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NOVEL TREATMENT STRATEGIES AND REGULATION OF IgE-MEDIATED ALLERGIC DISEASE

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Stockholm 2008

Published by Karolinska Institutet. Printed by INTELLECTA DOCUSYS

ISBN 978-91-7357-440-2

There’s a light that never goes out

Steven Patrick Morrissey

ABSTRACT

Allergic symptoms such as rhinoconjunctivitis, asthma or gastrointestinal symptoms, triggered by inhaled or ingested allergens cross-linking allergen-specific IgE on mast cells or basophils, are defined as *IgE-mediated allergy*. The major allergens from birch pollen (Bet v 1) and cat dander (Fel d 1) are two common allergens eliciting allergic disease. Allergen-specific immunotherapy (SIT) is the only curative treatment for IgE-mediated allergy. It is long-lasting and involves repeated injections of crude allergen extracts. Successful SIT modifies a number of allergen-associated immunological responses. SIT has been shown to induce IL-10 producing regulatory T-cells (Treg), allergen-specific T- and B-cell anergy as well as blocking antibodies. Although effective, SIT is associated with a risk for treatment side effects. This has led to the development of novel treatment strategies, such as modified recombinant allergens with reduced allergenicity (hypoallergens) and new means of antigen delivery. The general aim of this thesis is to investigate regulation of allergic immune responses and how novel strategies for SIT affect those responses.

The first article describes an eight injection short-course SIT study with Bet v 1 hypoallergens; where 27 birch pollen allergic patients participated. The major findings were that SIT with genetically modified Bet v 1 hypoallergens induced allergen-specific neutralizing antibodies and reduced immediate skin reactivity as well as the number of IL-5 and IL-13 producing cells. Even though rBet v 1 hypoallergen treatment exhibited typical immunological features of successful allergen-specific immunotherapy, there was no increase in the number of IL-10 producing cells after treatment. In the second study we therefore decided to evaluate the role of the suppressive cytokines IL-10 and TGFβ as well as natural $FOXP3^+$ Treg cells in immune-regulation of allergic immune responses. We found that unlike Treg cells from non-allergic controls, Treg cells from birch pollen-allergic patients displayed an impaired ability to suppress birch-pollen stimulated effector cells. Neutralization of IL-10 in $CD4^+CD25^+$ Treg cell and $CD4^+CD25^-$ effector cell co-cultures induced a significant increase of TNFα secretion, suggesting that IL-10 and TNFα may have counter-acting properties in the periphery, where IL-10 promotes tolerance and suppression by Treg cells and TNFα promotes inflammatory responses.

In the third and fourth article, recombinant (r) Fel d 1 was coupled to the novel adjuvant carbohydrate based particles (CBPs) and investigated in a mouse model sensitized to Fel d 1. Pre-treatment with CBP-rFel d 1 was able to induce antigen-specific T-cell tolerance and shift immunoglobulin production from an IgE to an IgG2a type of response. Antigen-coupled CBPs also demonstrated improved antigen depot-effects with prolonged antigen-exposure, when compared to the most commonly used adjuvant in vaccine preparations for humans; aluminum hydroxide. Furthermore, CBP-rFel d 1 was tested in a treatment protocol for SIT, where it was able to modulate the allergic immune response in rFel d 1 sensitized mice without adverse effects. Thus, CBPs ability to promote induction of potent immune responses and to deliver allergens without risk of systemic allergen spreading are beneficial properties of an adjuvant aimed to be used in allergen-specific immunotherapy. Possibly, CBPs coupled to infectious or auto-immune antigens could be applied as an adjuvant to prevent other types of diseases.

In conclusion, the work presented in this thesis has shed new light on *in vivo* function of two conceptually different approaches to improve allergen-specific immunotherapy. The thesis has also contributed to increased understanding regarding regulation of allergic immune-responses, thus providing a basis for further research.

LIST OF PUBLICATIONS

I. Gafvelin G*, Thunberg S*, Kronqvist M*, Grönlund H, Grönneberg R, Troye-Blomberg M, Akdis M, Fiebig H, Purohit A, Horak F, Reisinger J, Niederberger V, Akdis CA, Cromwell O, Pauli G and van Hage M.
"Cytokine and antibody responses in birch pollen-allergic patients treated with genetically modified derivatives of the major birch pollen allergen Bet v 1"
Int Arch Allergy Immunol 2005;138: 59-66

II. Thunberg S, Akdis M, Akdis C.A, Grönneberg R, Malmström V, Trollmo C, van Hage M and Gafvelin G.
"Immune regulation by $CD4^+CD25^+$ T cells and interleukin-10 in birch pollen-allergic patients and non-allergic controls"
Clin Exp Allergy 2007;37: 1127-1136

III. Thunberg S*, Neimert-Andersson* T, Cheng Q, Wermeling F, Swedin, L., Dahlén, SE, Bergström U, Arnér E, Scheynius A, Karlsson MCI, Gafvelin G, van Hage M and Grönlund H.
"Pro-longed antigen-exposure with rFel d 1-coupled carbohydrate based particles prevents induction of allergic immune responses"
Submitted

IV. Neimert-Andersson T, Thunberg S*, Swedin L*, Dahlén SE, Wiederman U, Jacobsson-Ekman G, Scheynius A, Grönlund H, van Hage M and Gafvelin G.
"Carbohydrate-based particles reduce allergic inflammation in a mouse model for cat allergy"
Allergy, in press

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Publications not included in the thesis:

1. Cirkovic Velickovic T, Thunberg S, Polovic N, Neimert-Andersson T, Grönlund H, van Hage M and Gafvelin G.
   "Low levels of endotoxin enhances allergen-stimulated proliferation and reduce the threshold for activation in human peripheral blood cells"
   Int Arch Allergy Immunol 2008;146: 1-10

2. Kaiser L, Cirkovic Velickovic T, Badia-Matinez D, Adedoyin J, Thunberg S, Hallen D, Berndt K, Grönlund H, Gafvelin G, van Hage M and Achour A.
   "Structural characterization of the tetrameric form of the major cat allergen Fel d 1"
   J Mol Biol 2007;370: 714-727

3. Akdis A, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber C.B, Blaser K and Akdis C.A.
   "Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T-regulatory 1 and helper 2 cells"
   J Exp Med 2004;199: 1567-1575

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid
Ag	Antigen
AHR	Airway hyperresponsiveness
Amb 1 a	Ambrosia artemissifolia (major ragweed pollen allergen)
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCR	B-cell receptor
Bet v 1	Betula verrucosa (major birch pollen allergen)
CBP	Carbohydrate based particles
CDE	Cat dander extract
DC	Dendritic cell
Der p 1	Dermatophagoides pteronyssinus (major dust mite allergen)
ER	Endoplasmic reticulum
Fel d 1	Felis domesticus (major cat dander allergen)
FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
KO	Knock-out
LN	Lymph node
LPS	Lipopolysaccharide
MΦ	Macrophage
MACS	Magnetic activated cell sorting
Mch	Methacholine
MHC	Major histocompatibility complex
NK	Natural killer
NKT	Natural killer T-cell
ns	Not significant
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
Phl p 5	Phleum pratense (major timothy grass pollen allergen)
PIT	Peptide immunotherapy
PRRs	Pattern-recognition receptors
r	Recombinant
RA	Rheumatoid arthritis
SIT (ASIT)	Allergen- specific immunotherapy
SLIT	Sublingual immunotherapy
SPT	Skin-prick test
STAT	Signal transducers and activators of transcription
TCR	T-cell receptor
TGFβ	Transforming growth factor beta
Th	T-helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T-cell

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

50 million years of co-evolution with pathogens, was the time the immune system of jawed vertebrates needed to develop an *acquired* or *adapted immune system*[1]. Thus giving rise to a specialized and effective defense system able to remember and store information about pathogens and tumor cells in order to strike back quickly at the next encounter and prevent disease. In contrast to the adaptive immune system, the *innate immune system* is shared by all multi-cellular organisms. It functions as a first line of defense against invasion, consisting of physical barriers, such as skin or mucus secretion and the complement system. Innate defense is executed by phagocytes, such as neutrophils, monocytes, macrophages (MΦs) or dendritic cells (DCs) and mast cells, eosinophils, natural killer (NK) cells or natural killer T-cells (NKT)[2]. Unlike the highly specific B- and T-cell receptors of adaptive immunity, recognizing specific protein structures and amino acid (aa) sequences, the receptors on phagocytic cells recognize pathogen-associated molecular patterns (PAMPs), often conserved structures of bacterial cell walls. Typical pattern recognition receptors (PRRs) are Toll-like receptors (TLRs), C-type lectins, mannose- and scavenger receptors. Interactions between PAMPs and their respective receptors activate phagocytes and trigger a number of important genes, transcribing inflammatory chemokines, cytokines and antimicrobial peptides, thus directing the immune response further[3]. A critical function of the immune system is to distinguish between self and non-self, e.g. endogenous proteins, produced within the body, or exogenous foreign proteins. However, most exogenous proteins are harmless.

Entry of a pathogen through skin or other epithelial barriers will cause tissue damage and distressed cells will send “danger signals” and activate surrounding phagocytes, mast cells and subsequent inflammatory processes[4]. Most infections are cleared by the innate immune system, but complicated infections require activation and function of B- and T-lymphocytes. B-cells or plasma cells produce antibodies, i.e. immunoglobulins, which neutralize bacterial toxins and opsonize pathogens in the extra-cellular compartments, while T-cells either “help” to orchestrate specific immune responses ($CD4^+$ T-helper cells) or directly kill infected cells ($CD8^+$ cytotoxic T cells). After pathogen clearance with both innate and adaptive immune systems activated, most antigen-specific B- and T-cells undergo apoptosis. However some cells remain and differentiate into memory cells. These cells will take part in the constantly growing pool of memory B- and T-cell, which will provide a faster immune response, compared to the first time of encounter, the second, third or eightieth time of encounter[2].

Immunoglobulins, consisting of a constant region as well as the antigen-recognizing variable region, can be secreted as antibodies or remain bound on the surface of B-cells, constituting the B-cell receptor (BCR). Depending on the localization of B-cells or type of pathogen, the immunoglobulin genes switch to one of five isotypes i.e. IgA, IgD, IgE, IgG and IgM. The T-cell receptor (TCR) differs from the BCR. Unlike the BCRs´ recognition of protein structures the TCR, which is membrane bound at all times, recognizes shorter peptide epitopes[2, 5]. Following positive selection in the bone marrow, BCR and TCR undergo negative selection in the bone marrow (B-cells) or thymus (T-cells), a process where both receptors encounter self antigens. Cells expressing receptors with strong recognition of self are deleted in order not to evoke immune responses towards self antigens in the

periphery[4, 6]. Cells not deleted during negative selection leave the primary organs of the immune system to encounter their specific antigens in the secondary immunological organs; the lymph nodes (LN), the spleen and lymphoid tissues in the intestines and nasal cavities[7].

1.2 ANTIGEN PRESENTATION

All nucleated cells in the body present peptides of its intra-cellularly synthesized proteins on the major histocompatibility complex (MHC) class I. Proteins in the cytosol are degraded by proteasomes to peptides, which are transported into the endoplasmatic reticulum (ER) where the peptides are bound to MHC class I. The complex is then transported to the cell surface. If the presented peptide comes from a non-self protein, antigen-specific $CD8^+$ T-cells will bind MHC class I and kill the infected cell[8]. However, only professional antigen presenting cells (APCs), most importantly DCs but also B-cells and MΦs, are able to present peptides on MHC class II. In their immature state DCs can internalize extra-cellular antigens through phagocytosis or macropinocytosis of fluids, after which the cells mature (morphological changes and up-regulation of co-stimulatory molecules) and migrate to draining LN. The content of the phagocytosed vesicles is degraded into peptides and the vesicle fuse with other types of vesicles carrying newly synthesized MHC class II molecules. The peptides are loaded on the MHC and transported to the cell surface where the peptides are presented to antigen-specific $CD4^+$ T-cells[2, 9]. Interactions between peptide bound MHC class II and the TCR of $CD4^+$ T-cells (signal 1) are not sufficient to induce effector function, such as cytokine production, or proliferation of $CD4^+$ T-cells. Rather, signal 1 alone induces apoptosis or a state of anergy. Activation of $CD4^+$ T-cells requires a combination of signal 1 and signal 2, i.e. co-stimulation. Co-stimulatory molecules are trans-membrane proteins that cross-link the TCR-MHC complex, inducing intra-cellular signaling cascades, resulting in gene transcription. Important co-stimulatory molecules on APCs are CD80, CD86, binding CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) on T-cells as well as OX40 ligand, binding OX40 [10].

