Ion exchange chromatography
IMAC	Immobilised metal chelate affinity chromatography
ISAC	Immuno Solid-phase Allergen Chip
LPR	Late phase response
LPS	Lipopolysaccharide
MAT	Modular antigen translocation
MHC	Major histocompatibility complex
NK cell	Natural killer cell
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
SEC	Size exclusion chromatography
SIT	Allergen-specific immunotherapy
SPT	Skin prick test
TGF	Transforming growth factor
TLR	Toll-like receptor
Treg	Regulatory T cell

1 INTRODUCTION

IgE-mediated allergic disease is a major health problem affecting a significant number of individuals in western countries [1, 2]. Symtoms include rhinitis, wheezing and hay fever, and the disease may progress into asthma, which can even be fatal in rare cases. The onset of allergic disease is a result of the immune system interpreting otherwise halmless antigens as foreign, leading to an imbalanced and harmful immune response. Knowledge of the immune system is the basis for the understanding of allergic reactions.

1.1 THE IMMUNE SYSTEM

The immune system of mammals is essentially a "work-in-progress", as it has evolved over millions of years to combat infectious agents of various types. This evolution has allowed the co-existence of mammals with rapidly evolving pathogens, such as bacteria, viruses, fungi or parasites. During courses of history, the human immune system has occasionally been subjected to high evolutionary pressure, e.g. during epidemics such as the plague of Athens (430 BC), where survival was based on the shape of the immune system of the host [3]. This is also the earliest known reference of the immune system, as Thucydides during that time observed that patients who had recovered from the plague would not develop symptoms a second time.

1.1.1 Initiation of the immune response

To face the broad and complex repertoire of different pathogenic agents, the immune system has developed a number of mechanisms to cope with our environment. These mechanisms are usually divided into two parts: the innate and the adaptive immune system [4, 5]. As a first line of defense, the innate immune system contains physical barriers, such as epithelial cell barriers, that prevent most pathogens from entering the body. However, in the event of foreign substances circumventing these barriers, a primary cell response immediately acts to control and in most cases eliminate the invading pathogen. This rapid defence mechanism is unspecific, and depends on recognition of common pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and peptidoglycan, by Toll-like receptors (TLR) on the surface of immune cells. Important innate immune cells are natural killer (NK) cells, neutrophils and phagocytic cells such as macrophages or dendritic cells (DC).

1.1.2 The adaptive immune system

DCs also play a pivotal role as antigen presenting cells (APC) in the initiation phase of the adaptive immune system, monitoring the periphery and engulfing foreign antigen, transporting it to peripheral lymphoid tissues such as lymph nodes and the spleen, and presenting it to T lymphocytes (T cells) [4, 5]. The adaptive immune system is considerably slower than the innate immune system, though it is highly specific for the presented antigen. A key mechanism of the adaptive immune system is the presentation of linear peptides on major histocompatibility complex (MHC) molecules, typically situated on macrophages, DCs, B lymptocytes (B cells) and other APCs. This antigen presentation is facilitated by either MHC class I molecules, presenting digested peptides to cytotoxic T cells expressing the co-receptor CD8, or by MHC class II molecules, expressing proteolytically digested proteins to T cells carrying the CD4 co-receptor. The latter mechanism is the onset of formation of immunologic memory; involving differentiation

of CD4+ T cells into T helper type 1 (Th1) or T helper type 2 (Th2) cells that ultimately help to activate B cells.

1.1.3 T lymphocytes

Both Th1 and Th2 cells are considered to be derived from the same naïve T cell subset, originally able to produce all cytokines that later are restricted to each subset post differentiation. These naïve T cells then proliferate into mature subtypes by cytokine stimulation, such as IL-12 (Th 1 cells) and IL-4 (Th 2 cells). In order to distinguish Th1 cells from Th2 cells, these cell populations have traditionally been divided based on their different effector cytokine profiles and functional roles [6]. Th1 cells typically produce interferon gamma (IFN-γ), and are involved in immunity against intracellular pathogens. The subsequent cascade involves activation of macrophages and an increased T-cell proliferation [4, 5]. Th2 responses are mainly directed towards extracellular pathogens such as fungal and helminth parasites, possibly initiated by IL-4 production by eosinophils, mast cells and basophils once activated with the parasite-associated polysaccharide chitin. Effector responses of CD4+ Th2 cells involve the production of cytokines like IL-4, IL-5, IL-13 and IL-33, and the class switch of B cells to produce IgE and further clonal expansion of these cells to form memory B cells. Recently, novel Th subsets have been described, i.e. Th17, Th9 and Th22 cells [7]. Th17 cells have shown to be involved in protection against extracellular pathogens, as well as to promote tissue inflammation. Th9 and Th22 cells mainly produce IL-9 and IL-22 cytokines respectively, and have been proposed to be involved in tissue inflammation, although their exact function is still unclear.

1.1.4 B lymphocytes

Different subsets of B cells are defined by the type of surface-bound Ig B cell receptor they express, the type of antigen recognized and by which type of Ig they produce [4, 5].
B cells are formed and matured in the bone marrow, where they undergo surface immunoglobulin re-arrangement that is essential for establishing a diverse B cell repertoire. This recombination of different germline-encoded segments will result in the production of a surface-bound Ig with a pre-defined specificity. A crucial step in the maturation of B cells is the negative selection of clones that recognize self antigens, leading to apoptosis of these autoreactive cells. Mature B cells that are transported to the periphery initially express membrane-bound IgM, designed for early recognition of foreign surface antigens. However, upon antigen recognition and co-activation with Th cells, the B cell undergo class switch to a more specialized isotype, such as IgG or IgE. This class switch is then closely followed by affinity maturation of the produced antibody, differentiation into effector cells such as plasma cells, and ultimately to formation of long-lived memory cells that may rapidly respond and re-expand upon subsequent antigen encounter.

1.1.5 Self-tolerance

Although DCs, T cells, B cells and other immune cells all play their own crucial roles in protecting us from foreign pathogens, a proper regulation of immune responses is essential in order to avoid unnecessary damage to host cells, especially to prevent the development of chronic conditions, such as allergy or autoimmunity. Several mechanisms of both the innate and adaptive immune system exist to provide the ability to distinguish self from non-self, i.e. self-tolerance. Patrolling DCs in the periphery continuously take up and process foreign or self antigens, hence presenting these in lymphoid tissues. Usually, this presentation is carried out without the co-stimulatory molecules CD80/CD86 on DCs, resulting in clonal deletion or induction of anergy (tolerance) [4, 5].

Both B and T cells undergo re-arrangement of receptor genes to allow for binding to a large variety of antigens and peptides. Unlike B cells that undergo negative selection in the bone marrow, T cells mature in the thymus where clones that recognize self peptides are eliminated. Although some autoreactive B and T cells make it to the periphery, their presence will in most cases not cause any injury, due to other regulatory mechanisms [5].

1.1.6 Regulation of the immune response

During the initiation of the adaptive immune response, DCs play a general role for the differentiation of naïve T cells into Th cells of different subtypes [8]. The overall cytokine profile and neighbouring cell milieu influence whether Th1, Th2, Th17, Th9 or Th22 cells are formed. It is generally accepted that different Th subset induce their own regulation of other subsets and control the immune response [9, 10]. As an example, induction of Th1 transcriptional regulator T-box expressed in T cells (T bet) inhibits migration of Th2 and Th17 cells to the airways [11].

Not many years ago, a novel T regulatory (Treg) cell subset was described that increased the understanding of how peripheral tolerance is maintained [12]. This CD4+ CD25+ subset is characterized by expression of the transcription factor Foxp3 [13], and distinguished by its IL-10 and TGF-β production [14]. The exact mechanism of peripheral tolerance exhibited by Tregs is not completely understood; however, several pathways have been suggested. Such mechanisms involve direct inhibitory contact with neighbouring DCs [15], inhibition of T-cell responses by secretion of CTLA4 and IL10 [16, 17], and by tolerance induction through TGF-β [18].

1.2 ALLERGY

Allergic disease arise when the immune system interprets harmless antigens, i.e. allergens, as pathogenic, hence eliciting a specific immune response against the antigen. The allergic response can be either antibody or cell-mediated. Each allergic reaction is distinguished from the normal response to allergens in healthy individuals, where an active immune regulation ensures tolerance to the allergen [1]. Allergic symptoms include immediate reactions, such as rhinitis, conjunctivitis, urticaria and angioedema, to more chronic conditions such as asthma, and in rare cases even fatal reactions, e.g. anaphylaxis. During the last decades, the prevalence of IgE-mediated allergies have increased, affecting roughly 25-30% of the population [19, 20], and up to 50% of children [21], in industrialized countries, although the increasing trend seems to have stabilized [21]. Onset of allergic disease may already begin *in utero* [22], and mostly develop during childhood with the outbreak of food allergies and atopic eczema, which often progress into allergic rhinitis to inhalant allergens and asthma of the upper airways [23]. Particularly in children with asthma, sensitization to airborne allergens occurs early in life [24, 25]. The exact aetiology of allergic disease is not completely understood. It is evident that both genetic and environmental factors play important roles [26]. Individuals with a genetic susceptibility for development of allergic diseases are called atopic [1]. Genome-wide association studies have attempted to predict the risk of developing allergy and asthma, based on common susceptibility alleles [27, 28], although such studies have failed to a large extent due to the complex nature of development of chronic inflammatory disease [29]. It has become clear that allergic disease and asthma rely on separate sets of genes and polymorphisms related to regulation of the immune response as well as to epithelial function [29]. Due to observations of the dramatic increase of prevalence of allergic disease during recent decades, and the fact that the genetic profile population-wise does

not change within this time frame, the escalation has prominently been attributed to environmental factors [30].

Numerous environmental factors have been correlated with an increase in allergic disease, such as air pollution, post-natal tobacco smoke and pre-natal maternal tobacco smoking [31]. However, life style changes in "western industrialized countries" have been attributed to the drastic increase in allergies, suggested by the "hygiene hypothesis" [32]. Simplified, the hygiene hypothesis states that by a decrease in incidence of infectious diseases, the immune system lacks necessary stimuli needed for skewing towards Th1 responses, promoting a Th2 shift instead. This connection was proposed in 1989, when Strachan described an inverse correlation between the number of older siblings and hay fever [33], or in other words, the more childhood infections, the fewer allergies. Since then, several epidemiological studies have been published, supporting the findings by Strachan [34], implying that individuals lacking necessary Th1 stimuli during the neonatal period, have an increased risk of developing autoimmune and allergic diseases. Suggested mechanisms include a "cleaner lifestyle" in developed countries, an increased usage of antibiotics and fewer numbers of siblings leading to a decreased exposure to infections in the household. Another theory states that the lack of microbial stimuli, mainly in the gastrointestinal tract, prevents the formation of Tregs that are necessary to maintain tolerance and prevent Th2 development [35].

1.2.1 The allergic immune response

When an allergen enters the body for the first time, an unfortunate set of events may occur, and lead to sensitization. The term "allergy" was introduced over 100 years ago, when von Pirquet described a quick and malicious immune response directed towards the host, which occurred in some subjects after a second injection of smallpox developed in horses [36], a response that was separated from the protective immune response induced by the vaccine. However, the serum component responsible for allergic reactions, IgE, was not identified until 1966-1967 by Ishizaka and Johansson [37, 38]. The immunoglobulin was characterized in 1972, and shown to be relatively stable, and contain 2.42 ng IgE per unit [39].

1.2.1.1 Allergic sensitization

DCs that are located at mucosal surfaces like the lungs, the gut or the mucosal surface of the skin, may initiate the sensitization phase upon encountering a new allergen (Fig. 1a) [2]. Antigen presentation to naïve T cells occurs in draining lymph nodes, provoking a Th2 response, leading to clonal expansion and the production of cytokines like IL-4, IL-5 and IL-13. This reaction is separated from the tolerogenic state induced by T cells from healthy individuals, and as previously discussed; this might occur for many reasons. Particularly, IL-4 might be an important contributor, as both mast cells and especially basophils can produce and secrete high amounts of IL-4, implicating them as actors in the initiation phase of allergy [40]. Neighbouring naïve B cells, carrying complexes of IgM recognizing the allergen, will take up the antigen and present it to primed T cells. Supported by stimuli from IL-4 and IL-13, this will promote class switching of the B cell from IgM to IgE, and the formation of latent memory cells [41]. The clonal expansion of allergen-specific B cells causes an increased production of allergen-specific IgE that will bind to the high and low-affinity receptors for IgE, FcεRI on mast cells and dendritic cells, and FcεRII (CD23) on B cells respectively, contributing to both immediate and late allergic responses [2]. In addition, the production of IL-5 by Th2 cells increase the production and survival of eosinophils, contributing to downstream allergic reactions [42].

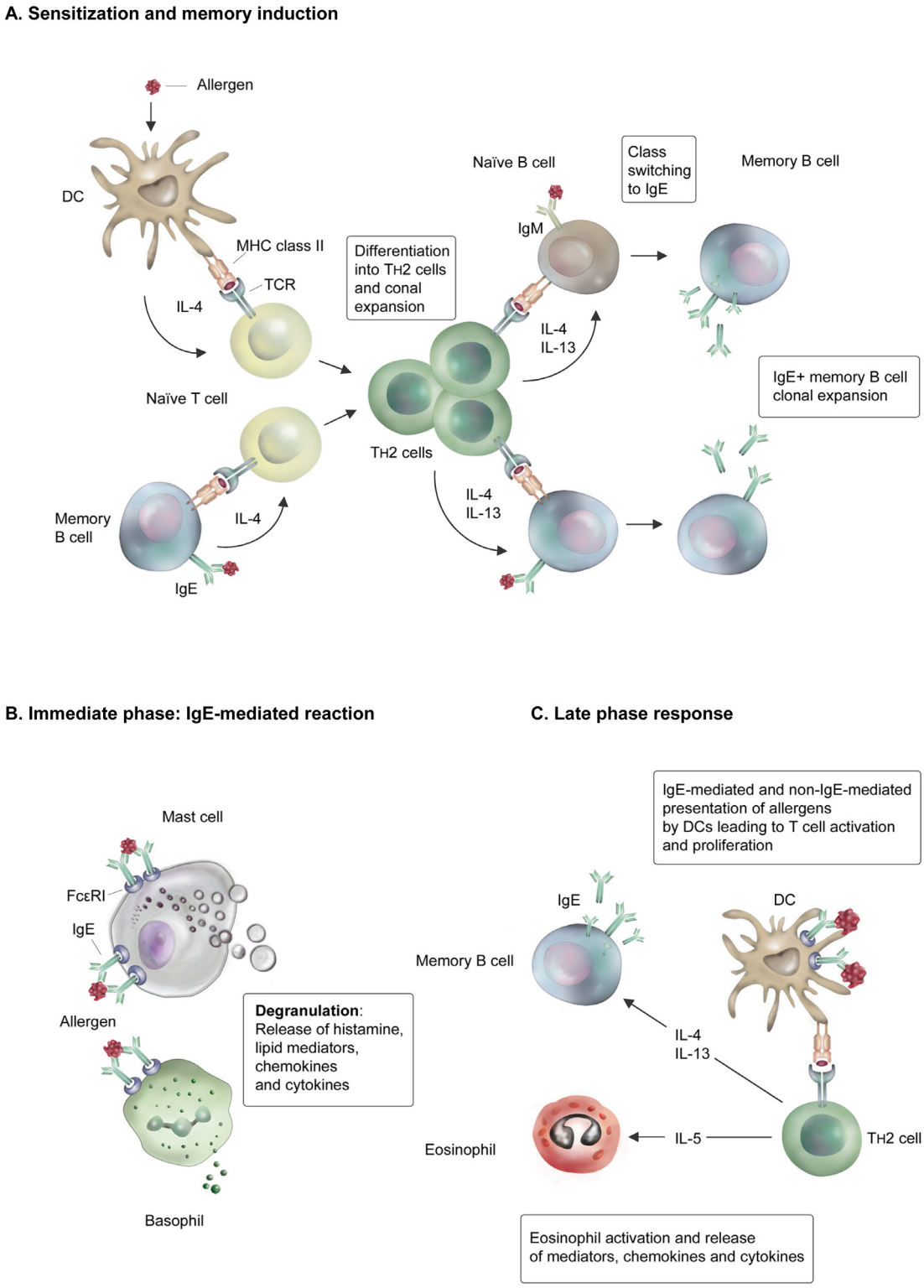

Figure 1. Overview of the allergic immune response. (A) Allergen uptake by mucosal DCs and antigen presentation to naïve T cells. T cells are differentiated into T_H2 cells that may induce class switch of naïve B cells to IgE-producing B cells and memory B cells. (B) The immediate phase reaction induces degranulation upon allergen cross-linking of surface bound IgE on mast cells and basophils. (C) The late phase response is initiated by allergen presentation to T_H2 cells. They become activated and produce inflammatory cytokines that promote the recruitment of eosinophils and stimulate IgE production by memory B cells. DC, dendritic cell; IL, interleukin. (Modified from [43])

1.2.1.2 The immediate-phase reaction

Subjects previously sensitized to allergens carry the risk of eliciting an immediate allergic reaction upon repeated allergen exposure (Fig. 1b). Allergen cross-linking of IgE on mast cells and basophils also cross-link FcεRI upon activation of two neighbouring receptors, leading to an activation cascade, initiating degranulation from the activated cell [2]. Typical mediator release include histamines, lipid mediators (Prostaglandin D, leukotriences, platelet-activating factor), chemokines and the cytokines IL-4, IL-5 and IL-13 [43]. Symptoms arise within minutes after allergen encounter, including oedema caused by increased vascular permeability, rhinitis, conjunctivitis and mucus production in the upper airways. In addition, asthmatic individuals might suffer from increased bronchoconstriction and oedema of the lower airways, inflicting wheezing, cough and anaphylaxis [2].

1.2.1.3 The late phase response

Unlike the immediate response that occurs within minutes, a second late phase reaction (LPR) might evolve several hours and last for several days after allergen encounter (Fig. 1c) [44]. The LPR has been attributed to a re-activation of T cell responses, e.g. antigen presenting cells such as DCs and B cells bind the allergen through FcεRI and FcεRII, thus stimulating antigen uptake and presentation to specific T cells [2, 44]. Support for the theory of IgE-mediated allergen presentation as inductive agent for the LPR can be derived from recent studies, where treatment of allergic patients with anti-IgE antibodies, i.e. Omalizumab, has been shown to reduce late phase responses [43, 45]. Additionally, immediate mediator release by basophils and mast cells may promote cellular activation, as well as promote recruitment of additional immune cells to the site of allergen encounter through increased blood flow and vascular permeability. Cellular recruitment involves basophil recruitment and migration to tissues by IL-4 and IL-13, and eosinophil recruitment by IL-5, resulting in further degranulation and promotion of allergic responses. Finally, LPR may play a central role in the development of asthma, since elevated levels of allergen-specific T cells have been detected in BAL fluid, bronchial mucosa and sputum of asthmatic patients [46].