Cross-presentation occurs when extra-cellular antigens are presented on DCs or MΦs on their MHC class I (instead of MHC class II) to $CD8^+$ T-cells. This pathway enables detection of certain types of tumor antigens or of viruses that do not infect APCs. Depending on how the antigen is taken up and presented by APCs, cross-presentation results in activation of immune responses or induction of tolerance, i.e. "cross-tolerance". The latter happens when antigens are presented in the absence of activated co-stimulatory molecules or signals of danger[8, 11].

1.3 CYTOKINES AND T-CELL DIFFERENTIATION

Activated cells within the immune system produce chemokines and cytokines. Most chemokines are chemo-attractants, secreted to facilitate recruitment of neutrophils, MΦs, eosinophils and lymphocytes to sites of infection[12]. Cytokines have more vast functions, ranging from induction of fever and isotype class switching to immune suppression with both autocrine and paracrine effects[2, 13]. The effector function of cells expressing cytokine receptors can be enhanced or suppressed, depending on the types of cytokines secreted and also due to interactions among them[14]. Cytokines can be grouped as "pro-inflammatory", like interleukin- (IL) 1, 6 and tumor necrosis factor alpha (TNFα)[15] or as anti-inflammatory or "suppressive", such as IL-10 and

transforming growth factor beta (TGFβ)[16]. TNFα stimulates endothelial cells to express adhesion molecules, thus initiating inflammation and subsequent activation of both innate and adaptive immune responses[15, 17]. Due to its potency, TNFα is also involved in the pathogenesis of inflammatory diseases, such as asthma[18], inflammatory bowel disease (IBD)[19] and rheumatoid arthritis (RA)[20]. IL-10 on the other hand suppresses lymphocytes, mast cells and other types of effector cells[21]. Cytokine production by $CD4^+$ T-cells is suppressed by inhibition of signal 2, i.e. interactions between the co-stimulatory molecules CD28 and CD80/CD86, expressed on T-cells and APCs respectively[22, 23].

Cytokines can also be labeled as Th1- or Th2-cytokines depending on the type of differentiated T-helper cell responsible for its secretion. Th1-differentiation of naive T-cells involves the proteins signal transducers and activators of transcription (STAT) 1 and 4, which leads to activation of *T-bet*, the "master switch" for Th1-differentiation. Similarly, STAT 6 initiate Th2-differentiation regulated by the master switch *GATA 3*[18, 24, 25]. Typical Th1-cytokines include IL-2, 12, TNFβ and interferon gamma (IFNγ), while Th2 cells produce e.g. IL-4, 5, 9 and 13. Yet, the boundaries are floating; DCs and monocytes also produce IL-6, 10 and 12[2] and NK-cells produce IFNγ[24]. Simplified, Th1-cytokines activate MΦs and promote class-switching to IgG and defense against intra-cellular pathogens, while Th2-cytokines promote IgE class-switching, recruitment of eosinophils and defense against parasites and helminths[2, 13]. Furthermore, Th1-cytokines are able to inhibit secretion of Th2-cytokines and vice versa[14, 16].

To complicate matters further, there has been the recent entry of the Th17 cell lineage. Th17 cells, distinct from Th1 and Th2-cells, are responsible for production of the pro-inflammatory cytokines IL-6 and 17, secreted in response to acute bacterial infections and extra-cellular bacteria and fungi. Naive $CD4^+$ T-cells differentiate into Th17 cells in the presence of TGFβ and IL-6, which activates the Th17 lineage master switch, the transcription factor *RORγt* (*RORC2* in humans)[26]. In humans Th17 cells are identified by the latter as well as surface expression of IL-23R and CCR6[27]. Th17 cells are also associated with increased disease severity in mouse models for auto-immune diseases[24, 25] (**Table I**).

Table I
Differentiation of naïve $CD4^+$ T-cells into lineage-specific cells in the periphery[25, 28]:

Initiating cytokines	*Transcription factors*	*Type*	*T-cell response*	*Effector mechanism against*
IL-12, IFNγ	STAT1 & 4 T-bet	Th1	IL-2, IL-12, IFNγ	Intra-cellular pathogens
IL-4	STAT 6 & GATA3	Th2	IL-4, IL-5, IL-13	Extra-cellular parasites and helminthes
TGFβ, IL-6	RORγt (RORC2)	Th17	IL-17, IL-6, IL-22	Acute bacterial infection Extra-cellular bacteria and fungi
TGFβ, IL-10	FOXP3	Treg	TGFβ, IL-10	Uncontrolled immune responses

1.4 REGULATORY T-CELLS

1.4.1 Natural FOXP3$^+$ CD4$^+$CD25$^+$ regulatory T-cells

The final described master switch regulating T-cell differentiation is the forkhead-winged helix transcription factor *FOXP3*, which is specifically expressed in thymus derived natural regulatory T-cells (Tregs)[29]. The *natural* regulatory T-cells are $CD4^+$ cell abundantly expressing the IL-2 receptor α-subunit (CD25). Those cells are also denoted as $CD4^+CD25^{bright}$ and were first discovered when Sakaguchi and colleagues depleted peripheral $CD4^+CD25^+$ cells from normal mice. The $CD4^+CD25^-$ fraction was transferred to recipient mice lacking thymus (nude mice) resulting in severe auto-immune disease, affecting a number of different organs. Reconstitution of $CD4^+CD25^+$ cells, through a second transfer, was able to prevent the development of auto-immune disease. *"Taken together, these results indicate that $CD4^+CD25^+$ cells contribute to maintaining self-tolerance by down-regulating immune responses to self and non-self Ags in an Ag-nonspecific manner, presumably at the T-cell activation stage"*[30]. Thus, $CD4^+CD25^+$ cells control/suppress effector function and proliferation of circulating self-reactive T-cells in the periphery.

Tregs not only suppress self-reactive T-cells, they regulate proliferation of and effector function by B-cells, DCs, NK-cells and $CD4^+$ and $CD8^+$ T-cells[31]. Among the $CD4^+$ T-cells, Th1 cells are more susceptible to suppression by Tregs than Th2 cells[32]. In addition, it was recently shown that GATA3, the lineage differentiation marker for Th2 cells, acts as an inhibitor of FOXP3 expression in early T-cell differentiation as well as in already differentiated Th2 cells, through binding to the FOXP3 promoter[33]. Thus, it seems that Th2 differentiated cells are not able to express FOXP3 as well as being less sensitive to Treg suppression, compared to their Th1 counterpart.

The mechanism of suppression *in vivo* is probably a combination of a number of different mechanisms, such as modulation of stimulatory and inhibitory co-stimulatory molecules on APCs, secretion of suppressive cytokines, inhibition of IL-2 secretion as well as CTLA-4-induced T-cell anergy[6, 31]. Natural Tregs have T-cell receptors with diverse antigen specificities[6, 34]. Early in Treg-history (1995), the suppressive function was thought to be dependent on cell to cell-contact as well as being antigen non-specific[30]. It is no longer as clear cut, since antigen-specific Tregs have been shown to more effectively suppress cells when their target antigens are present, particularly in graft verses host disease (GVHD) and in transplantation tolerance[35]. However, although non-distinguishable from natural Tregs, those Tregs

are thought to be $CD4^+CD25^-$ effector cells induced to become $CD4^+CD25^+FOXP3^+$ regulatory T-cells in the periphery[34, 36, 37] (**Table I**). In humans it has been postulated that those *induced* or *adaptive* Tregs are generated from the memory T-cell pool throughout life[38].

The link between the transcription factor FOXP3 and natural Treg cells, was apparent when the mutation behind the *scurfy* mice phenotype was discovered. Scurfy mice develop spontaneous lymphoproliferative disease, associated with fatal auto-immune manifestations. The mutation, resulting in loss of function, was located to the X-chromosome in the foxp3 gene[28]. Mutations in the FOXP3 gene were also found in young boys displaying the human counterpart of the scurfy phenotype. X-linked auto-immunity-allergic dysregulation syndrome (XLAAD) or immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) does not affect heterozygous female carriers, but for hemizygous males it is fatal with symptoms such as severe systemic auto-immune disease as well as allergy, eczema, elevated IgE-levels and eosinophilia[28, 39, 40].

The differentiation to the Treg lineage takes place in the thymus, specifically in the Hassal's corpuscles for human natural Treg cells[41]. It is believed to be driven by high-affinity TCR and self peptide-MHC interactions, meaning that Treg cells recognizes self-antigens but escape deletion through negative selection, instead FOXP3-expression is induced[6, 31, 40]. There are some controversies regarding the role of FOXP3 as a master switch for Treg lineage. Experiments, using mice with a non-functional fusion protein of foxp3 and green fluorescent protein, show that non-functional foxp3 does not change the fate of thymocytes programmed to become Treg cells. They share typical characteristics of functional Treg cells, but lack suppressive function, suggesting that foxp3 is not responsible for Treg cell lineage commitment, but vital for Treg function in mice[42]. However, this is not certain for human Treg cells. Two recently published papers show transient expression of FOXP3 in activated $CD4^+CD25^-$ T-cells from human donors. Wang *et al*[43], showed that polyclonal activation of human $CD4^+CD25^-$ cells *in vitro* resulted in up-regulated FOXP3-expression. Like natural Treg cells, these FOXP3-induced cells were anergic, but the FOXP3-expression did not correlate to suppressive function. Further analysis revealed that the expression of FOXP3 in the "activated" $CD4^+CD25^-$ cells was transient in contrast to functional Treg cells with stable FOXP3-expression. On the other hand, Pillai *et al*[44], reported a typical anergic Treg phenotype as well as suppressive function by $CD4^+CD25^-$ T-cells with transient expression of FOXP3, induced after both polyclonal or allogeneic stimulation. Interestingly, during peak mRNA expression of FOXP3 the allo-stimulated $CD4^+CD25^-$ T-cells also peaked in mRNA-expression of IL-10 and TNFα, suggesting that effector functions and regulatory functions can occur simultaneously in activated T-cells[44]. Lately, the epigenetic regulation of FOXP3, i.e. chromatin modifications affecting gene transcription, has gained much attention. Histone/protein deacetylases (HDACs) are proteins that dampen gene expression; inhibitors of HDACs thus promote transcription. Administration of HDAC inhibitors to mice resulted in increased foxp3-expression and increased number of Treg cells as well as improved function of those cells compared to Treg cells from control mice[45]. Moreover, demethylation status of the FOXP3 locus can differentiate between natural Treg cells with stable FOXP3-expression and activated effector cells with transient FOXP3-expression, as shown by Baron *et al*[46]. Only natural Treg cells displayed demethylation of DNA in the FOXP3 locus, which remained stable throughout extended *in vitro* expansions[46].

Clearly there are differences between Treg cells in the human and murine systems. Since not all FOXP3$^+$ cells isolated from and detected in humans are natural Tregs with stable FOXP3-expression, there are methodological problems for Treg analysis in diseases involving highly activated T-cells. Thus, when it comes to defining human Treg cells, unless epigenetic analyzes of methylation or acetylation status of FOXP3 are at hand, functional analyses are crucial.

1.4.2 Adaptive regulatory T-cells

Antigens drive induction of Treg cells in the periphery. However, not all adaptive Treg cells are CD25$^+$ or express FOXP3. T-regulatory 1 (Tr1) cells, which secrete high amounts of the suppressive cytokine IL-10, has been described as well as the TGFβ-secreting Th3-cell. Regulatory T-cells differentiated from both the Th1 and Th2 lineage, expressing different surface receptor characteristics, have also been suggested[16, 31, 47]. In addition, CD1d-restricted NKT-cells, expressing an invariant TCR recognizing glycolipids, are also considered to be regulatory, due to their rapid production of IL-4 and/or IFNγ[48].

The possibility to induce antigen-specific adaptive Treg cells opens a new therapeutic field, which may have important clinical implications in auto-immune diseases, transplantation tolerance, asthma and allergy.

1.5 IgE-MEDIATED ALLERGY

1.5.1 Sensitization and allergic immune responses

Allergic symptoms such as rhinoconjunctivitis, asthma or gastrointestinal symptoms, triggered by inhaled or ingested allergens cross-linking allergen-specific IgE on mast cells, are defined as *IgE-mediated allergy*[49]. There has been an increasing incidence of allergy and asthma as well as auto-immune diseases in the "capitalistic" part of the industrialized world during the past 50 years[50]. This increase is inversely correlated to the incidence of infectious diseases, especially in childhood, and exposure to microbial components such as endotoxins. These findings have contributed to the so-called *hygiene hypothesis* stating that a highly hygienic environment in early childhood, leading to fewer challenges for the immune system, increases the risk for developing inappropriate immune responses and hypersensitivity reactions. A study comparing allergy prevalence in Eastern and Western Germany showed significantly lower prevalence of asthma, wheezing and allergic rhinitis in East Germany, although the genetically identical populations on different sides of the iron curtain had been separated for only 40 years. Moreover, the prevalence of allergy in the eastern part of the now reunified Germany is increasing[51, 52].

How infections and endotoxin exposure protect against development of allergic and auto-immune diseases is not fully known. Yet, immune responses to infections induce effector cells to clear infection along with IL-10 and TGFβ-secreting regulatory T-cells, which can act directly or through bystander effects thus dampening more than just antigen-specific effector cells. A second mechanism is antigen competition, where responses to one antigen (e.g. an allergen) is diminished due to simultaneous immune responses to an unconnected antigen (e.g. a virus protein)[50, 53]. Thus the hygiene hypothesis proposes that early childhood infections protects pre-disposed individuals from becoming sensitized to food- or aeroallergens, proteins that are completely harmless for most people.

Primary exposure to an allergen, e.g. pollen or cat dander, initiates the *sensitization process*, as demonstrated in **Figure 1**. The inhaled allergens bind allergen-specific naïve B-cells directly or are taken up and processed by APCs followed by presentation to naïve allergen-specific T-helper cells[16, 54]. Un-like pathogens, which provide strong stimuli to PRRs, allergens are associated with low PRR-stimuli, which initiate DCs to prime Th2-differentiation of naïve T-cells[13]. B-cell proliferation and class-switching to IgE requires help from allergen-specific Th2 cells, which provides IL-4, IL-13 and the co-stimulatory molecule CD40L, connecting to CD40 on the B-cells' surface[5]. The activated B-cells form germinal centers, in spleen and LNs, where they differentiate into IgE producing plasma cells. The released IgE-antibodies bind the high affinity IgE-receptor (FcεRI) on mast cells and basophils[21]. However, there are some exceptions to the rule, high dose exposure to cat dander has been reported to induce class-switching to IgG1 and IgG4, rather than IgE, consequently protecting pre-disposed individuals from becoming sensitized to cat dander[55, 56].

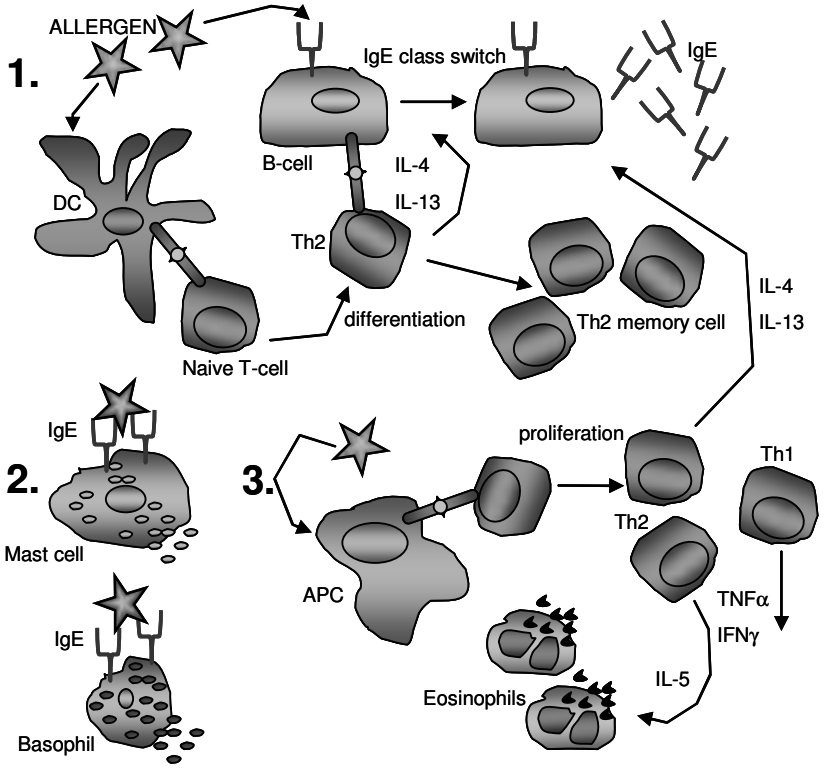

Figure 1, Sensitization and immune response

1. Primary exposure to allergens initiates activation of allergen-specific T-cells. Differentiated Th2-cells activate B-cells, leading to isotype class-switch, plasma cell differentiation and production of IgE. Allergen-specific Th2 cells differentiate into memory T-cells. **2.** Cross-linking of IgE on mast cells and basophils induces mediator-release and immediate-phase reactions. **3.** Late-phase reactions appear hours after allergen-exposure. Infiltration of inflammatory cells and eosinophils to the airways causes airway inflammation and epithelial damage.

The sensitization process ends with the formation of allergen-specific memory T and B-cells. Subsequent allergen-exposure activates effector functions of allergen-specific memory T and B-cells, which start to proliferate. The allergic symptoms are divided into two stages. First the *immediate-phase reaction* appears where the allergen cross-links IgE on mast-cells and basophils, inducing degranulation and release of

histamine, leukotrienes and other mediators. This causes rhinoconjunctivitis and/or asthma symptoms within minutes after exposure. Hours later the *late-phase reaction*, induced by T-cells, follows[54]. Activated allergen-specific T-cells infiltrate sites of allergen-exposure, like the upper and lower airways, where they release IL-4, IL-5 and IL-13 as well as pro-inflammatory cytokines, such as TNFα, leading to infiltration of eosinophils, epithelial damage and airway hyperresponsiveness (AHR)[18].

IgE-mediated allergy is diagnosed by skin-prick testing (SPT) or measurement of allergen-specific IgE in serum. Skin reactivity and rhinoconjunctivitis have been shown to be more closely linked, whereas high serum IgE levels correlate better to asthma-like symptoms[57].

Asthma and allergic diseases are influenced by a number of known and unknown genes. The complexity of the diseases makes it difficult to determine the genetic mechanisms leading to pathogenesis. Genetic analysis of asthma-related genes so far has focused on genes involved in immune-regulation, inflammation, airway remodeling and lipid mediators. Many asthma susceptibility genes, such as ADAM 33, DPP10 and HLA-G have been proposed, but subsequent studies in diverse cohorts have given conflicting results as to what role the genes are playing for asthma pathology[58]. Lately the genes GPRA/NPSR1 (coding the Neuropeptide S receptor 1)[59] involved in airway remodeling and VDR (coding the Vitamin D receptor) involved in immune-regulation have been scrutinized in association studies and meta-analysis and remain candidate genes for asthma pathology[58].

1.5.2 Allergen-specific immunotherapy

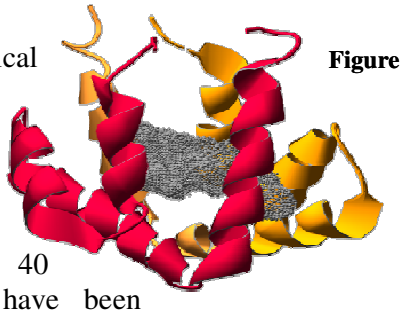

Figure 2

The difference between structure and biological functions of proteins that are classified as allergens is surprisingly large. Proteases, structural proteins, profilins, pathogenesis-related proteins and calcium-binding proteins are among the proteins that have been described as allergens[60]. Although around 40 three-dimensional structures of allergens have been determined[60], among them the cat-[61] *Fel d 1* (**Figure 2**) and birch pollen-[62] *Bet v 1* allergens, there are as yet no structural motifs that can predict the IgE-binding nature of an allergen[63]. Fel d 1 and Bet v 1 are major allergens, i.e. more than 50 % of cat dander- or birch pollen-sensitized individuals have allergen-specific IgE, recognizing those allergens[64]. Despite structural and functional differences, allergens have one common feature, i.e. to induce allergic symptoms in sensitized individuals.

The age of allergen-specific immunotherapy (SIT) began with Dr Noon's novel allergy treatment, consisting of subcutaneous injection of increasing doses of grass pollen extract (published 1911)[65]. SIT is still the only causative treatment for IgE-mediated allergies[66]. Conventional SIT, involves repeated injections of crude allergen extracts over a period of 3-5 years. It is effective in treating, or rather modifying, the immune responses in seasonal allergies, using grass, birch or ragweed extracts, but the treatment of perennial allergies, such as allergy to house dust mite or pets, is not as effective[66-68]. Successful SIT modifies a number of allergen-associated immunological responses. The APCs secrete more IL-10 as well as induce IL-10 secreting Treg cells[69, 70], leading to inhibition of IgE-induced degranulation of human

mast cells[71]. Moreover, allergen-specific Tregs are able to exclusively suppress allergen-specific effector cells of the Th2-lineage[72]. SIT also induces allergen-specific B- and T-cell anergy as well as shifts antibody secretion from IgE to secretion of the *blocking antibodies* IgG1 and IgG4[21, 68]. Blocking antibodies compete with IgE for allergen binding sites[54]. Collectively, these mechanisms contribute to decreased number of cells that mediates allergic immune responses resulting in reduced allergic symptoms.

One alternative to conventional SIT (with subcutaneous injections) is sublingual immunotherapy (SLIT). The route of administration is non-invasive; drops or tablets with allergen can be self-administered under the tongue. Since the allergens reach the body through the oral mucosa, SLIT is considered to be less harmful than SIT[73]. However, although clinical efficacy has been shown, the treatment benefits are about half of those obtained from subcutaneous SIT. Mechanistically, SLIT induces the same local effects as SIT, but the systemic effects seem to be less pronounced[21]. Yet, Dahl *et al* (article currently in press), report significantly reduced rhinoconjunctivitis symptom and rhinoconjunctivitis medication scores as well as increased grass pollen-specific IgG4 antibodies in SLIT treated patients, compared to placebo. The actively treated group (n=189) received grass allergen tablets for approximately 22 months[74]. Moreover, data from a birch pollen SLIT study shows induction of IL-10 producing Treg cells and increased Bet v 1-specific serum IgG4, resulting in improved nasal provocation scores. The treatment had very limited effects on food-related cross-reactivity, which was also investigated[75, 76].

IL-10 seems to be of major importance for regulation of established allergic immune responses in sensitized individuals, but there is conflicting data. IL-10 knock-out (KO) mice in an OVA-allergy model exhibited reduced IL-5 production, followed by reduced eosinophilic infiltration and mucus production in the airways as well as increased production of IFNγ, compared to wild type mice[77]. Similar results were recently reported in ragweed-immunized IL-10 deficient and wild type mice[78]. Collectively, those data suggests that IL-10 is necessary for the development of IL-5 producing Th2-cells and subsequent eosinophil infiltration and AHR. However, a mouse is not a human being and vice versa.