1.3 ALLERGENS

Allergens are defined as antigens (usually proteins) that may induce IgE-production and elicit IgE-responses in sensitized individuals [47]. Most allergens are derived from plant, fungi or animal families, although they can be found in several other sources. As of May 2012, more than 700 allergens have been submitted to the official list of allergens, maintained by the World Health Organization (WHO) and International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee [48], and the number is steadily increasing. In addition, the Allergome project attempts to gather all current knowledge of allergenic molecules from various sources, and as of May 2012, more than 2300 allergenic molecules are listed in the database, not counting isoforms and epitopes [49]. The names of allergens are determined by the first three letters of the genus in Latin, followed by the first letter derived from the species name, and the number of appearance by discovery. Thus, the sixth allergen discovered in the domestic dog, *Canis familiaris*, is named Can f 6 [47, 50]. When an allergen is characterized, much focus is on whether the allergen is a major or a minor allergen, i.e. whether it is recognized by more or less than 50% of the patients sensitized to that allergen source [51, 52]. The designation of whether an allergen is a major or minor allergen is not absolute, and depends on the characteristics of the study population used to determine the IgE profile. As such, it is

likely that an allergen designated “minor” by one group, may be labelled as “major” by another.

1.3.1 Allergenic and structural characteristics of allergens

Details on allergenicity of proteins, or “what makes an allergen an allergen” varies for different protein families with their structural and functional characteristics. In most cases, the allergenic characteristics of proteins are puzzling or inconclusive, and we are only beginning to understand the underlying mechanisms [47]. It is clear that the dose, route of exposure and function relates to the potency of the allergen. In general terms, exposure to high allergen doses seem to induce a tolerogenic response through Tregs and IgG4-production, rather than low dose allergen exposure, causing IgE synthesis and allergy [53, 54]. This relationship may be heavily influenced by differences in MHC class II haplotypes across individuals, and the resulting effect on presentation to T cells. In other words, high allergen doses for some subjects may be presented as low allergen doses by others, due to varying affinities of different MHC molecules [47]. The link between dose-dependent allergen exposure and risk of sensitization is controversial, as several studies have come to different conclusions. As exemplified by allergy to cat, studies in areas with low exposure to cat (ownership frequency below 5%), have shown that an increased risk of sensitization is associated with cat ownership or exposure to cat dander [55-57]. On the other hand, a study in an area with higher ownership frequency found that cat ownership was inversely correlated with allergic symptoms, suggesting a possible protective effect by cat allergen exposure [58]. Allergen encounter often happens due to inhalation of, ingestion of, bite and skin contact with the antigen. Carrier molecules and other non-allergenic substances that accompany the allergens might affect the resulting immune response, e.g. by binding to PAMPs such as TLRs or c-type lectins [47]. A major amount of allergens are delivered as airborne particles such as dust, pollen grains or animal dander, enabling exposure to mucosal surfaces of the airways. It would also seem that inhalant allergens are required for development of asthma, since allergenic epitopes, such as galactose-alpha-1,3-galactose (alpha-gal), present for instance in red meat and on cat IgA [59, 60], do not associate with the development of asthma, while inducing positive skin responses and severe reactions such as anaphylaxis in allergic patients [61].

Not all proteins or glycoproteins carry the risk of eliciting IgE-mediated allergies, and in fact, less than 2% of all protein families constitute known allergens [47]. Most allergens are stable, hydrophilic, negatively charged and relatively small proteins (<70 kDa) [50]. Several allergens are stabilized by disulfide bonds and post-translational mechanisms such as glycosylation. Even though these properties might enhance folding and stability of the allergen [62], they do not necessarily affect the IgE binding. In fact, most carbohydrates present on allergens, although diagnostically relevant in terms of IgE binding [63], do not induce allergen-specific degranulation [64]. However, this is not always true, as exemplified by the carbohydrate IgE-epitope alpha-gal, which has been shown to provoke anaphylaxis and urticaria in meat allergic patients [65].

The biological functions of allergens might relate to their allergenic activity. Several allergens with enzymatic activity have been identified in extracts of the house dust mite and subsequently cloned, e.g. Der p 1, Der p 3, Der p 6 and Der p 9 [52]. Examplified by the major mite allergen Der p 1, its activity as a cystein protease may relate to its potency, as it has the ability to cleave tight junctions between cells, thus increasing the permeability for allergen entry [66]. Nevertheless, several potent mite allergens are not enzymes, including the major allergen Der p 2, indicating that enzymatic activity is not required [50]. On the other hand, Der p 2 share structural homology with a lipid binding

protein, and has been determined to bind LPS [67], possibly relating to allergenic activity. In addition, Der p 2 binds and facilitates signaling through TLR4. This underlying mechanism might be central for Der p 2 sensitization, as experimental allergic asthma was induced following Der p 2 challenge of normal mice, but not in TLR4-deficient mice [68].

1.3.2 Epitopes

Linear amino acid sequences and three-dimensional characteristics of allergens are perhaps the most important features for allergenicity. By structural comparison of allergenic molecules with similar proteins, we can predict allergenicity based on shared structural motifs that are recognized by T cells or B cells, e.g. T or B cell epitopes [69]. T cell epitopes of allergens are linear peptides, presented on MHC class II molecules by APCs, and are recognized by the T cell receptor. Basic knowledge of human T cell epitopes and how short linear peptides could be used to induce tolerance through vaccination, existed even before basic mechanisms of antigen presentation was fully understood [70]. However, the term epitope is more frequently used to define 'specific antigenic determinant sites on a molecule', recognized by different immunoglobulins produced by B cells [71]. For each epitope, there is a corresponding paratope on the antibody, i.e. the recognition site of the combined variable heavy and light chains (Fig 2). It has been shown that one epitope may be recognized by several paratopes. Also, one monoclonal antibody might recognize several epitopes that share similar characteristics, which most likely is due to cross-reactivity (discussed below). At the same time, it has been shown that one single antibody may contain several paratopes that may interact with multiple epitopes, also defined as multi-reactivity of that antibody [72]. As such, it may be troublesome to distinguish cross-reactivity from multi-reactivity.

IgE-epitopes may be either conformational (i.e. dependent on structural fold of three-dimensional domains or dimerization of whole allergens), or linear (single chain of amino acids) [73]. As such, linear peptides containing motifs of both linear and conformational epitopes, as well as unfolded proteins, still carry the risk of inducing IgE responses, though they are likely to be much weaker than those elicited by fully folded allergens [74], since conformational epitopes are lost when three-dimensional structures are disrupted. On the other hand, vaccination with adjuvants might partially unfold the

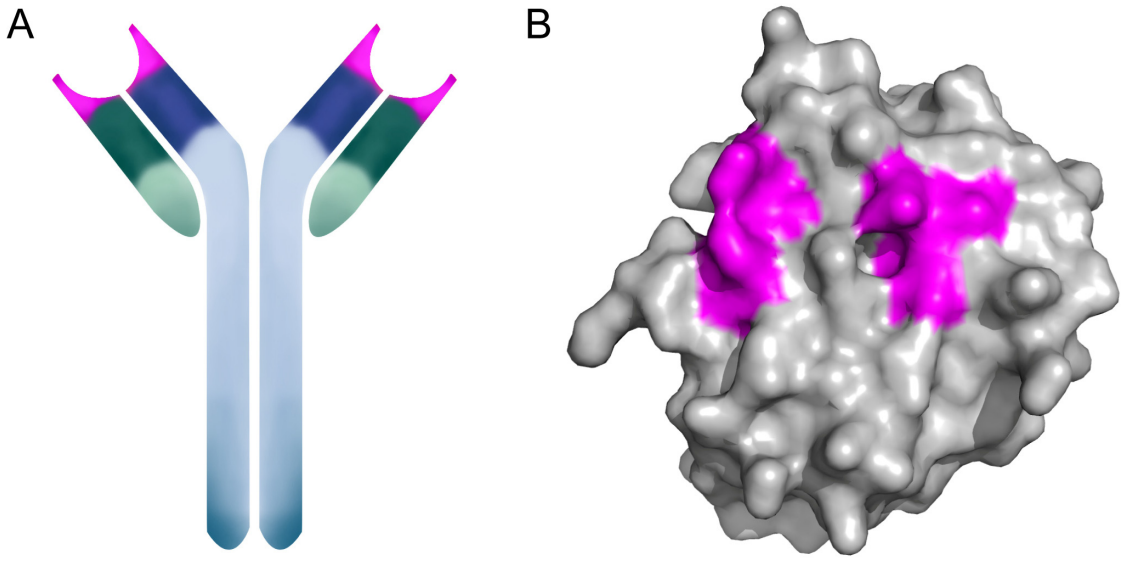

Figure 2. Structural features of IgE-binding. (A) Schematic overview of an IgE antibody, with the paratope coloured in magenta. (B) Modelling of protein surface areas of Fel d 1, with two suggested epitope regions coloured in magenta (from **paper III**).

protein, indicating that linear epitopes might be important considerations as well [75]. Identification of both linear, as well as conformational IgE-epitopes serves as basis for postulation of different allergy vaccines, discussed in section 1.6.2.

As a general rule, two IgE-binding epitopes, must be present on an allergen to cross-link two FcεRI receptors on basophils and mast cells [52]. Hypothetically, by adding more epitopes to an allergen, either by adjustment of folding or oligomerization, an allergen should be able to crosslink FcεRI on mast cells and basophils more efficiently, making it more allergenic [74]. This theory is also supported by recent data, describing dimeric and monomeric forms of the cow milk allergen β-lactoglobulin, Bos d 5, where the dimeric form had an increased degranulation capacity in comparison with the monomeric protein [76]. Additional evidence for this mechanism is provided in paper IV of this thesis, where linkage of four allergens into the same molecule resulted in an increased sensitivity for basophil degranulation, as compared with a mix of the same four allergens. In other words, the conformational organization of epitopes into one molecule, rather than the total sum of immunoreactive IgE-epitopes, enhanced the overall basophil degranulation capacity.

1.3.3 Cross-reactivity

Distinguishing cross-reactivity from co-sensitization can sometimes be confusing, due to different definitions being used to define cross-reactivity in separate studies. In this thesis, the term cross-reactivity is used both to define antibody-mediated cross-reactions, i.e. when the same paratope of an antibody recognizes two similar epitopes on separate antigens, as well as T cell mediated cross-reactivity, e.g. when identical or similar linear peptides are being presented to CD4+ T cells on MHC class II molecules [71, 77, 78]. However, some studies use the term cross-reactivity to describe co-sensitization; when allergic subjects develop immunological reactions to allergens sharing structural motifs [79]. While it is likely that many of these associated reactivities (also defined as multi-reactivities [71]) in fact are due to actual B and T cell mediated cross-reactions, this does *per se* not make it so, unless additional evidence such as antigen competition assay data or T cell proliferation data is provided.

Cross-reactive allergens harbour similar three-dimensional structures that are likely to relate to shared IgE-epitopes, as well as similar T cell epitopes, accounting for the described cross-reactivities. Clinically, cross-reactivity has implications for induction of allergic symptoms, since allergic patients may suffer from hypersensitivity to allergenic sources they have not encountered previously [80]. Additionally, features of cross-reactivity can be used to simplify diagnostics of related allergies, due to an abundance of shared epitopes between very similar allergens [81, 82]. The degree of common allergenic motifs shared by closely related allergenic sources, such as cat and dog dander, is illustrated when performing inhibition studies with allergenic extracts, as a major fraction of total IgE reactivity to cat dander among cat allergic patients can be abolished by pre-incubation with dog dander [83]. In addition, T cell mediated cross-reactivity might impact clinical responses to allergens to the same extent as antibodies [78, 84]. However, these effects may be considerably more difficult to measure, and as a result, T cell-mediated cross-reactivity has been studied less frequently than antibody-mediated cross-reactivity.

Several allergen families with groups of cross-reactive antigens have been described so far [77]. One of the most studied group of cross-reactive proteins are similar to the major birch allergen Bet v 1, often termed Bet v 1-like, PR-10 proteins. Allergens belonging to this family are found in several allergenic sources, including several tree pollens,

hazelnut, apple, and other fruits and vegetables. Other allergen sources that comprise highly cross-reactive allergen families include grass pollens, weeds, mites, fish, nuts, plants and seeds. In fact, some cross-reactive protein families, such as profillins [85] and tropomyosins [86], are found in a substantial number of allergenic sources, hence designated panallergens [77]. Cross-reactivities between mammalian species such as cats and dogs, have been attributed to albumins [87, 88], and more recently lipocalins [89].

1.3.4 Allergenic extracts

Ever since Leonard Noon used a grass-pollen extract to treat hay fever in 1911 [90], aqueous allergenic extracts have been used both for diagnostics and treatment of allergic patients [43, 91-93]. To create an extract, whole allergenic sources, such as animal dander, pollen or foods are dissolved to obtain a soluble phase of their protein content. However, due to heterogeneity found in nature, these extracts will be difficult to standardize, i.e. both their allergenic and non-allergenic content will vary [94]. Also, the preparation of allergenic extracts vary, and some might be unmodified (natural), chemically modified, adsorbtion-modified or allergoids [94]. Due to sometimes extreme variability of allergenic extracts, their quality is labeled in biological units, based on skin tests on allergic subjects [95]. However, these biological units are not comparable between extracts due to mentioned differences between extracts and variations in allergic profile and number of subjects tested in different patient populations [94-96]. Also, due to variations between manufacturers, or even differences between how extracts are handled in Europe and the United States, doubts may be raised against the use of crude allergenic extracts, especially for global allergy vaccination [97]. Recent quality assessments of the composition of different allergenic extracts reveal major drawbacks, such as low or even lacking major allergenic contents or contamination with allergens from other sources, of extracts from some manufacturers [98-102].

1.3.5 Recombinant allergens

In 1988-1989, the first cDNAs of recombinant allergens were cloned, thanks to advances in recombinant DNA technologies [103, 104]. Three years later, the first studies with recombinant allergens for allergy diagnostics were published [91], and today, they are used for both diagnostic as well as vaccination purposes. The escalating interest in cloning and characterization of individual allergen components, has lead to the current situation, where most of the important allergens from the most common allergen sources have been cloned [91]. A majority of all characterized allergens have been produced in *Escherichia coli* (*E. coli*), a highly versatile prokaryotic expression system known for rapid expression of recombinant proteins with high yields [105]. *E. coli* is usually the first choice, due to easy cloning and production of proteins, either as unsoluble proteins (inclusion bodies) or fully folded proteins. However, *E. coli* lacks natural glycosylation, as well as chaperones found in eukaryotic systems, that may be necessary for protein folding. If a protein cannot be folded correctly in *E. coli*, or if glycosylation is a desired feature of the natural allergen (i.e. if natural glycosylation affects IgE-binding), other expression systems may be selected, such as *Pichia pastoris* [106], or in rare cases, mammalian cell systems [107].

The use of recombinant allergens when characterizing novel allergens serves many advantages compared to their natural counterparts [105]. High yields of the protein can be produced, and high purity (>99%) can be achieved by the addition of affinity tags, such as the 6xHIS tag. The purity can be crucial when performing immunological studies, as well as when attempting to crystallize an allergen for determination of its three-dimensional structure through the use of X-ray crystallography [108]. Indeed, the introduction of recombinant allergens to the field of molecular allergology allowed for determination of

three-dimensional structures, which has contributed significantly to the understanding of the characteristics of allergens [105, 109]. Knowledge of primary and tertiary structures of allergens can be used to determine B and T cell epitopes, as well as to improve diagnostics and construct allergy vaccines. The question of whether recombinant allergens are advantageous over natural allergens or allergenic extracts is a recurring discussion, as both alternatives are still used extensively [91, 94]. This will be addressed in further sections.

1.4 ALLERGY TO PETS

Allergy to pets, such as dogs, cats, horses, guinea pigs and mice are common, and both sensitization and ownership frequencies for cats and dogs have increased over the last decades [110-112]. Pet allergens are usually found in dust and on airborne particles in homes, as well as in public facilities such as schools or day care centers [113, 114]. Although a majority of epidemiologic studies of pet allergies have focused on the major cat allergen Fel d 1 [112], dog allergen levels may actually be higher in schools and day care centers [115], and levels of cat and horse allergens found in mattresses on display in stores correlate strongly with the time of display [116]. In young children, increased IgE-levels to allergens from cat and dog have shown to be correlated with an increased risk of developing allergies later in childhood [117, 118]. Sensitization to common pet allergens was recently found to be associated with bronchial inflammation in children with severe asthma [119]. Also, a strong correlation has been demonstrated between allergies to cat, dog and horse in children [120, 121], and the latter study showed that sensitization to horse alone (mono-sensitization) was uncommon, possibly reflecting a common route of exposure or cross-reactivity between these allergen sources.

1.4.1 Pet allergen families: the dominance of lipocalins

Allergens from dog, cat and horse belong to a few allergen families, and most of these belong to the lipocalin family, harbouring major allergens in all three species [122]. The only other major allergens reported are Fel d 1, a secretoglobin [123], and Can f 5, a kallikrein [124]. In addition to major allergens from cat, dog and horse, major allergens from rabbit, guinea pig, mouse, rat and cow belong to the lipocalin family. Allergies to rabbit and rodents are especially relevant for workers in animal facilities, where 20-30% of all exposed individuals become sensitized to mammalian allergens [125, 126], and guinea pig ownership was associated with a three-fold increased risk of developing atopic eczema compared with other pet allergies [127]. The only other unifying family of allergens that connect pet allergies are albumins [87, 88], where described cross-reactions between different mammalian allergens may account for multi-sensitization to these sources. However, albumin allergens are recognized by relatively few patients (usually 5-35% depending on patient selection) [128-130], leaving a troublesome gap when attempting to explain the strong association between allergies to cat, dog and horse. However, in paper I and II of this thesis, a new route of poly-sensitization is described, as shown by the documented cross-reactivity between the lipocalin allergens Can f 2 and Fel d 4 (cat) [131], and between Can f 6 (new dog allergen), Fel d 4 and Equ c 1 (horse). Whether the high representation of lipocalins among mammalian allergens is linked to their allergenicity is not clear. The overall abundancy of the allergen in the source could be a reason, although this is contradicted by several findings where allergens of low concentrations are able to induce potent allergic responses [132, 133]. Another theory suggests that the allergenicity of lipocalins is linked to their biological function. Most lipocalins are ligand transport proteins, but some also have enzymatic activity or participate in cell homeostasis [133]. As an example, a human homologue of Can f 1, tear

lipocalin or Von Ebner's gland protein has been reported to exhibit endonuclease activity [134], and this feature could be shared by Can f 1, Equ c 1 as well as the major rat allergen Rat n 1 and the major mouse allergen Mus m 1 [133]. Lipocalins may also bind innate immune receptors such as the mannose receptor [135], suggesting that direct immunomodulatory effects might reflect allergenicity. However, a recent review discussed that T cell reactivity to lipocalins is generally low, possibly due to few T cell epitopes in the allergens, but also due to distinct differences in T cell repertoires of allergic individuals versus healthy individuals and the fact that lipocalins might not be interpreted as truly foreign by the immune system, since there are several endogenous lipocalins [133]. The weak T cell activation capacity of lipocalins could thus be linked to their allergenicity. A molecule which is totally foreign would elicit Th1 responses at low doses, and a high dose may imply a strong T cell activation and tolerance. However, a moderate T cell recognition of an antigen with partially endogenous T cell peptides, could induce Th2 responses.