The use of crude allergen extract in SIT is associated with some problems. First, standardization of allergen content between extract batches and manufacturers is difficult[64, 66]. Second, injection of allergen extracts can induce *de novo* sensitizations to components not previously recognized by the immune system[54]. Third, there is a risk for local and systemic side effects, with anaphylactic shock as a worst case scenario[68]. A multi-center study recently showed that the severity of side-effects differs between allergen-extracts. SIT with grass pollen or cat dander extracts were associated with a higher frequency of side-effects than SIT using wasp venom or birch pollen extract[79].

1.5.3 Novel treatment strategies

In order to evade the problems associated with SIT and the use of crude allergen extracts a number of novel treatment strategies have surfaced. The cloning and expression of most major allergens from animal dander, pollen, dust mites and foods has led to a new field of diagnostics and treatment of allergic diseases, using recombinant (r) allergens[60, 66, 80]. Unlike crude extracts, recombinant allergen preparations can be standardized and molecularly defined[21]. A randomized double-blind SIT study lasting for one and a half years, where an equimolar mixture consisting of five wild-type recombinant grass pollen allergens was used, resulted in improvements in medication and symptom scores for the actively treated patients, compared to placebo. Moreover, active treatment induced allergen-specific IgG1 and IgG4-secretion, while IgE-levels were reduced[81].

However, when wild type recombinant allergens are used, the risk for adverse side-effects still remains. The production of engineered allergen derivatives, or *hypoallergens*, is one attempt to overcome this problem. Hypoallergens are recombinant proteins with reduced allergenicity. Targeted disruption of defined B-cell epitopes is one means to do this and introduction of mutations through site-directed mutagenesis changing the overall 3D protein structure and epitopes, another. This results in reduced IgE-binding capacity with retained T-cell reactivity[82]. Many different allergen derivatives have been reported, e.g. the rBet v 1-trimer[83] and rBet v 1-fragments[84] as well as hypoallergens of rFel d 1[85] and the major grass pollen allergen *Phl p 5*[86]. Many hypoallergens have been tested in murine allergy models[87-89]. The rBet v 1-fragments and trimer were the first recombinant hypoallergens to be used in a human SIT study. 124 birch pollen allergic patients participated in the double blind multi-center SIT study[90-92].

Therapeutic administration of short soluble peptide sequences derived from allergens is another approach to induce non-responsiveness and symptom relieve. The peptides consists of amino-acid sequences covering allergen T-cell epitopes, which will activate T-cells but not be able to bind IgE and activate mast cells and basophils, thus preventing immediate phase reactions[21, 93]. Peptide immunotherapy (PIT), with peptides covering T-cell epitopes of phospholipase A2 (the major bee venom allergen), has been shown to induce allergen-specific IgG4 and allergen-specific T-cell anergy[94, 95] as well as reduce skin reactivity to bee venom in actively treated patients[95]. Moreover, the therapy increased production of IL-10, while the production of IL-13 and IFNγ decreased[95]. Similarly, overlapping Fel d 1-peptides (about 17 aa long) reduced allergen-specific proliferation of PBMC, increased IL-10 production as well as induced a $CD4^+$ T-cell population with suppressive function[96]. Adverse effects have been reported after peptide administration, most of which are due to activation of allergen-specific effector T-cells resulting in late asthmatic reactions. However, recent administration schemes with lower peptide doses have shown clinical benefits with significantly reduced adverse effects compared to high dose therapy[97].

Considering the increasing prevalence of allergic diseases, a future treatment aspect might be prophylactic allergen vaccination, i.e. a vaccination strategy to prevent allergen sensitization in pre-disposed individuals much like vaccination against infectious diseases. Recombinant wild type and modified allergens could both be suitable antigens for such allergy prevention[54, 98].

1.6 ADJUVANTS

Adjuvants were introduced to vaccine formulations about 70 years ago. The word adjuvant comes from the Latin word *adjuvare*, "to help". An adjuvant must be non-toxic and safe with regard to local and systemic side-effects and it shall function as an antigen depot. Slow release of antigen at the site of injection prolongs and enhances antigen presentation, which elicits stronger humoral and/or cell-mediated immune responses[5, 99, 100]. Aluminum hydroxide, the most commonly used adjuvant in vaccine preparation for humans[21, 99], induces high antibody titers and has defined depot-properties. Unfortunately, it is also associated with local side effects, such as contact hypersensitivity and granuloma formations[99, 101] as well as low ability to stimulate cell-mediated immune-resonses[102]. Antigens are adsorbed to aluminum hydroxide, through electrostatic interactions or, if the antigen contains a phosphate group, through ligand exchange[103]. Stability as well as amount of antigen adsorbed varies between preparations[104].

Over the past decades many new forms of adjuvants have been presented[100]. Antigen-loaded DCs are used in anti-tumor immunotherapy to boost tumor-specific $CD8^+$ T-cells[105]. Synthetic CpG-containing oligonucleotides are another approach. Bacterial DNA with un-methylated CpG-motifs binds directly to TLR9 on phagocytes, which in their activated state secrete the pro-inflammatory cytokines IL-12 and IL-18, which promotes Th1 responses[106]. Subcutaneous short-course immunotherapy with administration of CpG oligonucleotides covalently linked to the ragweed allergen *Amb a 1*, resulted in a shift from a Th2 cytokine profile to a Th1 profile and improved symptom scores[107, 108]. DNA vaccination is also able to induce Th1-responses. Injection of antigen-encoding plasmid DNA gives rise to spontaneous cellular up-take, followed by antigen-production by transfected cells. This in turn leads to prolonged antigen-exposure and CpG-mediated activation of phagocytes[106, 109]. Pre-immunization with DNA-plasmids, encoding the mite-allergen *Der p 2*, in a Der p 2-sensitized mouse model, reduced AHR and the levels of IL-4 and IL-13 in bronchoalveolar lavage (BAL) fluid [110]. Similarly, Bet v 1 DNA vaccination induced a shift in Bet v 1-specific Th2- to Th1-responses in Bet v 1-senzitised mice, which resulted in reduced IgE-mediated symptoms[111].

Another rationale is to use particulate adjuvants, such as poly-lactide glycolide (PLG) microspheres[112], saponin-complexes or ISCOMS[113], chitin[114] and chitosan particles[115], which structurally mimic pathogen size and shape thus enabling effective phagocytosis and antigen presentation. Oral administration of non-antigenic chitin particles prevented allergic airway inflammation in a ragweed-sensitised mouse model. Chitin-treated mice had decreased levels of ragweed specific IgE, while Th1-associated IgG2a-levels were increased, compared to control mice[114]. ISCOM particles with a formalin-inactivated influenza virus have been tested in a phase 1 study. Healthy volunteers received intra-muscular injections of influenza-ISCOM, which amplified $CD8^+$ T-cell cytotoxicity, compared to standard influenza vaccine[116].

Carbohydrate based particles (CBP), spherical particles 2μm in size, were recently introduced as a novel adjuvant with immuno-stimulatory properties. Recombinant Phl p 5, covalently coupled to CBP, elicited stronger immune responses in mice *in vivo*, compared to Phl p 5 adsorbed to aluminum hydroxide and un-coupled free Phl p 5 in combination with CBP[101]. Furthermore, CBP-rFel d 1 was readily taken up by, and stimulated a semi mature state in , monocyte-derived DCs[117], which has been suggested to be linked to immuno-regulatory responses[118].

2 AIM OF THE THESIS

The thesis' overall aim is to evaluate novel treatment strategies for allergen-specific immunotherapy.

More specifically, immuno-regulatory mechanisms in allergic patients and in allergen-sensitized mice have been investigated. The aims for the individual papers were as follows:

I. To evaluate cellular and humoral responses and changes in clinical manifestations in birch-pollen allergic patients after a short-course immunotherapy with Bet v 1-hypoallergens.

II. To investigate the regulation of immune responses to two different antigens, birch pollen and influenza, by $CD4^+CD25^+$ Treg cells and IL-10 in peripheral blood isolated from birch pollen allergic patients and non-allergic controls.

III. To track distribution of the novel adjuvant CBP *in vivo* as well as to evaluate its adjuvant properties, i.e. the ability to induce cell-mediated and humoral immune responses.

IV. To investigate allergen-specific immunotherapy with rFel d 1 covalently coupled to CBP in a mouse model for cat allergy.

3 MATERIALS AND METHODS

Each article holds a detailed material and methods section. The following list provides an overview of methods used in this thesis, with reference to the papers where they are applied:

Adoptive transfer [III]	Intravenous transfer of splenocytes or sera from treated donor mice to naïve recipients.
AHR measurements [III, IV]	Measurement of methacholine induced AHR, using a Flexi Vent small animal ventilator, after intra-nasal allergen-provocation.
Antigen-stimulated proliferation of PBMC or splenocyte cultures [II, III, IV]	PBMCs were prepared from peripheral blood and splenocytes were prepared from mouse spleens. Splenocytes, PBMC or Treg co-cultures were stimulated with +/- mitogens, anti-CD3 or specific antigen, followed by incorporation of [^{3}H]-thymidine to measure T-cell proliferation.
Bronchoalveolar lavage (BAL) [III, IV]	Mouse lungs were lavaged and cells and lavage fluid separated. The BAL cells were cytospun and stained with trypan blue or Grünwald-Giemsa, counted and analyzed. (Performed after AHR-provocation)
CAP system [I, II]	Diagnostic method to determine sensitization/allergic status. Allergen-specific IgE in sera is measured, ≥0.35kU/L is considered positive.
CBP-coupling [III, IV]	Covalent coupling of rFel d 1 or FITC to cyanogen-bromide activated CBP.
Cell viability [I]	Propidium iodide (PI) staining of dead cells analyzed by flow cytometry. Alternatively by trypan blue staining.
Cytokine blocking experiments [II]	IL-10 and TGFβ were neutralized with an anti-IL-10 antibody or a soluble TGFβIIR in allergen-stimulated Treg co-cultures.
Cytometric bead array (CBA) [II, III, IV]	Flow cytometry-based method used for detection of cytokines in antigen-stimulated cell culture supernatants or BAL fluid.
Enzyme-linked immunosorbent assay (ELISA) [I, III, IV]	Quantitative detection of allergen-specific immunoglobulin levels in sera from allergic patients or sensitized mice.
Immunization and sensitization of mice [III, IV]	Subcutaneous injections of CBP-rFel d 1, rFel d 1 alum or CBP-FITC in BALB/c mice
Isolation of $CD4^+CD25^+$ Treg cells [II]	Following PBMC isolation, $CD4^+$ cells were first isolated by negative selection using magnetic activated cell-sorting (MACS) and subsequently labeled with anti-CD25 conjugated magnetic beads and sorted by MACS.
Lung histology [IV]	Lung sections from mice were PAS (periodic acid Schiff) stained to detect mucus production in the airways.

Multi-color flow cytometry [II, III]	Detection of extra-cellular surface molecules and intra-cellular cytokines and FOXP3 with fluorochrome-conjugated monoclonal antibodies. Also used to track FITC-labeled CBP.
Quantitative real-time polymerase chain reaction (RT-PCR) [II]	Detection of mRNA-expression of FOXP3 and the house keeping gene GAPDH in isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ cells.
Rat basophil leukemia test [IV]	Degranulation experiments with rat basophil leukemia cells to determine the allergenicity of serum antibodies.
Skin prick test (SPT) [I]	Diagnostic method to determine sensitization/allergic status. A wheal and flare reaction to allergens is measured in the skin with histamine as reference.
Statistical analysis [I, II, III, IV]	Non-parametric; Wilcoxon matched pairs test, Mann-Whitney U-test, and Kruskal-Wallis ANOVA with Dunn's multiple comparisons test or parametric; one way ANOVA, followed by Bonferroni's multiple comparisons test.
Whole-body autoradiography [III]	[^{75}Se]-labeled rFel d 1 was coupled to CBP or adsorbed to aluminum hydroxide and injected in naïve mice. After different time points the mice were killed, freeze-sectioned and subjected to autoradiography in order to track the fate of the allergen *in vivo*.

4 RESULTS AND DISCUSSION

4.1 REGULATORY MECHANISMS [II] AND TREATMENT WITH rBet v 1 HYPOALLERGENS IN BIRCH POLLEN ALLERGIC PATIENTS [I]

Allergy to the 17 kDa major birch pollen allergen Bet v 1 (from white birch or *Betula verrucosa*) is one of the most common types of allergy in northern Europe[119]. Many Bet v 1-related allergens have been identified in various fruits and vegetables such as apple, cherry, apricot, carrots and celery, all of which are able to cross-react with Bet v 1-specific IgE and elicit oral allergic symptoms[75, 120]. As discussed in section 1.5.2, SIT is the only treatment that induces long-time relief of symptoms for IgE-mediated allergy. The mechanisms of action involve induction of T-cell anergy, skewing of T-helper responses, induction of IL-10 secreting Treg cells and blocking antibodies. It is generally successful, but time-consuming and associated with various problems, including treatment side-effects and difficulties with extract-standardization.

Hypoallergenic derivatives of Bet v 1 with 100-fold reduced allergenicity[83, 121] were recently produced aimed to decrease adverse effects during treatment. The rBet v 1 *fragment mix* consists of an equimolar mixture of two recombinant protein fragments of Bet v 1 (amino acid 1-73 and 74-159), while the rBet v 1 *trimer* consists of three Bet v 1 a.a.-chains, expressed as one large protein[83, 84]. The first double-blind placebo-controlled SIT study using hypoallergens started in December 2000 and ended before onset of the birch pollen season in March 2001[91]. Birch pollen allergic patients (n=124) with positive skin prick test to rBet v 1 and ≥3.5 kU/L of Bet v 1-specific IgE in serum participated. All patients received up to eight pre-seasonal injections of active treatment or placebo. Patients were recruited at three centers; Stockholm (Sweden), Strasbourg (France) and Vienna (Austria). Out of the 27 participants in the Swedish center, 10 received the fragment mix, 8 the trimer and 9 patients were given placebo (aluminum hydroxide alone) **[I]**. We sought to evaluate the cellular and humoral responses as well as clinical improvements, determined by immediate skin-reactions, after, compared to before, SIT.

In order to evaluate humoral responses, Bet v 1-specific IgG, IgG1, IgG2, IgG4, IgA and IgE was analysed before and after treatment with the rBet v 1 derivatives. Treatment with both fragments and trimer significantly increased production of IgG, IgG1, IgG2, IgG4 and IgE, compared to placebo-treated patients (**Figure 3B**, showing Bet v 1-specific IgG). The production of IgA did not change. The results are in agreement with those obtained from conventional SIT, showing increased levels of allergen-specific IgG-subclasses and IgE after treatment[69-72]. However, the increase in IgE-production in conventional SIT is transient with a slow decrease in IgE-levels over time[122-124], suggesting that allergen-specific IgE would decrease over time also after SIT with rBet v 1 derivatives. In fact, when Bet v 1-specific IgE was measured in sera from Swedish participants 11 month (including one birch pollen season) after treatment with Bet v 1 fragments and trimer the IgE-levels were back at baseline levels (unpublished data). Furthermore, although active treatment was performed with modified allergen molecules, the derivatives were nevertheless able to induce blocking antibodies recognizing the wild type allergen Bet v 1 both in serum as well as in nasal lavage[91, 92].

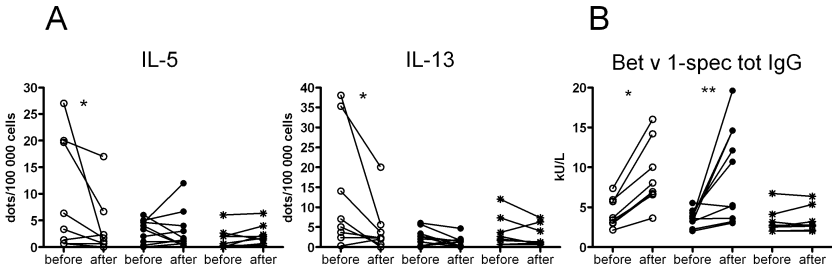

Figure 3, Immune responses after specific immunotherapy with rBet v 1 derivatives

Birch pollen allergic patients were treated with rBet v 1 trimer (○), rBet v 1 fragment mix (•) or received placebo (❋) in a short-course subcutaneous SIT study. **A.** Decreased number of allergen-specific IL-5 and IL-13 secreting cells after treatment with rBet v 1 trimer (ELISpot). **B.** SIT with rBet v 1 derivatives increases rBet v 1-specific total IgG (UniCAP). ≤0.05

The induction of neutralizing antibodies probably contributed to the decreased levels of Bet v 1-specific IgE detected in the actively treated groups in Austria during birch pollen season, compared to placebo[91].

Cellular responses before and after treatment were analysed by ELISpot (single-cell cytokine production) in rBet v 1-stimulated cell cultures with PBMCs obtained from patients receiving fragments, trimer or placebo. Treatment with trimer induced cell-mediated changes not detected after treatment with fragments. The number of allergen-specific IL-4 (ns), IL-5 and IL-13-producing cells were reduced, after trimer treatment, while the number of IL-12 producing cells increased (ns) (**Figure 3A**, showing IL-5 and IL-13). However, it is possible that the lack of cell-mediated responses among the patients treated with fragments is due to low allergen-specific cytokine responses before treatment, thus masking possible decreases in the number of Th2-cytokine producing cells. The number of IL-10 and IFNγ-producing cells varied considerably among all participants, regardless of treatment. Thus, the rBet v 1 trimer reduced the number of Th2-cytokine producing cells and increased Th1-cytokine production, although the latter was not significant. The reduction of Th2 cytokine producing cells after treatment with the trimer suggests that the trimer possess certain Th2-skewing properties *in vivo*, probably due to its size and structure. This is further supported by the fact that the trimer is more potent in reducing Th2- and increasing Th1-cytokines *in vitro*, when compared to wild-type Bet v 1[83].

Having established both cellular and humoral effects after treatment with Bet v 1 derivatives, the clinical benefits were evaluated from skin-prick test data. SPTs to rBet v 1 were performed before and 11 months after treatment. Patients treated with the fragment mix and trimer showed reduced immediate skin-reactions after treatment, compared to placebo-treated patients where no improvements were seen. Similarly, nasal challenges with the rBet v 1 derivatives, performed outside the pollen season on ten birch pollen allergic patients not participating in the immunotherapy study, resulted in fewer allergic symptoms and decreased mast cell and eosinophil activation, compared to challenge with wild-type Bet v 1[125]. Together these data suggested that rBet v 1 derivatives, especially the rBet v 1 trimer, were able to down-regulate allergen-specific Th2 cytokine-responses as well as induce IgG blocking antibodies, resulting in reduced allergic symptoms. One problem that interfered with the clinical interpretations of treatment outcome after SIT with Bet v 1 derivatives in Sweden was the poor birch-pollen season that followed after the end of the SIT trial. 2001, the year of treatment, the mean birch pollen count/m^3 was 10-fold lower,

compared to the year before♣. The low birch pollen exposure did not give rise to any prominent allergic symptoms, thus making it difficult to compare differences in symptoms between placebo and actively treated patients and evaluate whether the treatment was beneficial or not. There was no significant difference in clinical outcome, assessed by symptom scores and drug consumption, between placebo and actively treated patients during the birch pollen season 2001.

Although rBet v 1 hypoallergen treatment exhibited typical immunological features of successful allergen-specific immunotherapy, we did not detect an increase in the number of IL-10 producing cells after treatment. Thus, we could not support the finding from previous SIT studies, regarding induction of IL-10 producing Treg cells after SIT[70, 122]. A deficiency of another type of Treg cell in IgE-mediated allergic disease has been suggested. *In vitro* experiments with the "natural" $CD4^+CD25^+$ Treg showed impaired ability by $CD4^+CD25^+$ Treg to suppress allergen-specific effector cells´ proliferation and cytokine production[126-128]. In order to evaluate the importance of the suppressive cytokines IL-10 and TGFβ as well as $CD4^+CD25^+$ Treg cell for regulation and prevention of allergic symptoms we conducted a study outside the birch pollen season with ten birch pollen allergic patients (all with allergen-specific IgE to birch pollen, ranging from 4.0-39kU/L) and ten non-allergic controls (≤0.35kU/L) **[II]**. After isolation of PBMCs from peripheral blood, $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ effector cells were magnetically sorted and evaluated for their FOXP3-expression. Both mRNA-expression as well as expression of intra-cellular FOXP3-protein was higher in $CD4^+CD25^+$ isolated cells, compared to $CD4^+CD25^-$ cells. Moreover, there was no difference in FOXP3-expression in isolated $CD4^+CD25^+$ cells from allergic compared to non-allergic individuals. $CD4^+CD25^+$ cells from both allergic patients and controls were able to suppress poly-clonally stimulated (anti-CD3) $CD4^+CD25^-$ effector cells in co-cultures (**Figure 4**). Collectively, this suggests that the isolated $CD4^+CD25^+$ cells were indeed $FOXP3^+$ Tregs with equivalent suppressive function, regardless of donor.

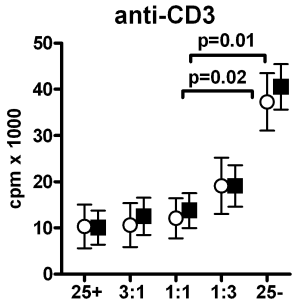

Figure 4, Suppression by $CD4^+CD25^+$ Treg cells

Anti-CD3 stimulated co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells from birch pollen allergic (○) and non-allergic controls (■). $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured alone or in ratios 3:1, 1:1 or 1:3. Treg cells isolated from allergic and non-allergic individuals were able to suppress effector cell proliferation equally well.

The ability of the isolated Treg cells to suppress antigen-specific effector cell proliferation and cytokine production was determined using cell co-cultures stimulated with birch pollen extract or influenza antigens, representing Th2 and Th1 responses respectively. Simultaneously, IL-10 and TGFβ were neutralized with an anti-IL-10 antibody or a soluble TGFβ-receptor (TGFβRII), in order to evaluate whether cytokine blocking could abrogate suppressive function. Treg cells from allergic patients and non-allergic controls suppressed proliferation of influenza-

♣ mean yr 2000: 383 birch pollen/m^3, mean yr 2001: 30 birch pollen/m^3, according to Palynological Laboratory, Swedish museum of Natural History, Stockholm, Sweden

stimulated effector cells equally well. In contrast, only Treg cells isolated from non-allergic controls were able to suppress birch pollen-stimulated proliferation of $CD4^+CD25^-$ cells, results which support previously published data[126-128].

Upon stimulation with influenza antigen, $CD4^+CD25^-$ effector cells from allergic patients and non-allergic controls produced IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα. Treg cells from non-allergic controls were able to suppress production of all detected cytokines, except IL-10, while Treg cells from allergic donors suppressed IL-2, IFNγ and TNFα, but not IL-4, IL-5 and IL-10. Thus, $CD4^+CD25^+$ cells from birch pollen allergic donors were able to suppress influenza-stimulated production of Th1, but not Th2-cytokines. Similar, birch-pollen stimulated production of IL-2, IFNγ and TNFα was suppressed by Treg cells from allergic donors and non-allergic controls, but not IL-4, IL-5 and IL-10. However, the production of IL-5 was significantly lower among effector cells isolated from non-allergic controls, compared to birch pollen allergic patients, implying that there was hardly any IL-5 production to suppress by $CD4^+CD25^+$ cells isolated from non-allergic individuals. Consequently, the difference in capabilities to suppress influenza-stimulated secretion of Th2 cytokines by Tregs cells from allergic and non-allergic donors could be explained by a more general Th2 pre-disposed immune response in allergic individuals. It has been suggested that Th2 clones are less subjected to Treg suppression, due to their ability to produce cytokines such as IL-4 and IL-9, which drives the Th2 cell cycle machinery, in the absence of IL-2[32].