1.4.2 Cat allergens

Allergy to the domestic cat, *Felis domesticus*, is referred to as the most common pet allergy by most studies, affecting around 10-15% of the population [136-138], and up to 20% of all children [139]. In Sweden, cat allergy is counted among the most common allergies, and it has been suggested as the most common cause of allergic asthma in Swedish children [137]. The link with allergic asthma has also been demonstrated in other studies [57, 140], and recently, the severity of asthma was shown to be associated with the basophil allergen sensitivity to cat allergens [141].

The major cat allergen Fel d 1, a secretoglobin [142], is found in the saliva and is transferred to the fur by grooming, and is spread to the environment via dander [143, 144]. Its role in cat allergy is dominant, as it has been shown to account for 60-90% of all IgE reactivity to cat dander [145, 146], and around 95% of all cat allergic patients react to Fel d 1 [146, 147]. The allergen is well-defined, and biochemical as well as immunological characterization has been thorough [148], since the allergen was identified in 1974 [149]. It is a tetrameric protein, joined together by two heterodimers consisting of two disulphide linked amino acid chains. The molecular weight of natural (n) Fel d 1 is approximately 38 kDa, while the recombinant (r) version has an observed molecular weight of 30 kDa [150]. The difference is most likely due to 10-20% N-linked glycosylation on nFel d 1. In addition, in the recombinant protein, the second chain is directly fused to the first chain, i.e. the protein is folded as a homodimer. Importantly, the folding of rFel d 1 mimics that of the natural protein, which is the basis for maintained allergenicity. Indeed the IgE-binding capacity of the recombinant molecule is nearly identical to that of nFel d 1, suggesting it can replace the natural protein for diagnostics and treatment of cat allergic patients.

Fel d 1 is unique compared to other allergens; not only by its dominant role in cat allergy, but also that no corresponding homologous allergen has been cloned in any closely related pet species. A human homologue to Fel d 1, Clara cell protein CC16 has been described [142], but the biological role of Fel d 1 is not known. In 2007, a paper was published describing a potential dog homologue of approximately 20 kDa detected in dander [151]. In several dog sensitized individuals, inhibition with rFel d 1 adsorbed more than 50% of all dog-reactive IgE antibodies, indicating that a majority of all IgE antibodies to dog detected in those individuals could be directed towards the homologue. However, no sequence of the allergen was identified, and the relevance of the described allergen is still disputable. Other cat allergens might account for the strong relationship with other pet allergies. The lipocalins allergen Fel d 4 was isolated from saliva by means

of phage display, and is recognized by up to 60% of cat allergic patients, hence designated as a major allergen [152]. The allergen shares 64% amino acid (aa) identity with Equ c 1, and 67% aa identity with the new dog allergen Can f 6. The close relationship between these allergens is demonstrated by the cross-reactivity, as shown in paper II. Another cat lipocalin allergen homologous to Can f 1 was recently described, Fel d 7, and is recognized by around 35% of all cat allergic patients [153]. It is likely that these proteins cross-react as well. Additionally, a few minor allergens have been described, i.e. Fel d 2 (albumin), Fel d 3 (cystatin), Fel d 5 (IgA), Fel d 6 (IgM) and Fel d 8 (latherin), recognized by roughly 20-35%, 10%, 40%, not determined (Fel d 6) and 20% of cat sensitized individuals respectively [153-156].

1.4.3 Dog allergens

Another important cause of pet allergy is the domestic dog, *Canis familiaris*. It is generally thought to affect 5-10% of all individuals in industrialized countries, although such numbers vary with study population and location [121, 157]. The current dog allergen panel leaves a troublesome gap regarding the frequency of dog allergic patients detected in diagnostic tests. For cat allergy, Fel d 1 efficiently binds IgE in almost all cat allergic patients, but for dog allergy, the picture is less clear. Two major dog allergens have been described so far, the lipocalin allergen Can f 1 [158], and the kallikrein allergen Can f 5 [124]. Can f 1 is found mainly in saliva, but also in dander [159, 160]. Can f 5 on the other hand is a prostatic kallekrein, being secreted mainly by male animals and is found in urine. The IgE-binding frequencies of Can f 1 and Can f 5 are 50-60% and 70% respectively, thus clearly being lower than the numbers accounted for by Fel d 1 in cat allergic patients. Additionally, as discussed in paper II of this thesis, the total IgE antibody levels to dog correlate poorly with IgE levels to Can f 1 in some patients, even though they are positive to the allergen. Only one paper has assessed the sensitization frequency to Can f 5, and among Spanish subjects with dog allergy [124]. Preliminary studies of IgE-binding frequencies with recombinant Can f 5 expressed in our lab do not nearly replicate the previous numbers reported. Also, the route of exposure to urinary allergens is controversial compared to saliva or dander allergens. Thus, the major allergens of the domestic dog leave a troublesome diagnostic gap that cannot be filled unless the allergen panel is extended. In addition to Can f 1 and Can f 5, four minor dog allergens have been described, i.e. the lipocalins allergens Can f 2, Can f 4 and Can f 6, and dog albumin, Can f 3. As shown in paper II of this thesis, IgE-reactivities to Can f 2, Can f 3 and Can f 6 are around 25%, 20% and 40% respectively [161]. A recent publication characterized Can f 4 and described an IgE-binding frequency of around 30% [128]. However, in paper IV of this thesis, we cannot replicate these numbers (15%). In contrast to cat allergy, each allergenic component of dog allergy, even the minor allergens, are occasionally detected in monosensitized subjects. In consequence, there are still a few dog allergens to be characterized before a complete dog allergen panel can be envisioned.

1.5 ALLERGY DIAGNOSTICS

Allergic disease may decrease quality of life in affected patients [162], and physician's diagnosis serve as basis for management of allergic symptoms as well as long term curative options, discussed in the next chapter. Traditionally, *in vivo* diagnostic methods such as skin prick test (SPT) or in some cases atopy patch test (APT) are used to diagnose allergic reactions or atopic eczema/dermatitis respectively [163, 164]. Both these tests depend on local allergen provocation on the skin surface, cross-linking IgE on mast cells and eliciting an immediate reaction. The skin area affected by the provocation is then

compared with a positive histamine control, and the outcome is deemed positive or negative. In the clinic, allergenic extracts are used for skin tests.

Measurements of allergen-specific IgE in serum are semi-quantitative (Immunoblot assay) or quantitative, and antibody levels can be accurately determined, also in long time stored serum samples. In research laboratories, enzyme-linked immunosorbent assay (ELISA) is continuously used to measure specific antibody levels either semi-quantitatively as optical density (OD), or quantitatively using a reference curve. In paper I of this thesis, a standard curve for IgE that uses a myeloma as reference is described. Several commercial systems are available for routine measurements of IgE antibody levels, and prominently, the ImmunoCAP™ system from Thermo Fischer Scientific is being used in laboratories worldwide [165]. Similarly to the ELISA, this system uses allergenic molecules coated to a solid phase, where IgE is allowed to attach, followed by detection with a secondary antibody coupled to an enzyme that emits fluorescence upon addition of substrate. Likewise to the reference curve used in ELISA, the system uses WHO-calibrated total IgE-levels as references. In addition to ImmunoCAP, other systems for determination of allergen-specific IgE are available. The ADVIA Centaur assay uses an IgE capture sandwich assay, which might be advantageous to solid phase coating with allergens [166, 167]. In this system, magnetic particles are coated with a monoclonal antibody specific for human IgE. After addition of sera, biotinylated allergen is added, and bound allergenic molecules are detected with streptavidin coated with acridimium-ester. One major advantage with this system is that there is no competition with allergen-specific IgG found in serum, which may compete with IgE for binding to the allergens, directly affecting the readout. On the other hand, total IgE-levels and the diversity of the IgE-repertoire of the patient may influence the result.

A diagnostic *in vitro* system which might be more clinically relevant is the assessment of the ability by allergenic molecules to cross-link IgE bound to circulating basophils, thus activating them [168]. The outcome may correlate better with the clinical picture, as a similar cellular mechanism underlie the readout of these systems. Modern basophil activation tests (BAT) rely on detection of allergen-specific activity exhibited by CD203c and CD63 expression on basophils by flow cytometry. CD203c is a basophil specific marker that is present on all basophils, but is upregulated on activated basophils [169]. Even though allergen-specific upregulation of CD203c may be sufficient to monitor histamine release by basophils, the myeloid cell marker CD63 is expressed upon degranulation [170], and the combined use of these surface markers is applied most frequently. The BAT is performed using a series of allergen dilutions added to whole blood samples of allergic patients. Interpretation of basophil results involve calculations of the total fraction of activated basophils (reactivity - usually analyzed as a percentage of positive basophils among all gated basophils), as well as observation of the lowest antigen concentration eliciting a positive basophil activation (sensitivity) [171]. When performing BAT using serial allergen dilutions, a dose-response effect is often observed, viewed as a bell-shaped curve [171-173]. Interestingly, excessive amounts of allergen decrease the fraction of activated basophils compared to lower concentrations. Several theories have attempted to explain this phenomenon. One theory describes cross-linking of the IgE-receptor FcεRI with the low affinity receptor for IgG, FcγRIIb, preventing allergen-specific degranulation [174, 175]. Support for this mechanism was described in a recent study, where an anti-IgE antibody unable to cross-link FcεRI with FcγRIIb failed to show a bell-shaped basophil curve, while a fusion protein specifically engineered to cross-link these two receptors was shown to inhibit basophil activation [176]. The described features of basophil curves may complicate the interpretation of BAT results, and consequently, the assay should only be performed in experienced laboratories [171].

Upon characterization of a novel allergen, BAT is an important complement to conventional analysis of specific IgE to determine the biological relevance of the allergen. Theoretically, unless allergen-specific activation of mast cells or basophils is demonstrated, the fraction of soluble antibodies detected may be entirely cross-reactive from another similar allergen, and if directed towards only one epitope, the antigen may not elicit IgE cross-linking. Thus it is required to demonstrate allergen-specific activation of immune cells to designate a protein as an allergen.

BAT or similar methods may be used in a clinical setting for routine diagnostics of allergic patients [177, 178]. In contrast to standard IgE measurements, BAT must be performed within 24 hours after blood has been drawn. However, it is not affected by conventional allergy medication such as anti-histamines or corticosteroids [178]. BAT may also be used to analyse the efficacy of anti-IgE treatment [177, 179] and allergen-specific immunotherapy [180], as well as a biomarker for allergen sensitivity in asthma [141, 181] and food allergy [182].

The various methods described above do not discriminate between natural or recombinant allergenic sources, and both natural allergen extracts as well as single recombinant or purified natural allergens are used in diagnosis of allergy. Traditionally, allergenic extracts have dominated both *in vivo* and *in vitro* diagnostic methods, and an obvious advantage of such extracts is that they ideally mimick the source of natural exposure, obtaining a positive result. High quality control of allergenic extracts is required to avoid contamination from other allergenic sources, which may produce false-positive results [102]. As an example, a recent study described contamination of a commercial dog extract with human serum albumin [98]. On the other hand, the non-specific nature of allergenic extracts may be advantageous for allergenic sources where all individual components have not been identified, such as dog allergy. In consequence, the outcome does not discriminate between specific allergens in the extract [183].

Recombinant allergens may serve as an alternative to natural allergenic extracts for diagnosis of allergic patients. Modern DNA technology also allows for assembly of several IgE-epitopes into one hybrid molecule, which may replace natural extracts [184]. In addition, artificial allergenic extracts may be prepared through combination of several recombinant allergens. However, the main advantage of recombinant allergens for diagnostic purposes is component-resolved diagnostics, where IgE-reactivities towards an allergenic source is divided into individual components [185]. Recent advances in routine diagnostics involve the use of microarray technologies in Immuno Solid phase Allergen Chips (ISAC) [186, 187], where a broad panel of different allergens from multiple sources are assembled to determine highly specific sensitization profiles of allergic patients. Often described as “the future of allergy diagnostics”, the results need to be interpreted with care due to the complexity of the readout. As such, ISAC diagnostics is more suited for research purposes, and as a valuable complement for allergologists.

1.6 ALLERGY TREATMENT

Management of allergic disease is based on aetiology of the allergy itself, but also on the perceived reduction in quality of life of the affected patient. As a first line of interventions, avoidance of the allergen source is a cost effective way of alleviating allergic symptoms [188-190]. Effectiveness of allergen avoidance is based on the allergen source. As an example, food allergies are mostly controlled with regard to the route of

their exposure, while seasonal pollen might be very difficult to avoid. Next in line for allergy management is alleviation of symptoms through medication with anti-histamines or corticosteroids. However, such treatment is limited to short-term palliative effects, and does not induce long-lasting protection. As an alternative, Omalizumab (Xolair) is a humanized, monoclonal antibody targeting circulating IgE antibodies, prohibiting them from binding to high and low affinity receptors for IgE (FcεRI and FcεRII) on basophils and mast cells. Omalizumab is used to treat patients with moderate to severe persistent allergic asthma [191], that otherwise respond poorly to treatment with corticosteroids. Although this treatment induces clinical effects that improve daily life of affected allergic and asthmatic patients, it is very costly, and may even cause anaphylaxis in rare cases [192, 193]. A long-lasting, curative option to allergic disease is SIT, which stands out to the above mentioned treatment strategies, as it may change the course of allergic disease [93]. Today, it has been more than 100 years since Leonard Noon used a grass pollen extract to treat allergic patients suffering from hay fever to timothy grass [90], a classic paper which demonstrated a reduction of acute immune responses. Over the next sections, possible mechanisms for SIT, as well as different strategies for the construction and administration of allergy vaccines will be discussed.

1.6.1 Mechanisms of allergen-specific immunotherapy

The mechanisms behind how successful SIT mediates symptom relief of IgE-mediated allergic disease are still not fully elucidated. De-sensitization towards allergens involves actions on both allergen-specific memory B cells and T cells [43, 194], and the outcome is likely to be affected by various pathways. However, a skewing of the immune system to protective responses, is likely to account for successful SIT [194].

Modulation of allergen-specific T cell responses has been implicated in beneficial outcomes of SIT. Memory T cells play a key role in the induction of LPR, and SIT has been shown to suppress these responses [195, 196]. Early on, much focus was directed to the balance between Th2 and Th1 responses, and Th1-responses have been considered a beneficial outcome of SIT [109, 197, 198]. However, this may be an oversimplified view, and Th1 induction in mice has been observed to fail skewing away Th2 responses, while instead causing airway inflammation and enhanced allergic disease [199, 200]. Instead, induction of FOXP3+ Tregs, characterized by their prominent secretion of IL-10 and TGF-β, has been a suggested feature of T cell suppression [12, 194, 201]. Although increases in the amounts of Treg cells in peripheral blood have failed to correlate with beneficial ouotcomes of SIT [202, 203], peripheral blood mononuclear cells (PBMC) stimulated with allergen *in vitro* show an increased IL-10 production [194]. It has been suggested that Treg cells may play a pivotal role during the initial phase of SIT [202, 204], and infiltration of IL-10 producing T cells have been detected in the nasal mucosa and mucosal surfaces following SIT with grass pollen [205, 206]. The induced T cell response may be either allergen-specific, or more non-specific, acting primarily through cytokine secretion [194]. Possible effector functions of Treg induction involve suppression of Th2 responses through histamine signalling, that result in a decreased production of IL-4 and IL-13 and a lower T cell proliferation [207]. Other effects are mediation of inflammatory responses [7], repression of Th1/Th2 cells [208], local tissue remodelling [209, 210], suppression of eosinophils, mast cells and basophils [211], and antibody class switching from IgE to IgG4 and/or IgA [212].

In addition to the effect on T cells, SIT has been demonstrated to directly affect allergen-specific immunoglobulin responses [194]. During the initial phases of SIT, a continuous increase of injected allergen dose (updosing) is performed to reach a maintenance dose. During this period, an increase in allergen-specific IgE levels is first observed, which

usually decrease during the course of treatment [213], and sometimes return to pre-treatment levels [214, 215]. However, the most dramatic change in immunoglobulin levels during SIT are increases in allergen-specific IgG4 and IgG1, that show 10-100 fold increases [216, 217]. IgG1 and especially IgG4 has been described as blocking antibodies, i.e. they compete with IgE for binding sites on allergens and inhibit IgE-mediated responses [218-221]. IgG4 may be advantageous over IgG1, since it does not activate complement, it does not bind Fc-receptors and does not form immune complexes, and therefore does not cause anaphylaxia [222]. However, a recent study with grass pollen immunotherapy suggested that the overall blocking IgG repertoire, rather than just IgG4 may correlate better with clinical outcome [213]. This finding is also supported by another recent study with cat-sensitized children, where increases in rFel d 1-specific IgG levels rather than IgG4 decreased the risk of wheezing [223]. Mechanisms for IgG-mediated improvement following SIT include prevention of mast cell and basophil activation [219]. This effect may be caused by direct hindrance of allergen-induced activation by Fc-epsilon receptors, but also by cross-linking of FcεRI with FcγRIIb, thus inactivating degranulatory responses (described previously). In addition, blocking IgG antibodies may prevent FcεRII (CD23)-mediated allergen presentation to T cells [224, 225], reducing Th2 responses and LPR. Finally, increases in allergen-specific IgA levels, together with IgG1 and IgG4 have been correlated with expression of IL-10 and TGF-β, suggesting a potential role for IgA in protective responses [201]. Despite the beneficial mechanisms of SIT-induced immunoglobulins, increases in allergen-specific IgG concentrations correlate poorly with clinical outcome, as measured by medication and symptom scores [226, 227]. A recent review suggested that functional, rather than quantitative levels of IgG might correlate better with clinical response to SIT [194]. In accordance, a paper was recently published that demonstrated how functional assays such as the FAB assay (measuring suppression of CD23-facilitated presentation to T cells) [228], and IgG blocking factor correlated with clinical outcome of grass pollen immunotherapy [213]. There was no correlation with allergen-specific IgG4-levels, supporting that functional assays may be advantageous over quantative assays.

1.6.2 Strategies for allergen-specific immunotherapy

Traditionally, SIT has been performed almost exclusively with crude allergenic extracts, despite obvious drawbacks discussed previously. Major shortcomings of extracts for use in SIT include a natural presence of non-allergenic content that has not been identified, low levels or even absence of important allergens, instability of included proteins due to contaminants or proteolytic activity and an uncontrolled immunogenicity of individual allergens [97, 229]. Immunotherapy with allergenic extracts is allergen-specific, although it may induce sensitization to new allergens, and even to other allergenic sources due to cross-reactive allergens [230-232]. These major hurdles cannot be overcome and viable alternatives to crude allergenic extracts in SIT are desirable.

1.6.2.1 Recombinant allergens in SIT

Recombinant allergens are attractive tools to combat the obstacles that are associated with allergenic extracts. With simplicity, a detailed sensitization profile of each patient can be determined with component-resolved diagnostics, and tailored treatment can be engineered using only the allergens a patient is sensitized to (also designated component-resolved immunotherapy) [185]. Using this strategy, natural extracts can be replaced and their problems avoided [229]. The complexity of necessary allergens that need to be included varies between allergenic sources. It might be sufficient to vaccinate with one single cross-reactive allergen source to treat multiple allergies [233], while other sources may require a combination of allergens [216]. Vaccination with recombinant allergens

may also improve allergy to other related sources, as studies with cross-reactive recombinant allergen sources have revealed that SIT with one allergen was as effective as a mix of all cross-reactive allergens [234-236]. However, SIT with recombinant allergens is a relatively new concept, and the first clinical study with recombinant allergens started only 11 years ago [237], using hypoallergenic derivatives of the major birch allergen Bet v 1. Since then, several studies with recombinant allergens have been performed, showing clinical relevance, evaluated by improvements in symptom and medication scores of treated patients [91, 229]. In 2002, a combination vaccine of the four major timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5 and Phl p 6 was administered to a group of 62 pollen allergic patients and compared with placebo [216]. Patients enrolled with the actual vaccine obtained strong IgG-responses towards the individual allergens, and had an improved combined symptom medication score compared with placebo. A central hallmark of successful SIT implies that the higher the allergen dose – the better efficacy of the treatment [213, 238]. However, treatment with high allergen doses is limited by acute side effects and LPR [239, 240], discussed in paper III of this thesis. To combat adverse events of SIT, different strategies have been applied to increase safety of the treatment, while maintaining efficacy needed for a beneficial clinical outcome.

1.6.2.2 Adjuvants

Similarly to normal vaccination, adjuvants may enhance immune responses to an injected antigen, allowing for usage of lower doses [241]. A classic adjuvant used frequently in SIT is aluminium hydroxide (alum), which is relatively safe to use and has a good efficacy [242, 243]. A key mechanism of alum and similar adjuvants is the targeting of APCs, such as as DCs [244]. However, the use of alum is not completely liberated from adverse events. Granuloma formation may be induced, and Th2 responses have been observed in mice [243]. Consequently, there has been interest in the development of alternative adjuvants. Such strategies have involved virus-like particles [245], or bacterial components such as LPS derivatives or DNA CpG-containing oligonucleotides [246, 247]. Another adjuvant that has shown promising results in a mouse model for cat allergy is carbohydrate-based particles, which may be covalently coupled to allergens [248]. Compared to alum, carbohydrate-based particles showed reduced allergic responses, and did not induce granuloma formation [249, 250].

1.6.2.3 Routes of delivery

During recent years, major focus within the field of allergy vaccination has been directed towards different routes of delivery for allergen vaccines. Traditional injection immunotherapy is administered subcutaneously (under the skin), by allergen updosing followed by a maintenance phase. Inconveniently, it is a time-consuming and costly treatment that involves 30-80 injections over 3-5 years [93, 251]. As previously mentioned, it also causes adverse events [252], and there is need for alternative routes of administration. As an alternative, administration of allergen sublingually [253] has proved successful in clinical practice, and exploits the natural tolerogenic state of the oral mucosa. A high allergen dose is used repeatedly over a course of several years, and thus, it is a costly treatment. Severe side effects of sublingual immunotherapy are rare, but local mild side effects are common [254]. More recently, a novel administration route was described, i.e. the intralymphatic route [255-257]. The allergen is introduced to the lymphatic system via direct injections into lymph nodes, thus ensuring efficacious delivery to APCs. Clinical trials with grass-pollen and cat allergic patients have demonstrated nasal tolerance, and an increase in allergen-specific IgG4 levels and IL-10 responses, after treatment with intralymphatic injections. A major advantage is the few number of injections needed to induce tolerance (basically 3 injections over a few weeks).

Also, low doses of allergen may be used and the treatment is deemed safe as no severe adverse events have been observed.

1.6.2.4 Hypoallergens

Formulation of recombinant allergen derivatives with attenuated characteristics, also called hypoallergens, is a widely explored strategy for efficacious and safe SIT. A general characterization of hypoallergens states that they have a reduced IgE-binding and a retained T cell reactivity, although this may vary for different strategies (Fig. 3) [91, 109, 229]. A central basis for the construction of hypoallergens has been developments in modern DNA technologies, allowing for modification of the primary structure of allergens. In the years following the cloning of the first recombinant allergens, several studies were performed to determine the specific allergenic capacities of individual allergens, leading to the discovery of B and T cell epitopes relevant for this activity [91]. Shortly, natural occurring hypoallergenic isoforms of the major hazelnut pollen allergen Cor a 1, and the major birch allergen Bet v 1 were described [258, 259], and genetically modified hypoallergens for safer SIT were described by several research laboratories.

A majority of all hypoallergens attempt to reduce IgE-binding epitopes by alteration of major IgE-binding epitopes, either by specific mutagenesis of linear epitopes or alteration of the three-dimensional structure of the protein [91, 109, 229]. However, such constructs need to maintain essential primary structure elements for T cell recognition, and preferentially also immunogenic epitopes for induction of allergen-specific IgG. One major exception is T cell peptides containing major allergenic epitopes for allergy vaccination [260]. Such peptides lack IgE-binding epitopes and are not able to cross-link IgE on basophils and mast cells, thus they do not induce acute allergic adverse events. Their mechanism of action is through the induction of T cell tolerance, which may improve immune responses towards the allergen mainly through induction of regulatory T cells. The complexity of peptide vaccines is a major drawback, and consequently, only a few have reached clinical trials. Such trials have demonstrated the induction of T cell tolerance, but since the protective effect is limited to T cells, acute responses are only partially or even unaffected by T cell epitope vaccines [260, 261]. In addition, peptide

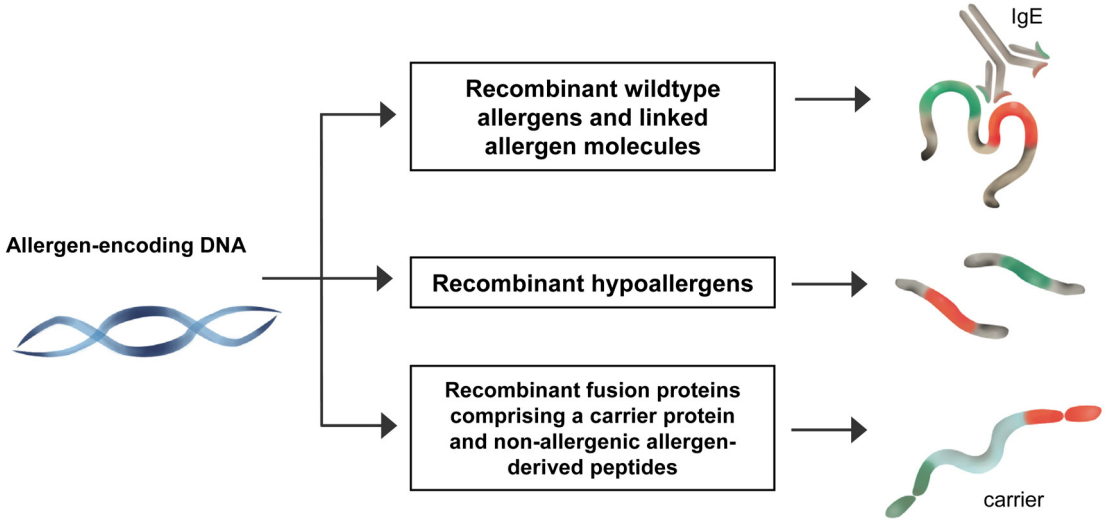

Figure 3. Overview of the common concepts of recombinant allergy vaccines. Allergen-encoding DNA sequences may be utilized to produce recombinant wildtype allergen constructs that retain conformational epitopes (red and green combined) and bind IgE. Modification of natural DNA sequences is performed to generate hypoallergens or fusion proteins with non-allergenic peptides for safer SIT. (Adapted from [229])

vaccines may induce LPR in vaccinated patients [262], and cannot be deemed totally safe for SIT. Vaccination with B cell peptides, also called haptens (naturally occurring sequences from fragmented allergens) or mimotopes (screened from peptide libraries), is an alternative approach for SIT that does not cause acute allergic responses [109, 229]. Per definition, a hapten only contains one IgE-binding site, and is therefore unable to cross-link IgE on its own [263, 264]. Positive effects of haptens involve neutralizing effects on mast cells and basophils by hindering IgE-crosslinking, but also by induction of blocking IgG antibodies. On the other hand, since many IgE-epitopes are conformational, it is difficult to form potent blocking antibodies using linear peptides, representing a major drawback for this approach. B cell peptides may also be fused to carrier molecules, such as viral proteins [91, 109, 229]. This approach strengthens the hapten approach by applying T cell help from the carrier protein, thus inducing stronger immune responses. Carrier molecules are attached chemically, or directly fused to the construct using recombinant DNA technologies [245, 265]. The latter approach may be advantageous to chemical coupling, since the process may induce end products which are hard to detect and standardize. Recombinant fusion of the viral proteins VP1 and the hepatitis B PreS protein has been performed with the major grass allergen Phl p 1 and the major cat allergen Fel d 1 respectively [265, 266]. These constructs potently induced blocking antibodies of similar quantities to recombinant allergens. It has also been speculated that these vaccines will be safer with regard to T cell mediated side effects, and clinical trials are currently in progress.

The vast majority of all hypoallergenic derivatives developed have been modified variants of full length recombinant allergens [91, 109, 229]. Such constructs carry T cell epitopes necessary for immunogenicity and immunomodulatory characteristics of the vaccine, while maintaining surface epitopes necessary for the induction of blocking IgG antibodies. Even though hypoallergens have the characteristics necessary for cross-linking IgE on basophils and mast cells, thus eliciting immediate hypersensitivity reactions, their IgE binding is lower than the original recombinant allergens, allowing for higher injection doses. Over 10 years ago, two individual hypoallergens to the major birch pollen allergen Bet v 1 were created, by fragmentation [267, 268], or oligomerization [269] of the primary structure, resulting in disruption of tertiary allergen structure. Both constructs had a reduced IgE-binding capacity *in vitro*, while major T cell epitopes were preserved, and were able to potently induce blocking antibodies in animal models. A multi-centre study using these hypoallergens was conducted, with a maintenance dose that was relatively high, 80 µg protein. Importantly, the reduced allergenic activity of these hypoallergens correlated with a reduction in immediate skin reactions to Bet v 1, and a trend towards an increased well being of patients treated in comparison with the placebo was observed [237, 270, 271]. Beneficial effects could be detected *in vitro*, by measuring the induction of allergen-specific IgG4 antibodies that inhibited basophil activation, as well as an attenuated Th2 cytokine responses in PBMC cultures from patients treated with the Bet v 1 trimer [271]. However, a drawback observed during the clinical trials, were LPR side effects [270]. It is likely that the occurrence of LPR in these studies were linked to the high administered maintenance dose, possibly by the same mechanism as observed for treatment with T cell peptides [240, 262]. Therefore, LPR may be a limiting factor for SIT with hypoallergens. In paper III of this thesis, a novel approach to combat recurring LPR during SIT is presented, where the primary structure of rFel d 1 was altered by random mutagenesis, and correctly folded mutants were selected by phage display.

Several other strategies have been used to develop hypoallergens, but only a few have reached clinical trials. A folding variant of rBet v 1 (rBet v 1-FV, [272]) has been evaluated in clinical trials, where it was deemed equally efficacious as the birch extract,

and shown to have a safe adverse events profile. A commercial vaccine based on rBet v 1-FV is expected on the market in the coming years [229]. In addition, a clinical phase I study using modified versions of the major peanut allergens Ara h 1, Ara h 2 and Ara h 3 is currently being conducted in the United States. Similar vaccine constructs have been formulated for other allergenic sources, but have not reached clinical trials. As an example, the sequence for the recombinant version of the major cat allergen Fel d 1 has been used to create hypoallergenic derivatives, by duplications of T cell epitopes and introduction of point mutations [273]. This approach was deemed effective, assessed by induction of potent blocking antibodies and a reduction of airway hyperresponsiveness and tolerance induction in a mouse model for cat allergy [274]. Additionally, the hypoallergenic version of rFel d 1 had a reduced SPT reactivity in cat allergic patients, compared to the wild type protein.

The approach of recombinant fusion of different molecules into combined constructs, similarly to the fusion of viral proteins with haptens, has been applied to full length allergens in order to create hypoallergenic constructs for SIT. The timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5 and Phl p 6 have been used to create hybrid molecules between these allergens, either in pairs [275], or as a quadruple fusion protein [184], or by fusing two hypoallergenic derivatives of Phl p 2 and Phl p 6 [276]. These vaccines induced blocking IgG antibodies in mice, and contained the epitope spectrum of the original recombinant allergens. The hypoallergenic hybrid also had a reduced allergenicity, similar to the individual counterparts. Other hybrid molecules have been attempted, using the major mite allergens Der p 1 and Der p 2 [277, 278], but with limited success due to improper folding of the individual allergenic components. Paper IV of this thesis brings up a similar strategy for the assembly of four dog lipocalin allergens into one molecule. Single recombinant allergens have also been fused with non-allergenic proteins or peptides with immunomodulatory functions. A fusion protein was created by joining rFel d 1 with a protein targeting human FcγRIIb [279], aiming to cross-link FcεRI with FcγRIIb, neutralizing allergen-specific mast cells and basophils by mechanisms discussed previously. This fusion protein did not induce mediator release on its own. Instead, it inhibited mediator release from basophils from cat sensitized individuals and mast cells from cord blood. Vaccine potential of the construct was demonstrated in a mouse model for cat allergy [280]. Another successful fusion-based strategy for an enhanced vaccine potential of recombinant allergens through recombinant fusion was recently described. By attaching a truncated invariant chain peptide, and a transactivator of transcription peptide to allergens, they were converted into cytoplasmic proteins, while also targeting the MHC class II pathway. The technology is called modular antigen translocation (MAT), and has been attempted with several major allergens [281]. MAT fusion of recombinant allergens allowed for 10-100 fold lower allergen doses for induction of PBMC proliferation, while also shifting T cell responses to Th1 and IL-10 production. MAT-Fel d 1 showed advantageous responses in immunized mice [282], and a recent paper described a clinical trial using this construct by intralymphatic injection immunotherapy [256]. The construct was safe to use in this setting, and tolerance to rFel d 1 was induced after only three injections. It was concluded that a combination of the MAT technology with intralymphatic injections is a major improvement over classical subcutaneous or sublingual immunotherapy, due to low allergen doses required, few injections, and a short incubation time.

2 THESIS AIMS

This thesis explores novel diagnostic and therapeutic concepts for allergies to pets, by using sophisticated molecular biology cloning, expression and purification systems, together with translational research involving serum and cells from allergic patients *in vitro*, and allergen testing in mice *in vivo*.

Specific aims:

Paper I: to determine the crystal structure of the lipocalin allergen Can f 2 from *Canis familiaris*, and to characterize its role in dog allergy, and the relation to other pet allergies

Paper II: to characterize the novel dog lipocalin allergen Can f 6, and determine whether its high homology with similar allergens from cat and horse underlies the basis for cross-reactivity

Paper III: to develop a new type of allergy vaccines applied to the major cat allergen Fel d 1, with reduced IgE-binding and T cell activation, using phage display

Paper IV: to generate a linked vaccine that comprises four dog lipocalin allergens, for improved diagnostics and vaccination of dog allergic patients

3 MATERIALS AND METHODS

This part of the thesis will discuss important methodology used in the four included manuscripts. Details upon specific experimental procedures can be viewed in the Materials and methods section of each paper.

Basophil activation test (BAT) [**I-IV**]	Stimulation of circulating basophils in whole blood from allergic patients with relevant allergens. Immunological staining of activated basophils with fluorescence-labelled and analysis of degranulated basophils by flow cytometry.
Biochemical characterization of proteins [**I-IV**]	Natural or recombinant proteins may be characterized by their biochemical behaviour, such as their oligomeric state, either by SDS-PAGE or analytical size-exclusion chromatography.
Circular dichroism (CD)-spectra [**I-IV**]	Spectrophotometric determination of secondary structure elements of proteins.
Cloning of DNA constructs with PCR-based techniques	
Gene detection [**II**]	By design of oligonucleotides specific for genomic DNA sequences, expression of the corresponding genes may be identified in cDNA libraries.
Gene fusion [**IV**]	To assemble multiple genes and create a fusion protein, overlapping oligonucleotides specific for each gene are used to create new template genes with specific nucleotide overhangs. The fused gene is synthesized in a final reaction, using the new template genes together with oligonucleotides specific for the genes situated at the ends of the fusion protein.
Gene synthesis [**I**]	Using overlapping oligonucleotides designed to span a complete gene sequence, the corresponding full-length gene may be assembled and cloned.
Random mutagenesis [**III**]	By incorporation of custom nucleotides (dNTPs), which may base-pair with several normal dNTPs, random mutations are introduced to a gene of choice.
ELISA	
Blocking [**III**]	Blocking IgG antibodies from immunized mice are utilized to demonstrate their ability to block allergen-specific IgE from allergic patients.