The production of IL-10 was not suppressed by Treg cells; in fact it was evenly distributed in wells with $CD4^+CD25^+$ or $CD4^+CD25^-$ cells alone as well as in wells with Treg and effector cells mixed in a one to one ratio. This implies that $CD4^+CD25^+$ cells in our co-cultures suppressed in an IL-10-independent manner. Moreover, the production of IL-10 was not confined to $CD4^+CD25^+$ cells, since IL-10 was detected in wells with $CD4^+CD25^-$ cells as well. Thus, it is likely that different regulatory mechanisms function simultaneously in the periphery.

In order to test this hypothesis, IL-10 and TGFβ was neutralized in birch pollen stimulated co-cultures. Addition of soluble TGFβRII increased IFNγ-production by $CD4^+CD25^-$ cells from non-allergic controls, whereas neutralization of IL-10 increased production of IFNγ by $CD4^+CD25^-$ cells from both allergic and non-allergic individuals. Hence, IL-10 in particular suppresses IFNγ-production by $CD4^+CD25^-$ effector cells. In contrast, production of the potent pro-inflammatory cytokine TNFα increased both for $CD4^+CD25^+$ and $CD4^+CD25^-$ cells in cultures with neutralized IL-10 regardless of donor. This was not seen in TGFβ-neutralized cultures, which indicate an important role for IL-10 in regulation of TNFα (**Figure 5**). It is possible that TNFα itself may modulate function and secretion of IL-10. In humans, the co-stimulatory molecule OX40L on APCs has been proposed to act as a master switch that converts regulatory IL-10 producing Th1 or Th2 cells into TNFα producing inflammatory cells in the absence of IL-12[129]. Moreover, blocking of OX40 and its ligand *in vivo* in mice results in decreased production of TNFα, compared to mice with functional OX40-OX40L signaling[130].

Natural and adaptive $foxp3^+$ Treg cells as well as activated effector T-cells express OX40. Recently published data indicate that *in vitro* stimulation of OX40 expressed on $CD4^+foxp3^+$ Treg cells abrogates both suppressive function and induction of adaptive Treg cells in the periphery, due to inhibition of foxp3 gene expression[131]. Thus, OX40-OX40L interactions seem to be of major importance to determine the effector functions of T-cells in the periphery, i.e. differentiation into a pro-inflammatory or a regulatory phenotype.

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During bacterial and viral infections IL-10 and Treg cells respond in order to dampen Th1-cytokine production, thus limiting tissue damage and leukocyte proliferation[132, 133]. Our results from co-cultures with isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from non-allergic individuals indicate that IL-10 and Treg cells contribute to maintain T-cell homeostasis during allergen-exposure in the periphery. Conversely, allergen exposure and activation of allergen-specific cells in Th2-pre-disposed allergen-sensitized individuals initiate effector functions, not sensitive to suppression by IL-10 or Treg cells.

In conclusion, data from both the Bet v 1 hypoallergen study **[I]** and the Treg study **[II]** suggest that desirable therapeutical mechanisms, after allergen-specific immunotherapy treatment, are generation of allergen-specific Treg cells, increased production of IL-10 as well as induction of Th2 cell anergy in order to successfully control cell-mediated immune-responses and allergic symptoms.

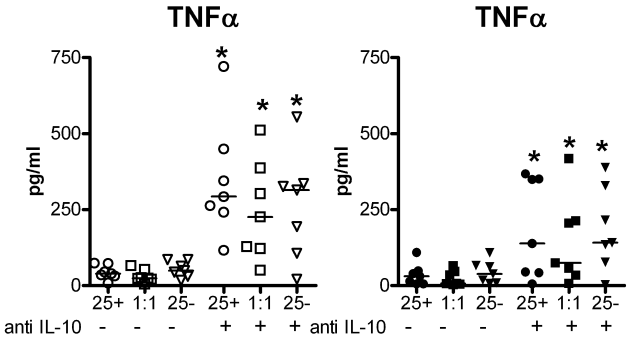

Figure 5, Neutralization of IL-10 increases TNFα production

Neutralization of IL-10 in birch pollen extract stimulated co-cultures with $CD4^+CD25^+$ and $CD4^+CD25^-$ from allergic and non-allergic individuals increased production of TNFα (CBA). ≤0.05

4.2 *IN VIVO* MECHANISMS AND ALLERGEN-SPECIFIC IMMUNOTHERAPY WITH CARBOHYDRATE BASED PARTICLES COUPLED TO THE MAJOR CAT ALLERGEN rFel d 1 [III & IV]

As an alternative to the manipulation of allergens for improvement of SIT, we choose to change target and focus on adjuvants, i.e. antigen-carriers and stimulators of immune responses. Aluminum hydroxide is a widely used adjuvant and antigen carrier for injections of allergen-preparations in conventional SIT. However, safety issues and other difficulties regarding preparations with aluminum hydroxide have led to development of new types of adjuvants[134]. CBP is such an adjuvant, with reported immuno-modulatory properties both *in vitro*[117] and *in vivo*[101]. Moreover, it is possible to covalently couple and co-couple proteins and other types of molecules to CBP.

Cat dander allergens are widely spread in areas where cats are not present, such as schools, work places and in public transportation. The allergens are typically carried in the clothing of cat owners and dispersed into the air, thus making allergen avoidance difficult for cat allergic individuals[135, 136]. This is further complicated by the close correlation between allergy to cat dander and asthma[137]. Almost all, or 90-95 % of cat allergic individuals have IgE antibodies to the major cat dander allergen Fel d 1[138].

Before investigating a treatment protocol for specific immunotherapy, using CBP covalently coupled to rFel d 1 (CBP-rFel d 1) in a mouse model for cat allergy **[IV]**, CBPs' intrinsic adjuvant properties were evaluated from a mechanistic point of view **[III]**. Mice were injected with CBP-rFel d 1, CBP alone, soluble rFel d 1 or a combination of CBP plus soluble rFel d 1 and bled before immunization (pre-immune) as well as days 3, 5, 8, 14 and 21 after immunization in order to determine kinetics of specific antibody production. Mice immunized with CBP-rFel d 1 responded earlier and with significantly higher levels of rFel d 1-specific IgM, IgG1 and IgG2a, compared to mice immunized with soluble rFel d 1, CBP or CBP plus soluble rFel d 1 **[IV]**. Similarly, IgM and IgG1-responses against CBP and CBP-rFel d 1 were detected in mice immunized with CBP-rFel d 1 but not in mice immunized with soluble rFel d 1. The CBP-specific IgG1 response started to decline after day 14 and was much weaker than the CBP-rFel d 1-specific IgG1 response, which had not reached its plateau at day 21 **[III]** (**Figure 6,** showing CBP-rFel d 1-specific IgG1). Thus, CBP-rFel d 1 induces rapid antibody production compared to soluble rFel d 1 or particles alone, suggesting that the covalent coupling of CBP to rFel d 1 is of major importance for CBPs' immuno-stimulatory properties. Previously published data reports that OVA coupled to polystyrene beads induced phagocytosis and antigen presentation 1000 to 100 000-fold more efficiently than soluble OVA[139].

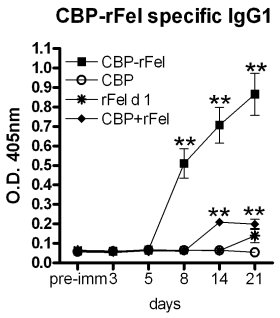

Figure 6

Having established induction of rapid antibody responses by CBP-rFel d 1, we sought to test whether vaccination with CBP-rFel d 1 could prevent IgE-sensitization in mice **[III]**. Ten mice per group were vaccinated three times with CBP-rFel d 1, CBP, PBS (sham) or un-treated (control). All mice were subsequently sensitized with rFel d 1 and challenged intra-nasally with cat dander extract (CDE) (groups CBP-rFel d 1, CBP and sham) or PBS (control) (**Table II**).

After allergen challenge, the mice were subjected to methacholine provocation and AHR (expressed as airway resistance) was measured with a Flexi Vent small animal ventilator. The presence of IL-5 and IL-13 and infiltration of MΦs, eosinophils and lymphocytes in BALF was also analyzed. Vaccination with CBP-rFel d 1 resulted in reduced AHR at the highest methacholine doses compared to sham treated mice (**Figure 7**). In addition, vaccination with CBP-rFel d 1 reduced the total BAL cell number, reflecting significantly decreased infiltration of lymphocytes, MΦs and especially eosinophils as well as reduced levels of IL-5 and IL-13 in BALF, compared to sham treated mice. Adoptive transfer of sera and splenocytes from CBP-rFel d 1 vaccinated donors also reduced AHR in sensitized and allergen-challenged recipients, suggesting that both humoral and cellular components contributed to the observed protective effect.

Table II
Vaccination and sensitization protocol

Group	*Vaccination*	*Sensitization*	*Challenge*
CBP-rFel d 1	100µg CBP-rFel d 1	1 µg rFel d 1	10 µg CDE
CBP	CBP	1 µg rFel d 1	10 µg CDE
sham	PBS	1 µg rFel d 1	10 µg CDE
control	no treatment	1 µg rFel d 1	PBS

Allergen-specific T-cell responses after vaccination with CBP-rFel d 1 were further analyzed in splenocyte cultures stimulated with rFel d 1 or CDE. Splenocytes from CBP-rFel d 1 vaccinated mice did not proliferate upon stimulation with rFel d 1, while splenocytes from CBP and sham pre-treated groups did (**Figure 7**). Furthermore, the levels of IL-5 in cell culture supernatants from CBP-rFel d 1 vaccinated mice, were lower compared to sham treated mice.

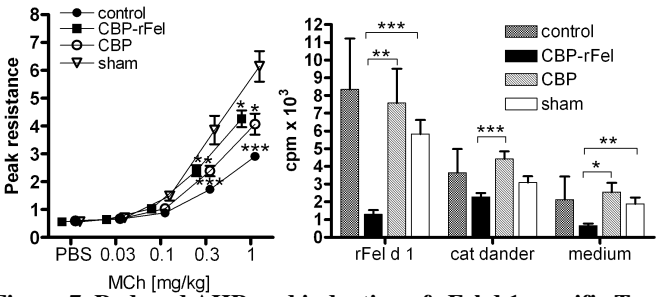

Figure 7, Reduced AHR and induction of rFel d 1-specific T-cell anergy.

After pre-treatment, mice were sensitized and intra-nasally challenged with CDE. Vaccination with CBP-rFel d 1 reduced AHR compared to sham treated mice (left) as well as induced rFel d 1-specific T-cell anergy.

Thus, vaccination with CBP-rFel d 1 seems to induce allergen-specific T-cell anergy. The allergen-specific non-responsiveness could not be explained by induction of Treg cells. In fact, a higher percentage of $CD4^{+}foxp3^{+}$ Treg cells were detected among splenocytes from sham treated mice compared to mice vaccinated with CBP-rFel d 1. The foxp3-expression in the CBP-rFel d 1 group was the same as in the PBS

challenged control group, indicating that CBP-rFel d 1 vaccinated mice maintain their effector T-cell homeostasis despite sensitization and challenge. It is possible that the increased foxp3-expression, observed in sham vaccinated mice, reflects normal regulatory responses in order to control systemic inflammation, in this case induced by sensitization and allergen challenge.

Our results showed that CBP-rFel d 1 clearly elicited both humoral and cell-mediated immune responses, but little was known about the fate of the particles and rFel d 1 *in vivo*. The following questions were formulated. First, what cell type/types were responsible for CBP-rFel d 1 phagocytosis and antigen presentation? Second, where were the particles to be localized after injection? Third, was rFel d 1 differently distributed when coupled to CBP, compared to adsorbed to alum?

Questions one and two were addressed using CBP labeled with the fluorescent marker FITC and question three by applying radioactively labeled [^{75}Se]rFel d 1. Groups of mice (n=5) were s.c. immunized with CBP-FITC and cells obtained from draining LN and the spleen were analyzed with flow cytometry 6 or 24 hours or 5 days after immunization. For control purposes, mice were also injected with PBS. Cells with intra-cellular CBP-FITC appeared in the draining LNs already 6 hours after injection, but were not detected in the spleen until 24 hours after injection, where they reached the highest detected level after 5 days. The phagocytes, carrying CBP-FITC, detected in both LNs and spleen expressed CD11c and F4/80 (a marker for MΦs and Langerhans' cells[140]), but not CD11b. Nevertheless, most FITC labeled particles (≈92 % of total cells obtained from skin samples) still resided at the site of injection 5 days after injection. Unlike cells detected in LNs and spleen, the cells in the skin showed high expression of CD11b, but only intermediate expression of CD11c, suggesting that tissue MΦs remain at the injection site, whereas DCs and Langerhans' cells migrate to peripheral lymphoid organs.

These data indicate that antigens covalently coupled to CBPs remain at the injection site, thus being subjected to phagocytosis by innate immune cells for a long period of time. In fact, radioactive [^{75}Se]-labeling of rFel d 1 confirmed the long-lasting depot effect by CBP observed with FITC-labeled particles. [^{75}Se]rFel d 1 was coupled to CBP or adsorbed to alum and the radioactivity was tracked in mice 24 hours or one week after injection. The majority of the radioactivity in CBP-[^{75}Se]rFel d 1-immunized mice was visible at the site of injection 24 hours and 1 week after injection, while it was spread throughout the body of [^{75}Se]rFel d 1-alum immunized mice at both time points (**Figure 8**, showing 24 hours data).

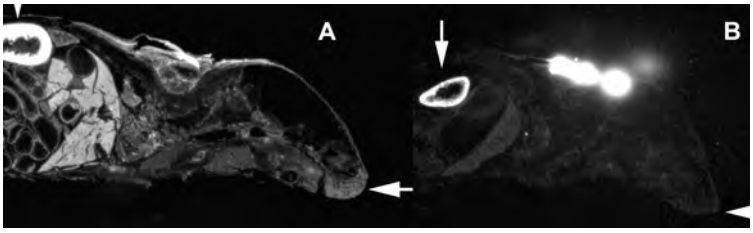

Figure 8, Tracking of [^{75}Se]rFel d 1 after 24 hours.

Fel d 1 was labeled with [^{75}Se] and covalently coupled to CBP (right) or adsorbed to alum (left) and tracked after 24 hours with whole-body autoradiography. The radioactivity was detectable throughout the body in animal receiving [^{75}Se]rFel d 1-alum, while the radioactivity in [^{75}Se]rFel d 1-CBP immunized resided at the injection site.

To summarize, allergen coupled to CBP elicits strong humoral and cellular immune responses, compared to un-coupled allergen or CBP alone. Moreover, tracking experiments, with both labeled CBP and rFel d 1, suggest that one important mechanism for induction of immune responses is prolonged antigen-exposure by CBP -coupled antigen, which enables effective phagocytosis and antigen-presentation.

CBP-rFel d 1 was next evaluated for use in SIT **[IV]**. In contrast to the vaccination protocol, where mice were treated before being sensitized **[III]**, mice in the treatment protocol were first sensitized to establish allergic immune responses, then therapeutically treated and allergen-challenged. Ten mice per group were randomly divided into six groups, sensitized with rFel d 1 and treated with CBP-rFel d 1, CBP, PBS, soluble rFel d 1, CBP plus rFel d 1 or un-treated (baseline control) (**table III**). All groups, apart from the un-treated mice, were intra-nasally challenged with CDE before AHR, BAL cells and cytokines as well as humoral and proliferative responses were measured.

Table III
Sensitization and treatment protocol

Group	*Sensitization*	*Treatment*	*Challenge*
A	1 μg rFel d 1	100 μg CBP-rFel d 1	10 μg CDE
B	1 μg rFel d 1	CBP	10 μg CDE
C	1 μg rFel d 1	PBS	10 μg CDE
D	1 μg rFel d 1	100 μg rFel d 1	10 μg CDE
E	1 μg rFel d 1	100 μg rFel d 1 + CBP	10 μg CDE
F	1 μg rFel d 1	no treatment	PBS

AHR measurements showed significantly reduced lung resistance in mice treated with CBP-rFel d 1, compared to CBP. In fact the airway reactivity in mice treated with CBP-rFel d 1 was similar to the PBS challenged control mice, indicating that, although allergen-challenged, CBP-rFel d 1 treated mice did not develop airway hyperreactivity, results that were in accordance with those obtained from rFel d 1-vaccinated mice **[III]**. Indeed, the number of eosinophils in BAL as well as airway mucus production in CBP-rFel d 1 treated mice were lower compared to mice treated with CBP alone. *In vitro* proliferation of rFel d 1-stimulated splenocytes from treated mice was also measured. Splenocytes obtained from CBP and PBS treated mice showed the most pronounced proliferative response among the different groups, which was reflected in elevated levels of IL-5 and IL-13 in the culture supernatants (ns).

Calculations of rFel d 1-specific IgG2a to IgE ratio showed increased ratios, reflecting elevated levels of IgG2a and lower IgE-values in CBP-rFel d 1 treated mice, compared to CBP or PBS treated mice (ns). Unexpectedly, the most pronounced IgG2a to IgE ratio was detected in mice treated with rFel d 1 alone or the mixture of CBP and rFel d 1, suggesting that rFel d 1 itself was responsible for induction of IgG2a. However, high doses of rFel d 1 was given (100 μg) and the mice treated with both rFel d 1 and the mixture reacted with adverse side-effects, such as pilo-erection and affected breathing rate, after injections. This was not seen in mice injected with CBP-rFel d 1, suggesting that high doses of rFel d 1 coupled to CBP can be administered without adverse effects. A recently performed clinical study with

virus-sized particles covalently coupled to the house dust mite allergen Der p 1 showed that high allergen concentrations elicited stronger antibody responses than low allergen concentrations did. Healthy volunteers participating in the study received 10 or 50 μg of Der p 1, which resulted in significantly higher levels of allergen-specific IgG when the higher dose was given, compared to the lower dose[141]. Thus, high allergen concentrations in SIT seem to have more beneficial clinical effects than low concentrations.

Collectively, data from mechanistic **[III]** and therapeutic **[IV]** investigations of carbohydrate based particles indicate that owing to CBP's ability to deliver high doses of allergen with low risk of allergen-spreading and adverse side-effects, allergen-coupled CBP is a promising candidate for application in allergen-specific immunotherapy.

5 CONCLUSIONS

The general aim of the articles presented in this thesis was to investigate regulation of allergic immune responses and how novel strategies for specific immunotherapy affect those responses. The first study describes a SIT study performed on birch pollen allergic patients, the second study was performed *in vitro*; using peripheral blood from birch pollen-allergic patients and healthy controls, and the last two studies were performed *in vivo* using an experimental mouse model for cat allergy.

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Here the major findings for each article are reported, we conclude that:

I. A new form of specific immunotherapy treatment with genetically modified Bet v 1 hypoallergens changes allergen-specific immune responses and reduces the immediate skin reactivity in birch-pollen allergic patients. The obtained results will aid development of new treatment strategies for allergic disease. Moreover, the novel hypoallergen concept may also be used in a prophylactic manner to prevent allergen-sensitization in pre-disposed individuals.

II. Regulatory T-cells from birch pollen-allergic patients display an impaired ability to suppress birch-pollen stimulated effector cells. In addition, neutralization of IL-10 in $CD4^+CD25^+$ Treg cell and $CD4^+CD25^-$ effector cell co-cultures induces significant increase of TNFα secretion. Thus, we propose that IL-10 and TNFα may have counter-acting properties in the periphery, where IL-10 promotes tolerance and suppression by Treg cells and TNFα promotes inflammatory responses.

III. Vaccination with the novel adjuvant CBP, coupled to the major cat allergen Fel d 1, is able to induce antigen-specific T-cell tolerance and shift immunoglobulin production in mice sensitized to rFel d 1. Antigen-coupled CBPs also demonstrate improved antigen depot-effects with prolonged antigen-exposure, compared to alum. Moreover, CBPs encompass immuno-stimulatory and inhibitory properties *in vivo*. Consequently, we believe that CBPs coupled to disease-specific antigens could elicit blocking antibodies and induce anergic allergen-specific and possibly anergic auto-reactive T-cells.

IV. CBPs modulate the immune response, allergic inflammation and airway hyperresponsiveness when for the first time used in treatment of rFel d 1 sensitized mice. CBPs' ability to induce potent immune responses and to deliver high doses of allergen without risk of systemic allergen spreading, are beneficial properties of an adjuvant aimed to be used in specific immunotherapy. Collectively, CBPs with its intrinsic adjuvant properties has a potential use in allergy vaccination and may possibly also be applied in treatment of auto-immune disease.

6 FUTURE PERSPECTIVES

The enticement of performing research lies in the spiraling process, where obtained knowledge always evokes new questions to answer and hypotheses to test. Studies performed and described in this thesis follow the same pattern.

Differences in immune regulation of allergic immune responses between allergic and non-allergic individuals still remain to be further elucidated. This is one focus of the ongoing "ERINA-study", or "Early inflammatory events in allergic asthma". Birch- or grass pollen allergic individuals with mild asthma and non-allergic controls participate in the study. All participants donate peripheral blood. In addition, non-allergic controls are subjected to bronchoalveolar lavage once, while the asthmatics are subjected twice, before and after bronchial allergen provocation. Thus, the ERINA-study will provide new and valuable information about regulatory mechanisms occurring in the airways as well as in peripheral blood, after allergen exposure. Preliminary data from BAL cell analysis, obtained with flow cytometry, show that the number of $CD4^+FOXP3^+$ Treg cells increases in the lung after allergen challenge. At the same time there is an increased expression of OX40 on $CD4^+$ T-cells and $CD4^+FOXP3^+$ Treg cells up-regulate the early activation marker CD69. Surface expression of CD69 on $CD4^+$ T-cells in the blood is virtually non-existent, while the expression on $CD4^+$ T-cells in BAL is pronounced[142, 143]. Analysis of mRNA-levels of FOXP3 in PBMCs, relative to house keeping gene EF-1, also show increased expression of FOXP3 after allergen challenge. Measurements of cytokines and analysis of RORC2, the lineage marker for human Th17 cells remains to be performed. The latter is of great interest, since the involvement of Th17 cells in asthma exacerbations remains to be elucidated[144]. Collectively the preliminary data from the ERINA-study suggest that bronchial allergen provocation activates both pollen-specific effector T-cells and regulatory T-cells to express homing receptors and migrate to the lung.

Another ongoing project aims to investigate the proposed counter-regulatory properties of TNFα and IL-10 in the periphery. In the study, we are fortunate to get PBMCs weekly during seven weeks from a group of patients diagnosed with IBD receiving anti-TNFα therapy. As previously mentioned, TNFα is a potent pro-inflammatory cytokine involved in the pathogenesis of inflammatory diseases, such as RA[145], asthma[18, 146] and IBD[19]. Anti-TNFα therapy has been shown to increase lung function in asthmatic patients in clinical studies[146-148]. But more studies needs to be performed in order to determine the clinical relevance of anti-TNFα therapy for treatment of asthma[149]. In RA treatment, where anti-TNFα therapy is common[150], the therapy seems to re-establish Treg cell function[20]. More specifically, the therapy induces a new population of adaptive Treg cells expressing FOXP3 and CD62L[151].

In our "TNFα-study" phenotypic markers for Treg cells, NK cells, APCs, Th1/Th2/Th17 differentiation as well as apoptosis are analyzed weekly with flow cytometry. Moreover, cytokines are evaluated with ELISpot and in culture supernatants and T-cell differentiation, determined by the lineage markers GATA-3, T-bet, FOXP3 and RORC2 are evaluated with RT-PCR. The patients are further divided into allergic and non-allergic in order to evaluate differences between allergic immune responses in allergic and non-allergic individuals in the absence of TNFα. Preliminary data from a small number of patients suggests that anti-TNFα therapy changes the omnipotent TNFα cytokine profile to a more balanced Th1/Th2 cytokine profile after treatment.