Inhibition [**I-IV**]	Serial dilutions of antigens and mixing with sera from allergic patients, may demonstrate that the soluble antigen competes with a similar solid phase-bound antigen for binding to IgE.
Quantitative [**I-IV**]	Allergen-specific IgE or IgG levels from allergic patients or immunized mice may be quantified or semi-quantified. To allow for absolute quantification, a reference standard must be applied to the analysis.
Immunization of BALB/c mice [**I**, **III**, **IV**]	By injection of allergens subcutaneously in the neck of female BALB/c mice, an immune response is induced.
Molecular modelling [**I**]	Modeling of novel protein structures using homologous proteins as templates.
Molecular replacement [**I**]	By using diffraction data from X-ray crystallography of allergen crystals, the structure can be determined by comparison with another crystal structure from a homolgous protein.
PBMC and splenocyte proliferation assays [**III**, **IV**]	PBMCs from the blood of allergic patients and splenocytes from spleens of immunized mice are prepared by Ficoll separation and cell straining methods respectively. Cultured T cell proliferation in the presence of allergens is measured by [^{3}H]-thymidine incorporation.
Phage display [**III**]	Decades ago, George P. Smith described a method using filamentous bacteriophages for rapid screening of highly diverse libraries for their binding specificities [283, 284]. In 1993, Reto Crameri and Mark Suter described the phagemid vector pJuFo, specifically developed for display of cDNA libraries by the Jun/Fos display system [285]. In paper **III** of this thesis, the pJuFo system was used for the development of novel hypoallergenic derivatives of the major cat allergen Fel d 1, with reduced B and T cell activation capacity.
Protein expression [**I-IV**]	DNA fragments of recombinant wild type allergens or modified variants are inserted into the expression vectors pET20b [**I-IV**] or pET28D [**IV**] and produced either as soluble proteins or inclusion bodies.

Protein purification [**I-IV**]	Bacterial pellets from growth cultures are solubilized in PBS (soluble proteins) or Guanidine-HCl (inclusion bodies) and purified by immobilised metal chelate affinity chromatography (IMAC), size exclusion chromatography (SEC) and ion exchange (IEC) chromatography.
Protein folding [**III**]	Inclusion bodies of proteins are refolded to naturally shaped proteins by dialysis buffer exchange.
Real-time analysis of protein interactions in Attana [**III**]	Antibodies in sera from immunized mice are analysed for their binding characteristics to a covalently coupled antigen on a sensor chip. Dissociation rates of antibodies from each serum pool are compared and ranked for their antigen specificities on an Attana A200 instrument.

3.1.1.1 Ethics statement

All animal experiments and human research conducted in **paper I-IV** were approved by local ethics committees at the Karolinska University Hospital and Karolinska Institutet. Written consent was obtained from all allergic patients enrolled in the basophil and the PBMC trials.

4 RESULTS AND DISCUSSION

4.1 STRUCTURAL AND ALLERGENIC CHARACTERIZATION OF THE LIPOCALIN ALLERGEN CAN F 2 FROM *CANIS FAMILIARIS* [I]

Lipocalin allergens have been identified in several species [133], and four dog lipocalin allergens have been described so far [161]. However, no 3D structure had been determined for a dog allergen. Although lipocalin allergens most often share only 18–25% sequence identity, they are defined by the striking overall similarity of their 3D fold as well as the presence of one to three structurally conserved regions (SCR) that act as a family signature. Lipocalins typically have a cup-shaped cavity within the central β-barrel formed by eight antiparallel β-strands (βA–βH) with a loop scaffold that might regulate access to the ligand-binding site. Determination of allergen structures may enhance the understanding of their underlying allergenicity and biological function. In addition, the role of Can f 2 in dog allergy had not been fully characterized prior to this work.

We have demonstrated that rCan f 2 displayed similar if not identical IgE-binding properties when compared to its natural counterpart (Fig. x). Quantification of specific IgE reactivities towards the three dog allergens rCan f 1, rCan f 2 and nCan f 3 as well as the cat allergen rFel d 4 in 89 dog-sensitized subjects with IgE-reactivities > 5kU$_A$/l to a commercial dog dander extract, revealed responses in 67%, 40%, 25% and 54%, respectively. Additionally, we identified a few subjects who only reacted to rCan f 2, demonstrating the importance of minor allergens in dog allergy. Previous studies have not demonstrated IgE antibodies towards Can f 2 in the absence of Can f 1 sensitization [158, 286].

The three-dimensional (3D) structure of the rCan f 2 allergen was determined to a resolution of 1.45 Å (Fig. 4). The asymmetric unit includes one rCan f 2 molecule (residues 7–157), one glycerol molecule and 129 water molecules. The final electron density map is of high quality with well defined polypeptide chains. The 3D structure of rCan f 2 displays a characteristic lipocalin fold [287], with a β-barrel structure with eight central, anti-parallel β-strands (βA–βH) interrupted by intermittent β-hairpin-forming loops (L1– L7) and with an adjacent α-helix (H1) localized at the C-terminus of the protein. The structure is stabilized by a disulfide bridge between Cys64 and Cys157. The open end side of the protein might act as a ligand-binding site and as an access to the calyx/cavity of rCan f 2, while the closed end might act as a binding site for surface receptors. We also identified a potential glycosylation site of Can f 2, localized on the L1 loop near the open end. In addition, several structural motifs important for the lipocalin fold, such as the Trp19 residue in the first structurally conserved region were identified.

The biological function of Can f 2 is still unkown, although it is likely to bind small hydrophobic molecules, similarly to other related lipocalins. A feature controlling the binding of molecules to the internal calyx of Can f 2 is the “lid”, formed by the loops L1 and L5, and this characteristic is also conserved among other related lipocalin allergens. We also confirmed that rCan f 2 is a monomer at neutral pH, similarly to other lipocalin allergens, excluding the dimeric major horse allergen Equ c 1. The sequence identity of Can f 2 to related allergens and human lipocalins is low. However, the structural resemblance of rCan f 2 to other lipocalins and is striking, as noted by comparison with Equ c 1, the major mouse allergen Mus m 1, and the major rat allergen Rat n 1. Upon

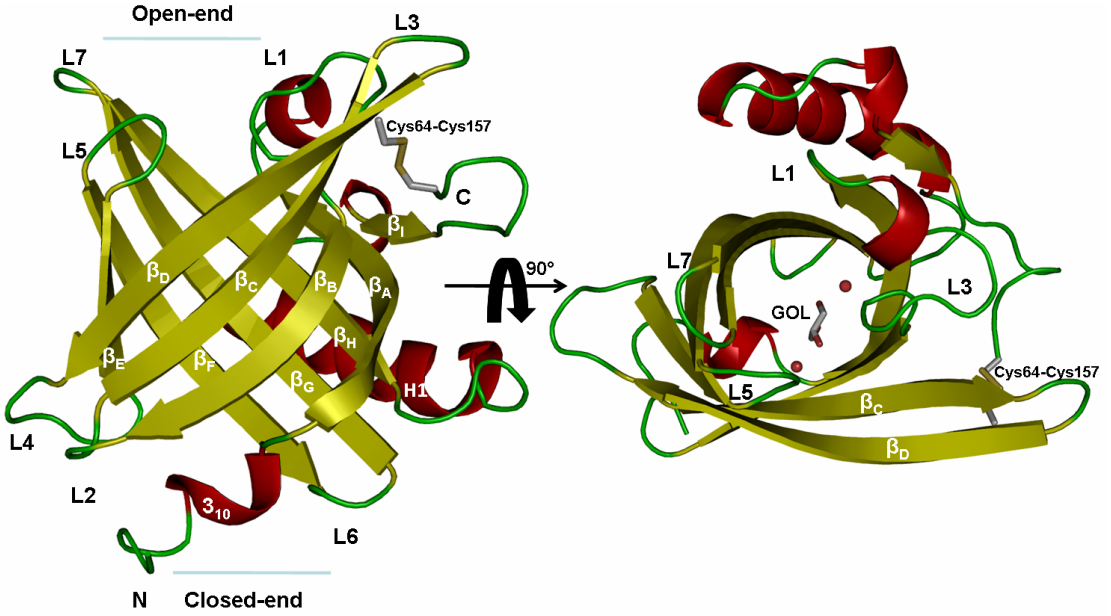

Figure 4. The three-dimensional structure of rCan f 2. Eight anti-paralell β-strands (yellow) compose the typical lipocalin β-barrel (left). The loops L1 and L5 (green) form the "lid" that control the internal calyx of Can f 2, viewed from above (right).

analysis of sera from 21 dog-sensitized asthmatic children with IgE antibodies to rCan f 2, we noted that several subjects also had IgE antibodies to a major cat lipocalin allergen, rFel d 4. Based on these results, we set out to investigate the IgE-mediated cross-reactivity of rCan f 2 with the lipocalin allergens rFel d 4, rCan f 1 and nEqu c 1, and the human lipocalins neutrophil gelatinase- associated lipocalin (NGAL) and retinol-binding protein (RBP) in sera from five subjects with high reactivities to rCan f 2. Although Can f 2 shares less than 22% sequence identity with the cat allergen Fel d 4, our assay clearly demonstrated a patient-dependent cross-reactivity (up to 58%) between these two lipocalins. In contrast, no cross-reactivity was observed with Can f 1, Equ c 1 or any of the human homologues RBP and NGAL, despite the high homology between Fel d 4 and Equ c 1.

The allergenic activity of rCan f 2 and rFel d 4 was also tested in vitro for the ability to induce basophil degranulation upon allergen cross-linking of FcεRI-bound allergen-specific IgE. Flow cytometric analysis of allergen-activated basophils from three asthmatic children sensitized to dog and cat revealed similar degranulation capacity by both allergens in all three patients, resulting in up-regulation of CD63 with antigen

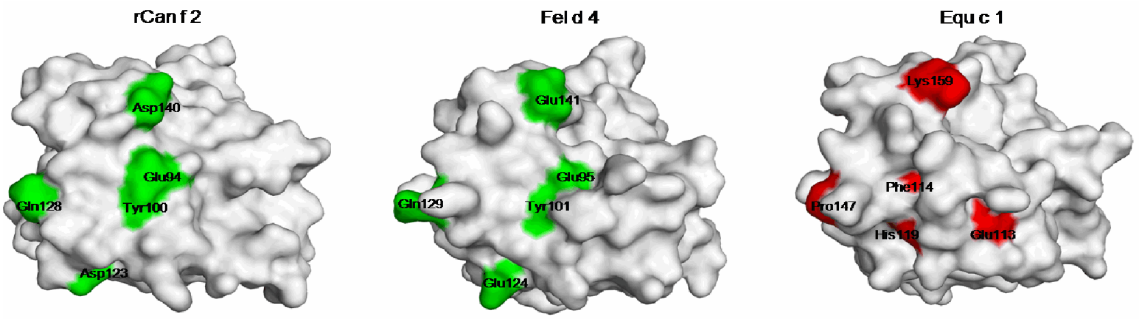

Figure 5. rCan f 2 cross-reacts with rFel d 4 but not with nEqu c 1. Molecular surface representation of the crystal structures of Can f 2 and Equ c 1 compared to a molecular model of Fel d 4. Similarities between rCan f 2 and rFel d 4 are highlighted in green, and dissimilarities in nEqu c 1 are highlighted in red.

concentrations as low as 10 ng/ml. Careful further comparative structural and sequential analysis of rCan f 2 with the cross-reacting Fel d 4 and the non cross-reacting Equ c 1 combined with epitope predictions was performed using the program Discotope (http://www.cbs.dtu.dk/services/DiscoTope/). This suggested a conformational epitope formed by the three stretches of the residues 94–104 (EVDPKSYLILY), 122–132 (RDLSRQQDFLP) and 134–141 (FESVCEDI) as a potential site for cross-reactivity between Can f 2 and Fel d 4 (Fig. 5). This solvent-exposed region, predominantly basic in Equ c 1, is more negatively charged in both rCan f 2 and Fel d 4. In addition, several other residues may play a central role in the formation of a conformational epitope that is conserved in Can f 2 and Fel d 4, but absent in Equ c 1. Other hypotheses for the observed cross-reactivity between Can f 2 and Fel d 4 may be the formation of neo-epitopes upon ligand-binding to the conserved calyxes of the two proteins, and the dimerization of Equ c 1 may also hinder the cross-reactivity with Can f 2.

4.2 IDENTIFICATION AND CHARACTERIZATION OF THE NOVEL CROSS-REACTIVE DOG LIPOCALIN ALLERGEN CAN F 6 [II]

Allergy to the domestic dog (Canis familiaris) affects 5–10% of the population in affluent countries. Dog dander harbours several dog allergens, including the lipocalin allergens Can f 1, Can f 2 and Can f 4, and the albumin allergen Can f 3 [128, 158, 288]. A majority of all identified mammalian allergens belong to the lipocalin protein family, including major allergens from dog, horse, cat, cow, rat and mouse [89, 289, 290]. Similarity in allergen tertiary structure can serve as a basis for polysensitization, and allergen-specific IgE antibodies may bind to similar epitopes across homologous allergens which is here defined as cross-reactivity [71]. Several studies have shown a strong association between sensitization to dog, cat and horse among young children [120, 121], where the latter study reported that mono-sensitization to horse was relatively uncommon. This relationship may resemble cross-reactivity between common allergens from these sources. Traditionally, the relationship between allergies to cat dog and horse has also been attributed to albumins [288], despite that few patients are sensitized to this family of proteins. We previously detected cross-reactivity between the dog allergen Can f 2 and the cat allergen Fel d 4, although they share a low sequence homology. However, no lipocalin allergen with high homology to the major cat and horse allergens, Fel d 4 and Equ c 1 respectively, had previously been detected in the domestic dog.

Previously, it has been indicated that a higher sequence homology may imply stronger cross-reactivity [291]. Thus, we identified the template gene for Can f 6 by BLASTP search, using template genes for Fel d 4 and Equ c 1 [152, 292]. The sequence similarity and identity of Can f 6 with Fel d 4, Can f 6 to Equ c 1 and Fel d 4 to Equ c 1 was indeed high, 80.0% and 67.4%, 72.5% and 55.1% and 79.5% and 64.2% respectively. The mature peptide revealed a predicted molecular weight (MW) of 20.193 kDa and an isoelectric point of 4.92. The gene encoding Can f 6 was amplified from canine skin and bladder cDNA libraries, while no amplification could be obtained from tongue and submandibular gland libraries. In support, we identified nCan f 6 in a commercially available dog dander extract using an in-house developed monoclonal antibody, demonstrating the natural expression of this protein.

The observed molecular weight of purified rCan f 6 was compared to the homologous allergens rFel d 4 and nEqu c 1 using size exclusion chromatography. The proteins eluted as symmetrical peaks, where nEqu c 1 appears as an expected dimer and rCan f 6 and rFel

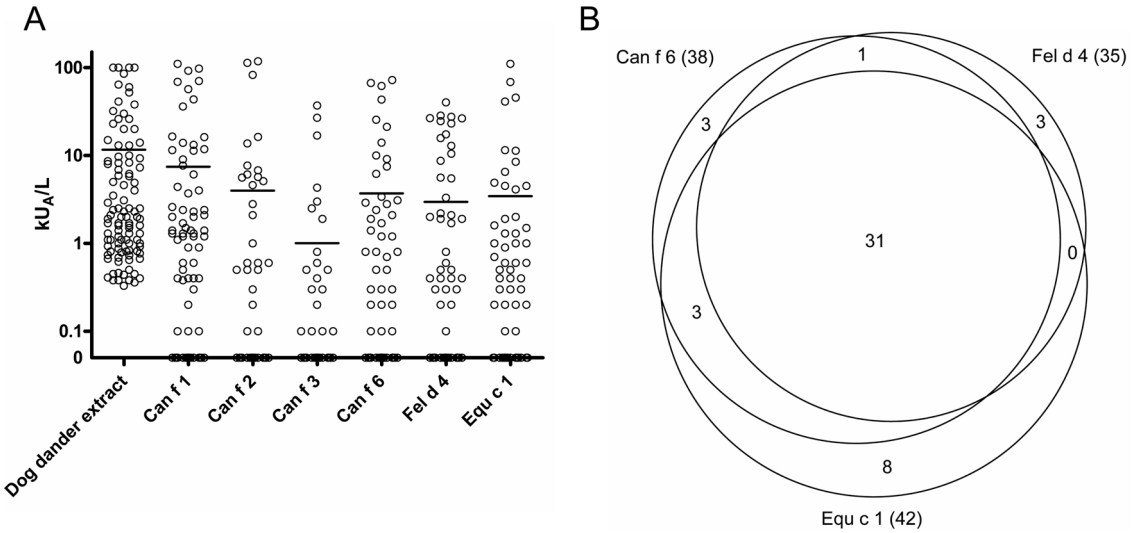

Figure 6. Assessment of IgE-reactivity to rCan f 6 and structurally related lipocalin allergens among 100 dog-sensitized individuals. (A) Specific responses to dog dander extract (ImmunoCAP e5), and the dog allergens rCan f 1, rCan f 2, nCan f 3, rCan f 6, rFel d 4 (cat) and nEqu c 1 (horse) by ELISA. Bars indicate mean values. (B) IgE-response overlap between rCan f 6, rFel d 4 and nEqu c 1.

d 4 as monomers. CD-spectra recorded for rCan f 6, rFel d 4 and nEqu c 1 revealed similar secondary structure elements, indicative of maintained fold.

The frequency of IgE-binding to rCan f 6 was 38%, analyzed in sera from 100 dog sensitized individuals. This suggests that Can f 6 is less frequently recognized than Can f 1 (55%), but more frequently recognized than the allergens Can f 2 (24%), Can f 3 (19%) and Can f 4 (32%, [128]), signifying Can f 6 as a key dog allergen (Fig. 6a). Importantly, we found several subjects with reactivity towards rCan f 6, who did not recognize rCan f 1 or rCan f 2. Moreover, quantification of IgE levels to rCan f 6 indicated that a significant fraction of total anti-canine IgE is directed towards this allergen, even though our results point to a low presence of the allergen in a commercial extract used for routine diagnostics of allergic patients.

There was a similar sensitization frequency to the allergens rCan f 6 (38%), rFel d 4 (35%) and nEqu c 1 (42%), among dog sensitizaed subjects, with median IgE levels of 1.7, 1.9 and 0.95 kU_A/L respectively. The majority were sensitized to all three allergens (Fig. 6b). Subjects sensitized to either rCan f 6, rFel d 4 or nEqu c 1 were 81.6%, 88.6% and 73.8% likely to be sensitized to all three allergens respectively. Pairwise correlation of IgE levels for the three allergens was $r = 0.42$ for Can f 6 and Fel d 4, $r = 0.41$ for Can f 6 and Equ c 1, and $r = 0.42$ for Fel d 4 and Equ c 1, $p < 0.001$. This strong relationship provides a basis for IgE-mediated cross-reactivity that may account for multi-sensitization to these allergies. In support of the cross-reactive behaviour between the three lipocalins, no correlation was found with a member from another cross-reactive protein family, the dog serum albumin allergen nCan f 3.

We investigated the allergenic potential of rCan f 6, rFel d 4 and nEqu c 1 in a basophil activation test, using blood from dog-allergic patients. Specific activation of CD203c and CD63-gated basophils was observed upon stimulation with all three allergens. As expected, there was an allergen-dependent variation in potency between the individuals tested, suggesting allergenicity, primary sensitization source and structural relationship between these allergens.

Finally, we investigated IgE cross-reactivity by means of antigen-titration inhibition ELISA. There was a common, but variable cross-reactivity between these allergens, and some subjects exhibited IgE inhibition at antigen concentrations as low as 1 ng/ml. A majority of subjects reacted with one or both of the two other allergens, and two subjects out of fourteen did not show signs of cross-reactivity. Interestingly, our results illustrate how certain antibodies to related allergens are more specific at lower concentrations to cross-reactive components than to the homologous antigen, and point to the main sensitizing source, which could be valuable information prior to initiating allergen-specific immunotherapy. Based on sequence similarity and identity between Can f 6, Fel d 4 and Equ c 1, Can f 6 can be expected to cross-react with the major rodent allergens Mus m 1 (mouse) and Rat n 1 (rat), similarly as demonstrated for Equ c 1 [291], but not with any human lipocalin.

4.3 MOLECULAR EVOLUTION OF NOVEL ALLERGY VACCINE CANDIDATES WITH REDUCED IGE-BINDING AND T CELL ACTIVATION CAPACITY, USING PHAGE DISPLAY [III]

The domestic cat (*Felis domesticus*) is one of the most frequent pets and approximately 10% of the general population in industrialized countries is sensitized to cat allergens [293]. The major allergen of the cat, Fel d 1, is a member of the secretoglobin protein family [150, 294], and ~95 % of cat allergic patients show elevated serum IgE levels to Fel d 1. Therefore, it is the primary target for the development of immunotherapeutic vaccines for the treatment of cat allergy [279, 295]. Allergen-specific immunotherapy (SIT), is the only treatment able to cure allergic diseases [296]. Successful SIT is thought to act through tolerance mechanisms induced by regulatory T cells and blocking IgG antibodies [43]. Induction of IgG antibodies may reduce clinical symptoms in several ways, by competition with IgE for binding epitopes on the allergens [297, 298]. Numerous studies have shown that crude allergen extracts currently used in SIT are clinically effective [93], and that a high allergen dose is more effective [238], although the potential risk of severe acute side effects is a limiting factor [239]. Attenuated allergenic molecules, i.e. hypoallergens or synthetic peptide fragments have been used as high dose and safer alternatives to conventional extract-based SIT [92, 299]. However, such treatments have also been limited by recurring side effects, such as LPR [270, 300].

In this study we used a novel strategy to engineer full sized and folded allergy vaccine candidates with reduced number of T cell epitopes and reduced risk of inducing anaphylaxis, while maintaining the immunogenic properties. The rational of the strategy is based on selection of IgE-binding allergens from randomly mutated phage-displayed libraries of the major cat allergen Fel d 1, used for proof of concept. Following error-prone PCR (epPCR), ligation and transformation, the

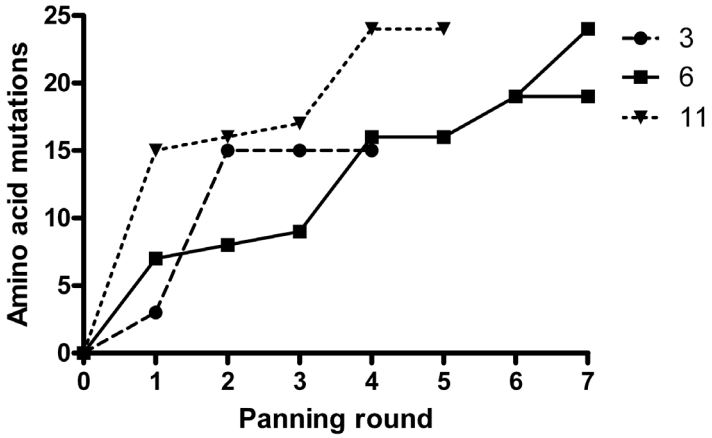

Figure 7. *In vitro* evolution of mutations by phage display. Number of amino acid exchanges (y-axis) in the four Fel d 1 mutants displayed as a function of panning rounds (x-axis).

generated mutated Fel d 1 phage libraries typically contained 10^5 individual clones. After the first round of panning and sequencing, 3 abundant clones (clones 3, 6 and 11) were identified and used as templates for further rounds of epPCR and panning. The iterative mutational procedure for clone 3 was stopped after 4, for clone 11 after 5, and for clone 6 after 7 rounds of panning, when no or few additional mutations could be detected (Fig. 7). The final clones, denoted 3.4.7, 6.7.1, 6.7.3 and 11.5.2 contained 15 (9.3%), 19 (11.7%), 24 (14.8%) and 24 (14.8 %) amino acid exchanges respectively, compared to wild type Fel d 1. Upon sequential comparison of the mutants with rFel d 1, we identified three regions that were less prone to mutations. Consequently, molecular evolution of recombinant allergens and selection of IgE-binding antigens with phage display may allow for prediction of IgE-binding epitopes. Indeed, the regions that were less prone to mutate were present on the surface of rFel d 1, supporting our initial hypothesis.

All mutant proteins were well expressed by *E. coli* either as inclusion body (clones 6.7.1 and 6.7.3) or as soluble cytoplasmic proteins (clone 3.4.7 and 11.5.2), re-folded well using established methods, and eluted as symmetrical peaks around 30 kDa by size exclusion chromatography as observed for Fel d 1. Circular dichroism analysis of the mutants showed spectra comparable to those of rFel d 1 except for clone 6.7.1 which displayed a less intensive maximum at 190 nm (Fig. 3). The similarity of rFel d 1 and the mutant spectra, with the possible exception of clone 6.7.1 is evidence that the mutants are indeed folded similarily as rFel d 1 allowing structural inferences to be made from sequence data.

The IgE-binding in serum from 20 cat sensitized subjects to the four mutants compared to the native-like rFel d 1 was investigated by indirect ELISA. We found that the mutants 3.4.7, 6.7.3 and 11.5.2 had a significantly reduced IgE-binding compared to rFel d 1, while the IgE-reactivity of 6.7.1 was not changed. Similar results were also observed when analyzing the capacity of the mutants to inhibit IgE from a pool of cat sensitized individuals to bind to solid phase bound rFel d 1 by ELISA. However, a more relevant assessment of the IgE-binding capacity of the mutants was performed *in vitro*, by analyzing their capability to release mediators upon allergen cross-linking of FcεRI-bound Fel d 1-specific IgE on basophils from six cat allergic patients. Analysis of the basophils by flow cytometry revealed variable reactivity and sensitivity to the allergen depending on patient and mutant tested, and the most potent up-regulation of CD63 was seen after stimulation with rFel d 1, followed by the mutant 6.7.1. The other mutants showed a weaker binding to IgE, with 3.4.7 generally showing the lowest degranulating capacity. Even though all mutants showed a hypoallergenic pattern in comparison to rFel d 1, the pattern varied between patients, which may call for evaluation when choosing the most optimal mutant if used for treatment.

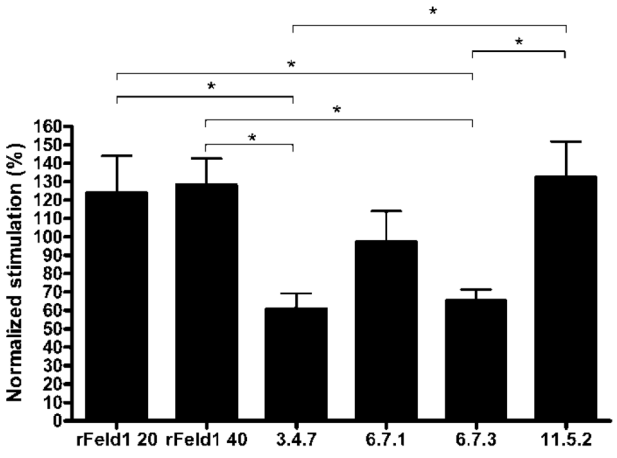

Figure 8. Analysis of allergen induced proliferation. Normalized proliferation (%, y-axis) measured by [3H] thymidine incorporation in cultured PBMC from 10 cat allergic patients after stimulation with mutants (LPS range 15–30 ng/mg protein) and two positive rFel d 1 controls containing 20 and 40 ng LPS/mg protein. Horizontal bars indicate standard error of the mean (SEM) values. * $p<0.05$ (Repeated measurements ANOVA with Newman-Keuls multiple comparisons test).

We also tested if the mutational procedure was able to generate proteins with reduced activation of allergen-specific T cells. Therefore, we measured the T cell proliferation of the vaccine candidates in PBMC cultures from ten cat allergic patients. Importantly, PBMCs stimulated with mutants 3.4.7 and 6.7.3 exhibited significantly lower mean proliferation indices in comparison to rFel d 1, ($p<0.05$) (Fig. 8). The mutant 6.7.1 showed a favorable trend towards lower induction of proliferation ($p<0.2$), whereas 11.5.2 showed proliferation comparable to rFel d 1. These results indicate that both mutants 3.4.7 and 6.7.3 may carry a lower risk of eliciting LPR upon treatment of cat allergic patients, which may be advantageous in comparison with similar vaccines.

Blocking antibodies of the IgG isotype have been suggested as an important mechanism behind successful SIT. In accordance, we immunized groups of mice with rFel d 1 or each of the four mutants. The immunogenicity of rFel d 1 and each of the mutants was investigated by ELISA. Interestingly, all mutants did induce higher titers of antigen-specific IgG than the wild-type protein, and surprisingly, all mutants except 11.5.2 induced higher titers of Fel d 1-specific IgG than rFel d 1. We also analyzed the specificity of the induced antibodies for rFel d 1 by real-time measurement of dissociation rates in Attana. After normalization of titer differences, the total shift (Hz) differed between the wild type and each of the mutants, with rFel d 1 showing the highest binding, followed by 6.7.1, 3.4.7, 6.7.3 and 11.5.2. Thus, a greater proportion of IgG antibodies induced by the wild type protein also bound to the wild type protein compared to antibodies induced by the mutants.

The capacity of the induced IgG antibodies to block serum IgE binding to rFel d 1 from 10 cat allergic patients was investigated by ELISA. rFel d 1 and the mutants 6.7.1 and 3.4.7 showed a similar ability to inhibit IgE-binding, whereas the blocking capacity of 11.5.2 was significantly lower than that of rFel d 1. (Fig. 9). The ability to block serum IgE binding to rFel d 1 was also compared using basophil activation test using basophils from three cat allergic subjects. In accordance with the ELISA results, rFel d 1 and the mutants 6.7.1 and 3.4.7 showed comparable capacity to inhibit IgE-responses, while 6.7.3 and 11.5.2 showed little if any blocking effect compared to mice immunized with PBS only. These results are supported by our off-rate screen of the induced sera in Attana, where similar dissociation rates between rFel d 1, 3.4.7 and 6.7.1 were obtained, while 6.7.3 and 11.5.2 dissociate more rapidly from rFel d 1. This indicates a strong cross-reactivity, similar to that of rFel d 1, by antibodies from 3.4.7 and 6.7.1.

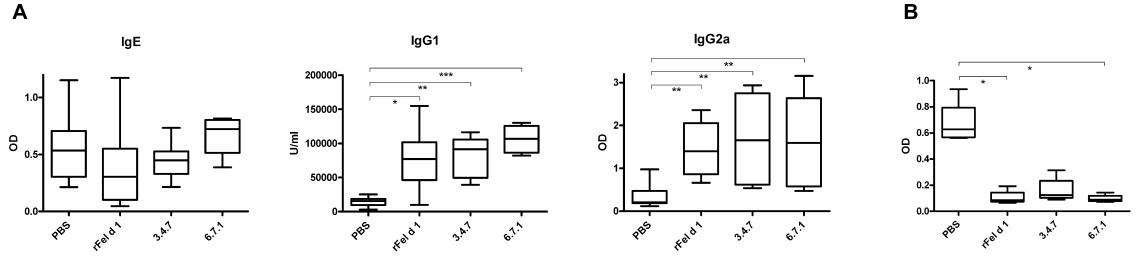

Figure 9. Evaluation of rFel d 1 and mutants in a mouse model for cat allergy. (A) Vaccination of rFel d 1 sensitized mice with rFel d 1 (n = 10), 3.4.7 (n = 7), 6.7.1 (n = 8) and PBS (n = 10) in an animal model for cat allergy. IgE, IgG1 and IgG2a-antibodies measured post treatment by rFel d 1 ELISA are presented as OD values, or for IgG1 in units/ml (U/ml). (B) IgE-blocking activity of pooled sera from sensitized mice vaccinated with either rFel d 1, 3.4.7 or 6.7.1 or PBS mice on sera from PBS treated mice by IgE-blocking sandwich ELISA. IgE responses are presented as OD values. Boxes with median values and horizontal bars denote 50% of values and 1 standard deviation respectively. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (Kruskal-Wallis with Dunn's multiple comparisons test).

The vaccine potential of the mutants 3.4.7 and 6.7.1 was compared to that of rFel d 1 in a mouse model for cat allergy. Mice were sensitized with low (1 µg) doses of rFel d 1 to obtain high levels of IgE antibodies, and were then vaccinated with higher (10 µg) doses of rFel d 1, 3.4.7 or 6.7.1. The treatment did not increase the IgE-responses in any of the four groups. However, vaccination with rFel d 1, 3.4.7 and 6.7.1 induced significantly higher titers of IgG1 and IgG2a than non-vaccinated mice, and no differences were detected between the mutants or the wild-type protein. The actively vaccinated groups potently blocked (rFel d 1 and 6.7.1, p-values < 0.05) or showed a favourable trend (3.4.7, p-value < 0.2) of blocking IgE-binding in sera from the PBS treated mice, indicating the usefulness of the mutants for efficacious treatment of cat allergic patients.

4.4 CONSTRUCTION OF A LINKED MOLECULE COMPRISING FOUR DOG LIPOCALIN ALLERGENS, FOR EFFICACIOUS ALLERGY DIAGNOSTICS AND THERAPY [IV]

The complexity of dog allergic sensitization distinguishes it from sensitization to cat, where the major allergen Fel d 1 is recognized by up to ~95% of all patients [147], and some dog allergic individuals only recognize minor allergen components [128, 131, 161]. The four dog allergens Can f 1, Can f 2, Can f 4 and Can f 6 all belong to the lipocalin family, thus sharing similar three-dimensional structures [131, 301] despite a low sequence homology (<25%). A low or no cross-reactivity may be expected between these dog lipocalins [161, 291]. Both diagnostics and SIT today rely on conventional allergenic extract, but the variability or even lack of allergenic content is a limiting factor. For dog allergy, commercial extracts may be of particularly low quality [98]. Modern biotechnology enables easy design, cloning and production of recombinant allergenic constructs, which may replace natural extracts for diagnostics and vaccination of allergic patients. Previous efforts to combine several allergens in one construct have been demonstrated for grass and mite allergies, but with variable success. Here, we present the design, production and evaluation of a linked construct (here designated Can f 1+2+4+6) comprising the four lipocalin allergens Can f 1, Can f 2, Can f 4 and Can f 6.

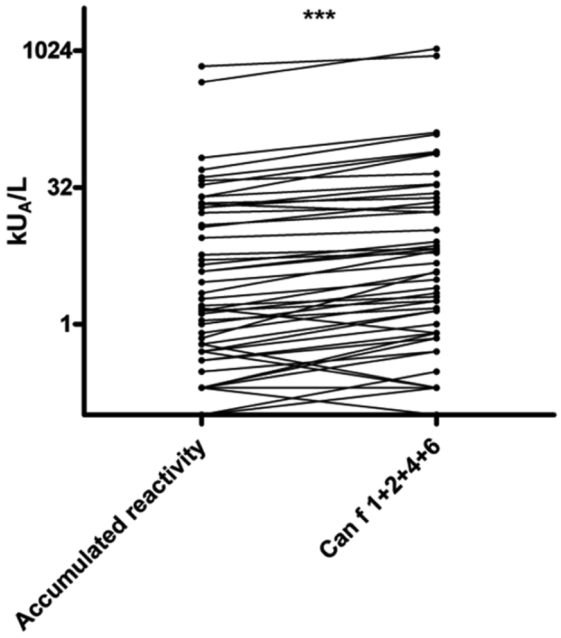

Figure 10. The linked molecule binds more IgE than the accumulated single allergens. Pair-wise comparison of accumulated IgE-reactivities to Can f 1, Can f 2, Can f 4 and Can f 6 among positive subjects with reactivity to Can f 1+2+4+6. *** p<0.001 (Wilcoxon matched pairs test).

We assembled the linked construct by overlapping PCR recombination, using oligonucleotides designed to introduce Gly-Ser-Gly-Ser linkers between the individual allergens. A corresponding recombinant fusion protein was produced, and after Ni2+ affinity chromatography, His-tag removal, ion exchange and size exclusion chromatography, a homogenous (>95%, by SDS-PAGE) and stable protein of approximately 75 kDa was obtained. The Can f 1+2+4+6 fusion protein eluted as a symmetrical peak, with an approximate molecular weight of ~90-100 kDa. The CD

spectrum of the construct closely resembled that of the equimolar mix of corresponding lipocalin allergens, indicating that the Gly-Ser link did not disturb the folding. Overall, the molecule appeared to behave as a soluble and well-folded protein, comprising the individual allergen components.

The IgE-binding properties of Can f 1+2+4+6 were analyzed, and compared to the individual allergens rCan f 1, rCan f 2, rCan f 4 and rCan f 6 or an equimolar mix of the four allergens, in sera from 100 dog-sensitized individuals from the greater Stockholm area. IgE-binding frequencies were 45% for rCan f 1, 16% for rCan f 2, 14% for rCan f 4, 39% for rCan f 6, 59% for Can f 1+2+4+6 and 53% for the mix. In most cases the IgE-levels to the linked construct was significantly higher than the accumulated reactivity of the four single lipocalin allergens or the mix ($p<0.001$) (Fig. 10), and in several cases, also to the extract. Occasionally, the construct also elicited an IgE-reaction, where all four allergens were negative. This may be an effect of avidity due to intrinsic cross-reactivity between the allergens in the construct, which otherwise appear negative as single entities. No case was recorded where Can f 1+2+4+6 was negative and a single allergen positive. Instead, the linked construct had an enhanced IgE-binding potential in comparison with the mix and the single allergens. There was a poor correlation between IgE-reactivities to the commercial extract and the linked molecule which may be explained by the serum selection based on IgE responses to the extract. Furthermore, 39% of the subjects that were positive to any lipocalin showed higher responses to the linked construct than to the extract, which may result from suboptimal exposure to patient IgE of lipocalins in the extract.