Prolonged antigen depot effect of CBP and its ability to deliver high allergen doses without adverse effects was reported in this thesis. There are a number of interesting future projects regarding mechanisms and treatment strategies involving CBP to be considered. The most obvious one is of course to test CBP-rFel d 1 in humans. However, in order to do so, toxicity tests and other types of safety tests must be performed on laboratory animals and CBP-rFel d 1 needs to be manufactured according to GMP standards.

Since CBP-rFel d 1 induces allergen-specific T-cell anergy, another interesting project would be to couple CBP to a self-antigen or other types of antigens used in experimental models for auto-immune disease, e.g. collagen type II or glucose 6 phosphoisomerase[152], to investigate whether treatment with "self antigen"-coupled CBP could elicit anergic auto-reactive T-cells and consequently reduce disease symptoms. The fact that vaccination with CBP gives high antigen-specific neutralizing antibody titers opens another possibility to use CBP in therapy for auto-immune disease. Spohn and colleagues[141] recently presented a novel anti-TNFα treatment strategy, where mice were immunized with virus-like particles "QB", covalently coupled to soluble TNFα or a 20 aa peptide of the N-terminus of TNFα. The immunization elicited anti-TNFα antibodies which prevented clinical signs of inflammation in a murine model for RA. Interestingly, mice immunized with the TNFα-peptide-QB were not immuno-compromised, unlike mice immunized with the whole protein because the anti-TNFα antibodies, induced after TNFα-peptide-QB immunization, only targeted soluble and not membrane bound TNFα[141]. Thus, CBP covalently coupled to TNFα peptides could possibly neutralize soluble TNFα in patients in need of anti-TNFα therapy, suffering from RA, IBD and possibly asthma, without risk of opportunistic infections.

Concerning CBP mechanisms *in vivo*, it would be interesting to investigate the means of phagocytosis and antigen presentation in MHC class I or MHC class II KO mice to evaluate whether cross-presentation and cross-tolerance are involved in the protective effects obtained after CBP-rFel d 1 vaccination. Another interesting mechanism to investigate regards the specific antibody production induced by CBP, whether it is T-cell dependent or independent or perhaps more likely, both types of antibody responses are involved. Furthermore, the adoptive transfer experiments in **[III]** were crude, i.e. un-fractionated splenocytes or sera from donor mice were transferred to recipients. A more precise approach would be to isolate certain cell types, such as T-cells, Treg cells or B-cells, from vaccinated donors and transfer those to recipients. Such experiments would provide a deeper understanding regarding the immuno-regulatory properties of antigen-coupled CBP.

7 SAMMANFATTNING PÅ SVENSKA

Vårt immunförsvar består till stor del av vita blodkroppar. Det skyddar oss från en rad sjukdomsalstrande mikroorganismer, t.ex. bakterier och virus. Immunförsvaret brukar delas in i en medfödd och en förvärvad del. Den senare består i sin tur av B- och T-celler, celler som mycket intrikat selekteras utifrån deras specifika B- och T-cellsreceptorers förmåga att känna igen speciella kroppsegna receptorer. De B- och T-celler vars receptorer starkt binder kroppsegen vävnad genomgår programmerad celldöd, eftersom dessa celler potentiellt kan skada kroppen. De celler som inte selekteras bort, cirkulerar i kroppen till dess att de träffar på det för just deras receptors specifika antigen (t.ex. proteiner eller kolhydratstrukturer från virus och bakterier). Receptorengagemang aktiverar B-cellen till att producera antikroppar och T-cellen till att antingen döda virusinfekterade celler eller producera s.k. cytokiner, vars effekter på olika sätt påverkar immunförsvaret.

Allergiska symtom uppstår när kroppens immunförsvar reagerar på, för de flesta människor helt ofarliga, proteiner som finns i vår närhet. Dessa proteiner brukar kallas ”allergener”. Två allergener, som vanligen ger upphov till hösnuva och astma i Sverige, är huvudallergenet i björkpollen, Bet v 1 samt huvudallergenet i kattmjäll, Fel d 1. Den enda behandlingen som botar allergi är allergen-specifik immunterapi (SIT), en flera år lång behandling som består av en mängd återkommande injektioner av allergenextrakt. Resultatet av behandlingen är en höjd toleransnivå vid allergenexponering och därmed minskade symtom. Även om SIT ger goda resultat är det förknippat med vissa problem. Behandlingslängden är problematisk, liksom de allergenextrakt som används. Dessutom finns det risk för både lokala och systemiska allergiska reaktioner inducerade av behandlingen. Gemensamt har dessa faktorer gett upphov till en rad föreslagna behandlingsstrategier för SIT. En sådan strategi är s.k. hypoallergen, dvs. allergen med reducerad allergenicitet skapade med hjälp av molekylärbiologisk teknik.
Syftet med denna avhandling var att undersöka hur allergiska reaktioner regleras samt hur nya behandlingsmetoder för SIT påverkar dessa reaktioner.

I den första studien behandlades 27 björkpollenallergiker, uppdelade i tre grupper, med två olika Bet v 1 hypoallergen eller placebo under tre månader. Resultaten visade att behandling med de modifierade Bet v 1 allergenen minskade en rad immunologiska attribut förknippade med allergi samtidigt som andelen skyddande allergen-specifika IgG-antikroppar ökade, vilket visar att en sådan form av terapi kan vara aktuell för framtida behandling av allergiker. I den andra artikeln valde vi att utreda hur regulatoriska T-celler (Treg) och de regulatoriska cytokinerna IL-10 samt TGFβ, d.v.s. celler och molekyler som reglerar andra immunologiska funktioner, påverkar den allergiska reaktionen. Regulatoriska T-celler, som kännetecknas av transkriptionsfaktorn FOXP3, samt IL-10 och TGFβ har inhiberande verkan på funktion och aktivering av en rad immunceller. När vi jämförde Treg-celler, isolerade från björkpollenallergiker och friska kontroller, visade det sig att Treg-cellerna från allergiker uppvisade en sämre förmåga att inhibera allergen-specifika T-celler än motsvarande Treg-celler från friska kontroller. Dessutom ökade produktionen av det starkt inflammatoriska cytokinet TNFα, när funktionen av IL-10 blockerades, vilket pekar på att TNFα och IL-10 kan ha motverkande funktioner i kroppen. Vår hypotes är att allergenexponering kan ge olika immunologiska svar beroende på produktionen av IL-10 och TNFα. Antingen inhiberas immunförsvaret av IL-10 tillsammans med Treg-celler eller så aktiveras immunförsvaret av TNFα till att ge ett inflammatoriskt svar och allergiska symptom.

Sarah Thunberg

I artikel tre och fyra utvärderade vi en ny behandlingsstrategi för SIT, men som alternativ till att undersöka behandlingseffekter i människa valde vi att behandla möss som på experimentell väg gjorts allergiska mot kattallergenet Fel d 1. Till skillnad från upplägget i den första studien där modifierade proteiner användes, valde vi istället att utvärdera ett nytt adjuvans för användning i SIT-behandling. Adjuvans fungerar som ”proteinbärare”, som hjälper till att förstärka ett immunologiskt svar vid t.ex. vaccination. Vi kopplade Fel d 1 till ”kolhydratbaserade partiklar” eller ”CBPs”, ett nytt adjuvans för SIT, och behandlade kattallergiska möss både profylaktiskt och terapeutiskt. Fel d 1-kopplat CBP (CBP-Fel d 1) visade sig ha mycket goda egenskaper som adjuvans utan att ge bieffekter. Dessutom visade kattallergiska möss, som behandlats med CBP-Fel d 1, minskad inflammation i lungan samt minskade allergiska immunsvar. Således har CBP-Fel d 1 mycket lovande egenskaper som adjuvant för SIT, vilket skulle kunna användas i SIT-behandling av allergiker.

8 ACKNOWLEDGEMENTS

A number of colleagues as well as family and friends have kept me company on my way to stardom, bitter unemployment or wherever this PhD quest may lead me. Thank you all!

With exclamation marks I would like to thank:
-My supervisor **Guro Gafvelin** for your great support and knowledge, your kindness and enthusiasm and your almost magical ability to tackle my pigheadedness and sometimes grumpy appearance with a smile.
-My co-supervisor **Marianne van Hage** for accepting me in your group, believing in me and keeping me focused (a rather difficult task).

-Everyone, past and present, in Marianne's group: **Guro**, **Marianne**, **Theresa** (for being a great friend and a research partner in crime), **Neda** (for all your help over the years), **Gerd** (for the ever present sunshine, except during pollen seasons), **Lotta K** (for being so bold and outspoken), **Jenny**, **Tiiu** (for Finish sisu), **Justus** (for being so mysterious), **Ola** (for keeping me up to date about the man, the myth, the legend, i.e. Morrissey), **Jeanette** (for your curiosity), **Hans** (for our friendship, scientific discussions as well as "the Swedish classic", which would have been boring without you), **Kurt** (for being inspiring and a tiny bit more confused than me), **Helena**, **Elin**, **Klas** (for Brunnsviken runt), **Tanja** and **Natalja** (for the laughs and the introduction to the Belgrade nightlife), **Sofia**, **Maria**, **Åsa** and **Nariman**.
-My master thesis student **Anna Bohman**.

-All my **co-authors** (quite the bunch and of major importance!!!!!) as well as research nurses **Helene Blomqvist**, **Marghita Dahl** and **Gunnel de Forest**.

-**Cezmi** and **Mübeecel Akdis** for a great collaboration and for welcoming me in Davos.

-**Lotta K, Lotta A, Susanne, Micke, Ulrika, Sara L, Sara J, Anna, Tiiu, Hans, Tanja, Natalja, Monica, Theresa, Ulf, Erika, Maaike, Guro, Marianne, Pierre** and **Emma** who have kept me company in ski slopes, eating and drinking, riding Baldur at Liseberg, watching Manhattans' skyline and keeping me dry in the Boston "wetlands" during the many hard and difficult research congresses poor PhD students must attend.
-My room mates **Helen, Erika, Christine, Justus, Maria** and **Monica**.
-Everyone in Olas group for providing me with excellent coffee, far more superior than anything the "coffee machine" has ever produced. The same group also houses the FACSaria-friendly amazons **Emma, Mona, Petra** and **Maria**, I owe you!

-**Everyone at L2:04, which thanks to you is a great place to work at**.

Tack till mina fantastiska vänner:
-**Pernilla** och **Ulrika**, lätt bäst i test och på ständig älgjakt, tack för en perfekt Thelma and Louise-semester! Jag förväntar mig ytterligare en, fast kanske ännu mer utomsocknes nästa gång, nu är ni ju trots allt två som kan köra……
-**Louise** och **Jonathan** (för all segling och kräftfiske), **Andreas** (för att jag nu kan allt om "manbags"), **Daniel** (för pensionat New York) och **Robert** (för alla spännande anekdoter

Sarah Thunberg
från Köpenhamn och snart Oslo….), **Jenny** (för hootchy kootchy), **H** (för att du är du), **Micke**, **Ingela** och **Roger** (för midsommar på Norröra), **Therese** och **Robban**, **Maria** och **Frasse** (för pensionat Åmål), **Emma** och **Fredrik** (för pensionat Åhus) **Emma** (my personal stop staring shopper), **Malin**, **Tobias**, **Kati** samt **Håkan**.

Ett STORT tack till mina naggande goda släktingar (**Gunilla** och **Johan**, (skräck)-moster **Ami**, **Lars**, **Astrid** och **Janne** och många många fler) för allt stöd under åren.

Ett OÄNDLIGT STORT tack till mina underbara föräldrar **Gudrun** och **Jörgen**, som trots något egendomliga namn har varit de bästa föräldrar en Sarah någonsin har kunnat önska sig. Införskaffandet av en jycke skulle göra er om möjligt ännu bättre…….

FACIT tackar och bockar!

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