We found that the linked molecule effectively covered the entire IgE-spectrum of the included lipocalin allergens, by means of competitive inhibition ELISA. The assembly of

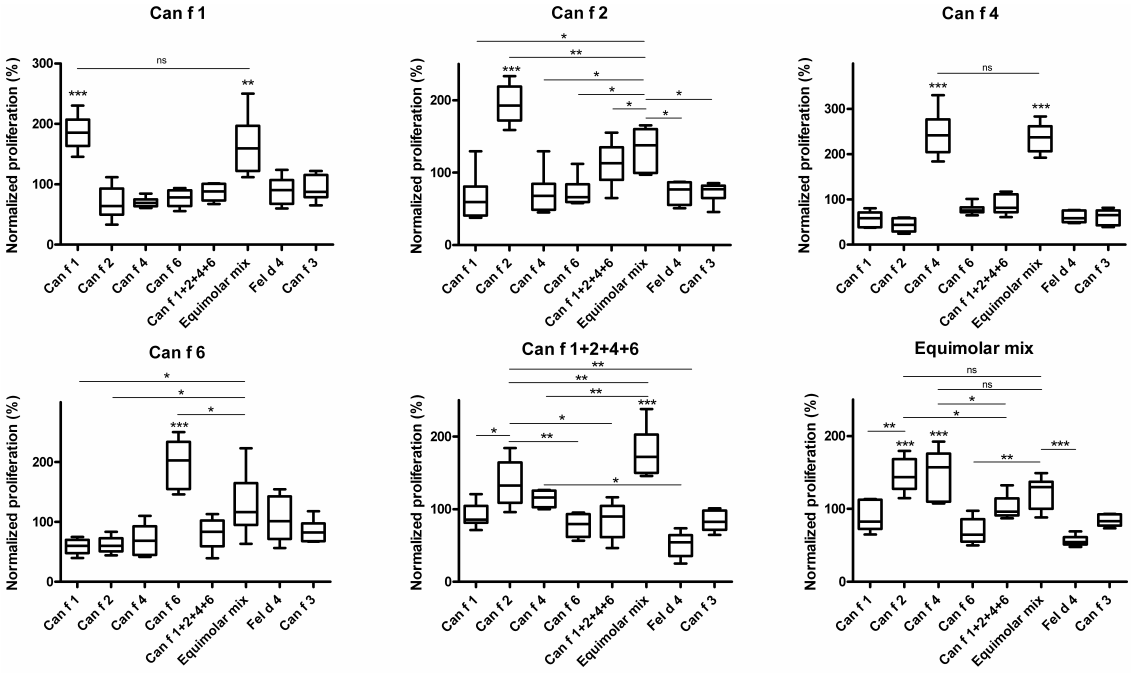

Figure 11. T cell proliferation of linked construct and related allergens in immunized BALB/c mice. Proliferative responses to stimulation of splenocytes from immunized mice with 10 µg Can f 1 or equimolar amounts of rCan f 2, rCan f 4, rCan f 6, an equimolar mix or Can f 1+2+4+6, measured by [3H] thymidine incorporation (y-axis). Annotations for significance (*) stated directly above boxes are true for all group comparisons, unless specified otherwise. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ns (non significant) $p>0.05$, analyzed with repeated measurements ANOVA with Tukey's multiple comparison test.

the individual allergens into one fusion protein did not negatively affect binding to either allergen in the linked molecule. There were no differences in binding to the linked molecule and individual components, because no cross-reactivity was observed between the allergens. Interestingly, we noticed a slight increased IgE-binding to Can f 1+2+4+6 at lower concentrations possibly due to an increase in antibody avidity by cross-reactive neighbouring epitopes, particularly to Can f 4.

The biological activity of the individual allergens was also maintained in the linked construct, as investigated by the ability to upregulate degranulation markers in a basophil activation test. The outcome depended on the sensitization profile of the patient. The sensitivity to the linked molecule was enhanced in comparison to the mix or the single recombinant allergens in a multi-sensitized patient. This is likely due to the multiplicity of IgE epitopes on the construct being able to induce stronger basophil degranulation in comparison to the single monomeric allergens with fewer epitpopes. This is also supported by results of enhanced allergenicity of dimers, in comparison to monomers [76]. On the other hand, as shown by the same patient, the mix exhibited a higher reactivity than the linked molecule. However, as observed with two other patients who were only reactive to one allergen in ELISA, the allergenic activity of Can f 1+2+4+6 was similar or even lower than the single recombinant allergen or the equimolar mix.

The usefulness of allergen linkage for vaccine purposes was investigated by immunization of BALB/c mice. Groups of 6 mice each were immunized with rCan f 1, rCan f 2, rCan f 4, rCan f 6, Can f 1+2+4+6 or the allergen mix. We then investigated the proliferative responses of splenocytes upon culturing with the single allergens, the mix or the linked construct. We found that the individual recombinant allergens potently induced high proliferative responses in mice (Fig. 11). In cases of rCan f 2 and rCan f 6, by an unknown mechanism, the mix exhibited significantly lower responses than the individual allergens. The linked vaccine construct did not induce significantly higher proliferative responses in any of the cultures. However, splenocytes from mice immunized with Can f 1+2+4+6 proliferated significantly higher with rCan f 2, rCan f 4 and more prominently the mix, but not with Can f 1+2+4+6 itself. This effect might reflect a different uptake of the linked construct, possibly due to the increased molecular weight. It was evident that rCan f 2 and rCan f 4 dominated the proliferative responses in cultures from mice immunized with the mix, and proliferative responses to rCan f 1 and rCan f 6 were not significantly higher than to the control allergens rFel d 4 and nCan f 3 in these mice. The mix also induced the highest proliferation in mice immunized with the mix, observed as overgrowth in the culture wells, indicating that the effect is additive in this case. These results show that we can expect the vaccine to modulate T cell responses for allergen recognition, without inducing strong proliferative responses. Consequently, the linked constructs used as a vaccine may be safer for use in SIT, by carrying a lower risk of eliciting LPR.

Surprisingly, the relationship observed in our proliferation assay was not completely mirrored by the immunoglobulin responses. We found that the linked construct induced high titers of allergen-specific IgG1 to all individual allergens, which were comparable with antibody levels induced by the single recombinant allergens or the mix. Interestingly, the difference between the linked molecule and the equimolar mix is evident when comparing the variability of the induced immunoglobulin levels. While the linked construct induced steady IgG1-responses towards all allergens (only rCan f 1-levels being higher), the mix induced variable levels towards the included allergens (Fig. 12). Both

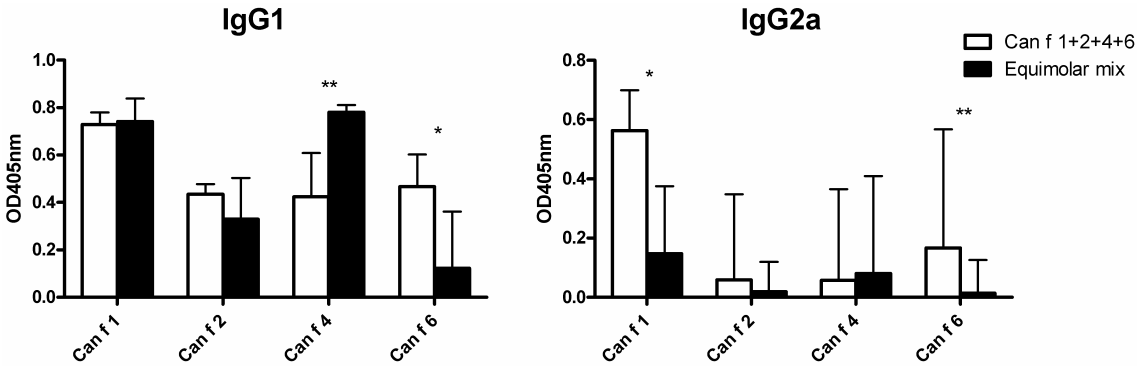

Figure 12. Allergen-specific IgG1 and IgG2a-responses induced by immunization of mice with Can f 1+2+4+6 or an equimolar mix. Comparison of IgG1 and IgG2a-antibodies to each single lipocalin allergen induced by either Can f 1+2+4+6 or a mix. * $p<0.05$, ** $p<0.01$, analyzed with Mann-Whitney test.

rCan f 1 and rCan f 4-specific IgG1-levels were significantly higher than the weak responses to rCan f 6. The rCan f 2-levels also showed a weak trend in comparison. We speculate that the weak cellular and humoral responses to rCan f 6 induced by immunization with the mix, may be accounted for by the close relationship with Mus m 1. It may be argued that the overall high allergen-specific IgG levels of the linked construct is preferential over very high and very low antibody responses towards some allergens. Notably, IgG1-levels to Can f 6 induced by the linked construct were higher ($p<0.05$) than those induced by the mix, and thus, the concept of allergen linkage appears to boost immunoglobulin responses to otherwise weak antigens. Can f 1+2+4+6 also induced significantly stronger IgG2a-responses towards rCan f 1 and rCan f 6, compared with the mix, indicative of a Th1-skewed response in these mice, which may a beneficial feature for SIT. Also, a strong T cell response might not a necessity for the induction of blocking IgG responses, which also favours a lower risk of inducing LPR.

5 CONCLUSIONS

Paper I: The crystal structure of the dog lipocalin allergen Can f 2 was determined to a resolution of 1.45 Å, and showed a classical lipocalin fold. The recombinant protein showed nearly identical IgE-binding features to the natural protein as investigated by inhibition ELISA. We identified several key features of the allergen structure, including the formation of a "lid" by the loops L1 and L5, which is likely to be related to the potential biological function of Can f 2 as a ligand binder. Using sera from Can f 2-sensitized patients, we identified an IgE-mediated cross-reactivity of Can f 2 to the cat lipocalin allergen Fel d 4, despite a low sequence homology between these proteins. A negatively charged conformational epitope conserved in these two allergens were suggested as the potential site of cross-reactivity.

Paper II: A novel dog lipocalin allergen designated Can f 6 with high homology to major lipocalin allergens from cat (Fel d 4) and horse (Equ c 1) was identified. The recombinant allergen was produced as a well-folded monomeric protein. rCan f 6 elicited a positive IgE response in nearly 40% of dog sensitized subjects, signifying it as an important allergen. Sensitization to rCan f 6 correlated with sensitization to the homologous allergens rFel d 4 and nEqu c 1, and most subjects recognized all three allergens. The biological activity of rCan f 6 was demonstrated by basophil activation test, and by inhibition ELISA, we confirmed an IgE-mediated cross-reactivity in several dog-sensitized patients. Thus, Can f 6 is implicated as a possible candidate for cross-species sensitization.

Paper III: As proof of concept, we utilized epPCR and phage display to create a novel type of allergy vaccines, with alterred primary structure, but maintained tertiary structure. Four vaccine candidates were isolated, and produced as soluble monomeric recombinant proteins with a conserved secondary structure in three of four proteins. We observed a reduced IgE-binding, measured by direct and competitive inhibition ELISA, and the allergenic capacity of the mutants was reduced in comparison with the wild type protein, assessed by BAT with blood from cat allergic individuals. Two of four mutants had an attenuated T cell activation capacity, investigated upon culturing of PBMC from cat allergic patients using the wild type protein as a control. All mutants but one induced higher titers of rFel d 1-specific IgG antibodies than the wild type protein in mice, and the antibodies were able to block IgE responses in sera from cat allergic individuals. Finally, the usefulness of our vaccine candidates was demonstrated in a mouse model for cat allergy, where mice sensitized to rFel d 1 had a potent induction of allergen-specific IgG1 and IgG2a that blocked IgE-responses in non-treated mice. We conclude that the described methodology may be used to generate similar vaccines from other allergens.

Paper IV: We assembled four of the six known dog allergens in one recombinant construct. The linked molecule was expressed as a soluble fusion protein that showed nearly identical secondary structure composition in comparison with a mix of all individually included allergens. By quantative measurements of IgE-reactivities in dog sensitized individuals, the linked construct bound significantly more IgE than the accumulated responses to the four individual allergens. The linkage seemed not to affect any of the IgE-binding epitopes, investigated by comparison with an equimolar mix of the included allergens in competitive inhibition ELISA. The biological activity of the individual allergens was also maintained in the linked construct, as shown by BAT. By vaccination of BALB/c mice, we demonstrated the advantages of the linked construct in comparison with a mix of the included allergens. The linked construct induced high

immunoglobulin responses to all four allergens, while the mix produced variable responses to each of the components. In addition, the linked molecule promoted IgG2a responses to two of the individual allergens, which may be a desirable feature for SIT. Humoral responses induced by the linked molecule were present despite that no significant proliferative responses were exhibited by the linked construct, measured by proliferation of splenocytes from immunized mice. Thus, the linked molecule may carry a lower risk of eliciting LPR. Our results show the usefulness of the linkage approach for generation of allergy vaccines covering epitopes from several allergens.

6 FUTURE PERSPECTIVES

This work uses molecular approaches to improve the quality of life of people affected by IgE-mediated allergies to pets. In Sweden, allergies to pet animals actually seem to be more common than allergies to pollens among school children [139], and are a prominent cause of asthma. Allergy to birch or timothy grass pollen, as well as allergy to cat, have been explored extensively during the last decades. Successful clinical trials with recombinant vaccines based on allergens from these sources have been published during recent years and are expected to enter the markert in the coming years [229]. Allergy to the domestic dog is among the most common allergies in Sweden, and is strongly associated with allergies to cat and horse [120, 121, 139]. Despite the major role of dog allergy, it has been studied less extensively than cat or pollen allergies.

The low research activity in dog allergy is likely connected with the complex sensitization profiles to dog allergens among sensitized patients. Two major dog allergens have been identified, Can f 1 and Can f 5, recognized by around 55% and 70% of dog allergic subject. However, the sensitization prevalence for Can f 5 has not been replicated, thus the number reported is uncertain. In addition, several dog sensitized subjects only recognize minor allergen components, adding to the complexity of dog allergy. In **paper I**, we focused on a minor dog allergen, Can f 2, and aimed to characterize its role in dog allergy on both structural and immunological basis. Previously, lipocalin allergens from horse, mouse and rat had been structurally characterized. The crystal structure of Can f 2 further adds to the panel of structurally determined lipocalin allergens. The potential mechanisms behind the allergenicity of lipocalins have been extensively debated, and our paper describes several structural features that may improve the understanding of this allergen family. For instance, the lid formation of loops L1 and L5 may play a pivotal role in the functional role of Can f 2. This may relate to its allergenicity, which could be investigated in future studies. Can f 2 also stands out among the other dog lipocalin allergens, since no homologous allergen with a high identity (>50%) has been identified in any other species. It is unclear whether this relationship relates to the low frequency of Can f 2-sensitized patients. However, among subjects sensitized to this allergen, relatively high allergen-specific IgE levels are not uncommon (>10 kU_A/L, **Paper I, II, IV**), and we detected several individuals with very high IgE levels to Can f 2 (>100 kU_A/L). Thus, the low frequency of sensitized patients may not be related to the allergenicity of this protein, but rather to a low expression of natural Can f 2 in various dog breeds. Furthermore, it would be desirable to quantify the expression of dog allergens in extracts from different dog breeds, e.g. by using monoclonal antibodies. This information could be used to match the sensitization profile of dog sensitized individuals who plan on purchasing a dog, with dogs which express low levels of matching. This concept would bring the term "hypoallergenic dog" to an acceptable scientific level, and improve allergen avoidance in dog allergy, as a primary low-cost intervention.

The importance of an allergen is most exclusively derived from its frequency of IgE-binding among patients allergic to that source. For cat allergy, a high interest has naturally been attributed to the major allergen Fel d 1, which is recognized by up to 95% of all cat allergic patients. On the other hand, the minor cat allergens Fel d 2, Fel d 3, Fel d 5, Fel d 6, Fel d 7 and Fel d 8 have all generated relatively low interest, and the majority of publications describing cat allergens are addressing the major allergens Fel d 1 or Fel d 4. Moreover, papers that characterize novel allergens may generate a higher interest if the allergen is recognized by more subjects, especially if the number is over 50%, hence the

allergen will be designated a major allergen. **In paper II and IV** we characterized the sensitization profile to a new dog allergen, Can f 6, which was identified by 38 and 39 of 100 respectively consecutively selected dog sensitized subjects. Thus it is a minor allergen. We have also shown the accuracy of the frequency, as it was replicated in two separate Swedish populations. However, the sensitization described for other allergens may not be as replicable, due to a narrow selection of sensitized individuals. As exemplified by the lipocalin allergen Can f 4 [128], the sensitization frequency was analyzed in 37 dog sensitized subjects only. Among those, 13 individuals recognized the allergen, and the sensitization frequency was described as 35%. As shown in **paper IV**, we cannot nearly reproduce this number among 100 dog-sensitized individuals, where only 15% recognized the allergen. On the other hand, we analyzed the IgE-binding frequency of rCan f 1, rCan f 2, rCan f 4 and rCan f 6 among 40 dog allergic patients from the Stockholm area (unpublished data). Here, we detected IgE-binding frequencies of 28% to Can f 1 and Can f 6, 18% to Can f 2, and remarkably 35% to Can f 4, making it the most frequently recognized allergen among these patients. This finding illustrates how a small patient selection may skew the outcome of an IgE-binding profile, and consequently, a higher number of subjects should be required for publications that characterize novel allergens. As far as it goes, we cannot draw any conclusions from our unpublished data on the 40 dog allergic patients, for reasons previously mentioned. However, we might speculate that Can f 4 may be particularly important in the Stockholm area. This may possibly be due to a specific selection of popular dog breeds in the area, or by housing conditions that may differ between larger cities and smaller communities. These results suggest that further investigation is warranted. It would also be convenient to be able to suggest a combined panel of dog allergens to diagnose virtually all dog allergic patients. However, component-resolved diagnostics of dog allergic patients is less sensitive, since the current dog allergen panel seems incomplete. Therefore, it is necessary to identify and characterize additional dog allergens for improved diagnostics and therapy of allergic patients.

Even though Can f 6 is a minor allergen, it may still be implicated as a key allergen in dog by its cross-reactive relationship with Fel d 4 from cat and Equ c 1 from horse. The study conducted in **paper II** characterizes Can f 6 based on dog-sensitized patients only and does not take into account cat or horse-sensitization. However, it is likely that IgE-binding frequencies of Can f 6, Fel d 4 and Equ c 1 is greatly enhanced in patients with a primary sensitization to all three of these animals, and preliminary data obtained in our lab support this assumption. Historically, the strong allergenic relationship between pets, the albumin allergens from these sources, i.e. Can f 3, Fel d 2 and Equ c 3, were credited with the role of cross-over allergens, causing poly-sensitization to these sources. The albumins may complement the lipocalins with this relationship. Also, it is not clear if other allergen families may add to the strong relationship between allergies to different mammalian species. One paper identified a dog homologue to Fel d 1 in dog dander. However, the protein has not been verified by others, and the occurrence of allergens homologous to Fel d 1 in other species is still disputable. The dog allergen Can f 5 is a kallikrein, and a BLASTp search with the corresponding template gene for this allergen identified potential homologues in both cat and horse. The role of kallikrein allergens from these sources however remains to be elucidated.

The term cross-reactivity is often used to describe a common relationship between structurally related allergens, and seldom discriminates co-sensitization from cross-reactivity or distinguishes cross-reactivity among B cell epitopes (antigen surface area) with those of T cell epitopes (the linear allergenic peptide interacting with the T cell receptor). In **paper I-II**, we focused on IgE-mediated cross-reactivity between Can f 2

and Fel d 4 and Can f 6, Fel d 4 and Equ c 1 respectively. It is unclear how the cross-reactivity observed between Can f 2 and Fel d 4 relate to the cross-reactivity identified between Can f 6, Fel d 4 and Equ c 1. It is clear that even very low levels of allergen may induce allergic sensitization, but to what extent IgE-mediated cross-reactivities influence neo-sensitization is not clear and should be investigated further. Furthermore, conserved linear sequences among allergens that do not implicate IgE-mediated cross-reactivity, but may promptly be recognized by the T cell receptor (i.e. T cell mediated cross-reactivity) may also play a central role in poly-sensitization to related allergen sources. To properly characterize the effects of IgE-mediated and T cell mediated cross-reactivity on the development of allergic sensitization to multiple pets, it would be attractive to perform a cohort study of children with at least one positive sensitization to one pet species. In a prospective study, blood samples would be taken for the analysis of allergen-specific IgE antibodies, and culturing of PBMCs with a selection of cross-reactive allergens. It would be particularly interesting to determine whether susceptibility to cross-reactive allergen components can be detected by serum or cellular analysis, before the development of allergic symptoms to new sources. In other terms, one could define an allergy prognosis, based on allergen-specific reactivity, which could be used for recommendations of allergen avoidance for these patients.

In **paper III-IV** we describe the development of conceptual vaccines for cat and dog allergy. The methodology is applicable for any allergen source, and does not discriminate for particular allergen families. Consequently, we could generate hypoallergenic mutants by epPCR of any allergen with a known primary structure as in **paper III**, given that the allergen is properly folded on the surface of the phage. It would also be possible to engineer linked allergen vaccine comprising any recombinant allergen, as in **paper IV**, assuming that the linkage does not disturb the protein fold, or that the total construct does not exceed a molecular weight limitation of *E.coli*.

The hypoallergenic mutants generated by epPCR were subjected to evolutionary pressure, by selection of clones with a maintained fold for IgE-binding in phage display. This term is also designated molecular evolution, and carries the advantage of being highly versatile for the selection of clones with a desired specificity, while requiring no background knowledge of the selected target allergen. The biopanning process selects for any mutation introduced to the primary structure that does not disturb important IgE-binding structures, while neglecting those that disrupt the entire protein fold. This technique effectively preserves conformational epitopes that could be required for the induction of blocking IgG antibodies. Instead, the selection process ensures that improperly folded variants are excluded by their diminshed IgE-binding. One can expect most clones to retain a certain amount of IgE-binding, either by fully or partially conserved conformational epitopes, but also linear epitopes that escape mutation will account for some IgE-binding. Over several biopannings, by selection with patients IgE antibodies, only the strongest binders will be retained as a result of the increasing stringency in the washing procedure. In fact, IgE may be particularly suitable for this selection process, in comparison with IgG. Specific IgG levels for a particular allergen may account for a very low amount of the total circulating IgG levels, and capture of allergen-specific IgG for the biopanning of allergens would be troublesome due to high-levels of non-allergen specific IgG. However, by the selection of patient sera with a high fraction of allergen-specific IgE of the total IgE fraction, we may overcome this problem. Using a similar approach, molecular evolution of allergens may also improve allergy diagnostics using recombinant allergens. Using affinity maturation by phage display, improperly folded recombinant allergens that are unable to take a natural shape in the recombinant setting, thus having a weak IgE binding profile, may be “improved” for better IgE binding.

In **paper III and IV**, we have characterized novel allergy vaccine candidates with reduced (**III**) and maintained (**IV**) IgE-binding capacity for production of IgE blocking IgG antibodies that are intended as disease modifiers by therapy of allergic patients. However, safety aspects must be addressed in a successful allergy vaccine. The described constructs carry the advantages of recombinant allergy vaccines over extract-based vaccines, i.e. they are possible to standardize, do not vary in allergenic content, and do not contain non-allergenic contaminants. While we expect the hypoallergenic mutants to have an improved safety profile for acute reactions in conventional subcutaneous immunotherapy, the linked molecule contain all conformational epitopes of the original allergens. In recent development, the use of alternative administration routes such as intralymphatic injections [255, 256] has shown that acute side effects can be avoided when using recombinant natural like allergens. This strategy also allows for the usage of lower injection doses, while maintaining, or even enhancing efficacy of the treatment. SIT with high doses of modified birch pollen allergens has demonstrated the induction of late asthmatic responses within hours of allergen administration [270], which it is a limiting factor of SIT. We have shown that both the hypoallergenic mutants and the linked vaccine construct may carry a lower risk of eliciting LPR, by their lower T cell activation capacity through the mutation of T cell epitopes (**III**) and possibly through an altered uptake (**IV**). It is likely that a certain amount of T cell activity is required for successful SIT, but whether a very strong T cell activation also implicates a better clinical outcome remains to be elucidated. In fact, only a fraction of the total T cell response towards an allergen might be necessary for the induction of tolerance, through the mechanism of epitope-linked suppression [302]. As demonstrated in **paper IV**, our linked construct induced high titers of allergen-specific IgG to all included allergens, compared with an equimolar mix of the same allergens. This finding was not replicated in our splenocyte proliferation assay, where the linked molecule did not induce significant proliferative responses, implicating that a strong T cell activation may not be required for the generation of protective responses in this setting. Future studies may demonstrate the advantages of linked allergen vaccines for therapy of allergic individuals.

Component-resolved diagnostics with recombinant allergens is a valuable tool for researchers and allergologists. We have demonstrated the usefulness of allergen linkage for diagnostics of allergic patients, shown as an increase in IgE-binding compared with the accumulated individual allergen components. Using this strategy, we may not only cover a broad spectrum of IgE epitopes within one molecule, but may also enhance the sensitivity of the assay. Hypothetically, multiplication of the same allergen in a linked construct should enhance the sensitivity to that component, and this may be particularly useful for chip diagnostics, such as ISAC. Linked allergen constructs should also increase the diagnostic sensitivity in BAT, where oligomeric allergens have shown to enhance basophil degranulation [76].

In **paper III and IV**, we have developed conceptual vaccine candidates for improved treatment of allergic patients. The hypoallergenic mutants showed a good safety profile *in vitro*, but it is necessary to conduct a clinical trial to fully elucidate the usefulness of this approach. Very few allergen vaccines reach clinical trials, mostly due to a very costly process before the treatment can start. In fact, we may have reached a point where it may be nearly impossble for academic departments to develop vaccines for clinical trials. Consequently, promising theurapeutic options that may improve the quality of life for a significant number of the population might never be tested. Long-term, this may correspond to a significant reduction of translational research conducted in Sweden. However, future vaccines based on our methodology may be formulated for other

allergens and perhaps in collaboration with other countries. Hopefully, hypoallergenic mutants may one day be used for safe and efficacious treatment of allergic patients.

The strategy with the linked allergen used in **paper IV** should also be used to generate similar molecules in the future. It would be convenient to formulate molecules that comprise allergens from multiple sources. This would allow for the creation of a new generation of general vaccines, which could be used not only for desensitization of allergic individuals, but also for prophylactic vaccination. It may be years before prophylactic allergy vaccination of children is achieved, due to safety aspects. However, it may easily be argued that prophylactic allergy vaccination should be the superior choice over classical SIT, particularly in children with a familial atopy. All individuals will ultimately be exposed to allergens from the most common sources during a life-time. The question is whether this encounter will lead to the allergen tolerance as in healthy individuals, or to allergic sensitization, rhinitis and asthma. Vaccination using major allergen components in a controlled way should ensure a tolerogenic state, while random un-controlled exposure could implicate allergic sensitization. In fact, prophylactic allergy vaccination may eventually put allergy researchers out of business.

To conclude this thesis, **papers I-IV** describe how we may use state of the art laboratory techniques within the field of allergy for an improved understanding of allergenicity, a sensitive and specific diagnostic platform, and safer and more efficacious therapeutics of allergic patients. In order to answer the question of "what makes an allergen an allergen", we must consider several factors, such as tertiary structure characteristics, primary structure motifs that are conserved across several allergens and functional characteristics which may determine how the allergen is interpreted by the immune system. We may then understand how concepts of cross-reactivity may translate into poly-sensitization to various allergen sources. Finally, we may use our knowledge of allergenic molecules in order to create vaccines, either unspecifically through molecular evolution, by careful design of linked constructs, or by a combination of the two. Ultimately, we may accomplish a reduced burden of allergic diseases on affected individuals, which not only would improve the quality of life of allergic patients, but also lighten the socio-economic burden on societies of the industrialized world.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Allergiska sjukdomar berör en stor del av befolkningen världen över, såväl barn som vuxna. Framförallt i västvärlden är allergier vanligt förekommande, och upp till 30% av befolkningen är drabbad i vissa länder. De allra flesta allergier beror på en missriktad immunologisk reaktion mot normalt ofarliga proteiner som då uppfattas som främmande av kroppen, s.k. allergen. En stor mängd djur, växter och födoämnen innehåller allergen, och vanliga allergenkällor för svenskar är katt, hund, häst, björkpollen, gräspollen och jordnötter. Den immunologiska reaktionen karaktäriseras av att sjukdomsframkallande IgE-antikroppar som sitter på immunceller reagerar med allergenet, och får cellerna att i sin tur reagera genom att frisätta en mängd ämnen som leder till allergiska symtom. Dessa symtom uppkommer vanligtvis inom några minuter vid allergen-exponering, och kan omfatta allergisk rinit (hösnuva) eller kliande eksem. I många fall går dock en allergi över i astma, vilket bland annat kan ge upphov till andningssvårigheter, och i sällsynta fall orsaka en så kallad anafylaktisk chock vilken kan ha en dödlig utgång. En vanlig rekommendation till allergiker är att undvika de pälsdjur, pollen eller födoämnen man är allergisk mot, vilket kan vara särskilt svårt under exempelvis pollensäsongen.

Allergier mot pälsdjur är bland de mest förekommande i världen, vilket är tydligt i Sverige där allergier mot katt, hund och häst är vanliga. Särskilt hos barn spelar pälsdjursallergi en stor roll, då det är den vanligaste orsaken till utveckling av barnastma. Effektiv diagnostik på ett tidigt stadium är viktigt för att säkerställa känslighet mot pälsdjur, vilket kan användas för att förhindra utveckling av astma. Traditionellt sett så har allergier diagnostiserats med hjälp av allergenextrakt som appliceras på huden, varvid man avläser en reaktion och ställer diagnos. Ett alternativ är att ett blodprov tas, som sedan analyseras i professionella laboratorier. Allergenextrakt används även för att inducera ett långvarigt skydd mot allergier, genom behandling med specifik immunterapi (SIT). Denna behandling skiljer sig från traditionell behandling med anti-histaminer eller steroider, vilka endast kortvarigt behandlar symtomen utan att åtgärda den underliggande orsaken. Vid SIT får patienten ett stort antal injektioner med ökande dos allergen-extrakt för att göra immunsystemet tolerant mot de allergener man är allergisk mot, s.k. tolerans. Vid lyckad tolerans ändras det skadliga immunsvaret, och de celler som vanligtvis orsakar en allergisk reaktion påverkas, vilket leder till färre allergiska symtom.

Både allergidiagnostik och SIT baseras idag på användandet av allergenextrakt, vilka innehåller ett stort antal olika allergena och icke-allergena ämnen. Blandningen av olika allergen i allergenextrakt är svår att kartlägga, något som kan vara till nackdel vid båda användningsområdena. Vid diagnostik vill man gärna veta exakt vilka enskilda allergen en patient reagerar mot. Detta kan avgöra vilka rekommendationer som skall delges patienten för att bäst behandla allergin. Vid SIT är det särskilt fördelaktigt att använda enskilda allergena komponenter. Detta är särskilt viktigt ur säkerhetsperspektiv, då man gärna inte vill utsätta patienten för andra allergener än de som han/hon reagerar mot, vilket kan leda till att man istället förvärrar allergin genom att introducera nya allergen. Med hjälp av DNA-teknik kan syntetiska, s.k. ”rekombinanta” allergen framställas, vilka kan ersätta extrakten för diagnostik och behandling av allergiska sjukdomar. Denna teknik kan även användas för att skapa försvagade allergenmolekyler, med färre biverkningar, som ska leda till en säkrare SIT. När denna avhandling skrivs har de allra flesta allergener från de viktigaste allergen-källorna klonats, även om det fortfarande saknas vissa pusselbitar för att täcka hela allergenpanelen. Avhandlingens första två delarbeten fokuserar på karaktärisering och identifiering av allergener hos hund, och sätter detta i

perspektiv till andra pälsdjursallergier. Delarbete tre och fyra beskriver framtagandet av två nya allergivacciner för behandling av kattallergi respektive hundallergi.

Allergenet Can f 2 ingår i proteinfamiljen lipokaliner, och bidrar till hundallergi världen över. **Delarbete I** visade för första gången den tredimensionella strukturen för ett hundallergen. Vissa ingående strukturella egenskaper hos Can f 2 beskrevs i arbetet, vilket är viktig information för att förstå proteinets biologiska funktion, samt ger möjlighet att utreda varför det är ett allergen. Vidare jämfördes den tredimensionella strukturen med liknande strukturer hos andra allergener och humana proteiner inom samma proteinfamilj. En viktig del i arbetet är karakteriseringen av Can f 2, där det påvisades att det rekombinanta proteinet som strukturbestämts, har samma egenskaper som det naturligt förekommande proteinet. Slutligen användes serum från individer med ett tidigare uppmätt positivt allergitest mot hund, för att påvisa liknande allergena egenskaper för Can f 2 med ett liknande allergen hos katt, Fel d 4, vilket skulle kunna förklara varför det finns en koppling mellan allergi mot katt och allergi mot hund.

Länken mellan häst-, hund- och kattallergi har länge varit känd, då det är vanligt att allergi mot ett pälsdjur senare går över i en eller flera ytterligare pälsdjursallergier. Förklaringen till detta har hittills tillskrivits proteinfamiljen albuminer, mot vilka endast en bråkdel av pälsdjursallergiker reagerar mot. I **delarbete II** beskrevs identifieringen och framtagandet av ett helt nytt allergen hos hund, samt dess roll i med allergi mot katt och häst. Hundallergenet Can f 6 identifierades, och det visade sig att detta allergen är väldigt likt både Fel d 4 från katt och Equ c 1 från häst. Vi påvisade att ungefär 40% av alla hundallergiker reagerar mot Can f 6-proteinet, och att de flesta av dem även reagerar mot de liknande proteinet i katt och häst. Naturlig förekomst av Can f 6 påvisades i ett proteinextrakt från hundmjäll, och en betydande biologisk aktivitet av allergenet demonstrerades efter stimulering av celler som ger upphov till allergiska symptom. Dessutom påvisades att den starka kopplingen till Fel d 4 och Equ c 1 beror på att samma antikroppar kan reagera med de olika allergenerna, vilket bidrar till att förklara den starka kopplingen mellan dessa tre pälsdjursallergier.

Allergi mot katt är vanligt förekommande, och berör runt 10% av alla svenskar. I **delarbete III** användes metoden phage display på ett helt nytt sätt, för att ta fram modifierade vaccinkandidater baserade på huvudallergenet för katt, Fel d 1. Syftet var att ta fram kandidater med färre bi-effekter för säkrare behandling med SIT. Efter 7 anrikningssteg erhölls 4 st vaccinkandidater som sedan framställdes på rekombinant väg. Vaccinkandidaternas biokemiska egenskaper liknade orginalproteinet, och vi kunde demonstrera en lägre IgE-bindning med hjälp av serum från kattallergiker. Vi kunde även påvisa att några av vaccinkandidaterna hade en lägre aktivering av immunceller, vilket skulle kunna betyda att de är säkrare för behandling av allergiker. Till sist kunde vi visa att vaccinkandidaterna behållit sin skyddande förmåga, och gav ett lovande immunsvar efter behandling av möss som gjorts ”allergiska”. Dessa kandidater är således intressanta möjligheter för framtida behandling av patienter med kattallergi.

Den nuvarande allergenpanelen för hund består av sex stycken allergen, varav fyra tillhör familjen lipokaliner. Nyligen genomförda studier har påvisat stora brister hos de hundextrakt som används rutinmässigt för allergidiagnostik och allergibehandling med SIT. **Delarbete IV** beskrev konstruktionen av ett nytt vaccin mot hundallergi genom molekylärbiologisk ihopkoppling av fyra hundallergener till ett helt proteinkonstrukt med hjälp av rekombinant DNA-teknik. Efter framrening av proteinet kunde vi fastställa att de biokemiska och immunologiska egenskaperna hos de ursprungliga allergenerna bibehållits i konstruktet. Vi fann också att de uppmätta IgE-nivåerna hos hundallergiker

mot konstruktet i de flesta fall också var högre än den samlade IgE-reaktiviteten mot de enskilda allergenerna i molekylen, vilket utgör en stor potential för förbättrad diagnostik. Slutligen utvärderades konstruktet som ett vaccin genom vaccination av möss. Vi fann att konstruktet gav potentiellt skyddande allergenspecifika IgG-antikroppar hos mössen, samtidigt som resultaten indikerade att det skulle kunna ha säkrare egenskaper vid behandling. Manuskriptet visar på "proof of concept" för vaccinstrategin av länkade vacciner, vilket möjliggör konstruktion av liknande molekyler för andra allergier.

Sammantaget visar de fyra delarbetena i denna avhandling hur rekombinanta DNA-tekniker kan användas för att bryta ny mark inom allergiområdet. Med hjälp av strukturbestämning av allergener kan vi förstå bakgrunden till varför de gör oss allergiska. Identifieringen av nya allergen gör också att vi bättre kan diagnostisera allergiker, samt förklara varför en allergi kan övergå i en annan. Till sist kan vi tillämpa vår kunskap om allergener för att skapa vacciner för säkrare behandling, vilket skulle leda till en ökad livskvalité hos behandlade patienter.

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