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DISSERTATION

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“Mucosal tolerance induction for treatment of type I allergy: Use of novel allergen constructs and adjuvants to improve treatment efficacy”

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ZUSAMMENFASSUNG

Mukosale Toleranzinduktion zur Behandlung von Typ I-Allergie: Verwendung neuartiger Allergenkonstrukte und Adjuvantien für verbesserte Behandlungsmethoden gegen Allergien

In den letzten Jahrzehnten wurde ein ständiger Anstieg der Prävalenz von Type I-Allergien beobachtet, so dass mittlerweile mehr als 25% der Bevölkerung in westlichen Ländern an Allergien leiden. Häufig werden Allergiker mit zunehmendem Alter gegen mehrere Allergene co-sensibiliisert, wobei diese Patienten schwierig zu behandeln werden. Goldstandard der Allergiebehandlung ist die Allergen-spezifische Immuntherapie. Jedoch birgt diese Therapieform auch einige Nachteile, wie zum Beispiel regelmäßige Injektionen über einen Zeitraum von 2-3 Jahre, Gefahr von anaphylaktischen Nebenwirkungen sowie eine geringe Erfolgsquote bei der Behandlung von Mehrfachallergien. Um die Compliance der Patienten zu erhöhen, aber auch um die Effizienz der Therapie zu verbessern, ist in den letzten Jahren an folgenden Ansätzen gearbeitet worden: Verwendung von rekombinanten Allergenen, die dem Sensibilisierungsprofil der Patienten entsprechen, Applikation effektiver Adjuvantien, und der Wechsel zu einer mukosalen (z.B. oral, nasal) Route der Applikation zur Umgehung der häufigen Injektionen.

Entsprechend wurden im Zuge dieser Doktorarbeit neue Behandlungsansätze entwickelt und neue Mausmodele für Einfach- und Mehrfachsensibilisierung etabliert, um Effektivität und Wirkungsmechanismen dieser neuen Ansätze *in vivo* und *in vitro* testen zu können.

(i) Eine vielversprechende Strategie zur Behandlung von Allergien sind rekombinant hergestellte Allergene. Die rekombinante Produktion garantiert eine genaue strukturelle wie auch immunologische Definition des Allergens. Die Identifizierung von Allergenen, wie auch die Herstellung als rekombinantes Protein sind die Grundsteine bei der Entwicklung von neuen Formen von Immunotherapeutika wie hypoallergene Varianten von Allergenen, Fusionsmoleküle oder Multiallergenkonstrukte. Wir beschreiben die Charakterisierung und rekombinante Herstellung eines neuen Allergens der Dörrobstmotte, das Thioredoxin Plo i 2. In *in vivo* Studien in Mäusen konnten wir das allerogene Potential dieses Allergens, im Vergleich mit einem anderen Mottenallergen (Plo i 1), untersuchen. Mittels Immunoblot-Experimenten mit

humanen Sera wurde die IgE-Reaktivität des Thioredoxins Plo i 2 analysiert, und so Plo i 2 als neue relevante Allergiequelle für mehrfachsensibilisierte Haus- und Meeresfrüchteallergiker, definiert.

(ii) Pollen-allergische Patienten sind häufig co-sensibilisiert gegen homologe Lebensmittelallergene; dies wird als sogenannte Pollen-assoziierte-Lebensmittelallergie bezeichnet und manifestiert sich klinisch als „orales Allergiesyndrom“ (OAS). Mehr als 70% der Birkenpollenallergiker leiden unter einer Pollen-assoziierten-Lebensmittelallergie, somit zählt diese Form der Birkenpollen-assoziierten-Lebensmittelallergie zu einer der häufigsten Varianten der Mehrfachallergien. Da das OAS im Rahmen der Behandlung gegen Birkenpollenallergie meist nur ungenügend beeinflusst werden kann, haben wir ein Multiallergenkonstrukt entwickelt, das eine gleichzeitige Behandlung der Birkenpollen- und Nahrungsmittelallergien ermöglichen soll. Dieses Multiallergenkonstrukt wurde gentechnologische hergestellt,, bestehend aus dem gesamten Hauptbirkenpollenallergen Bet v 1 verlinkt mit den immundominanten T-Zell-Epitopen der Birkenpollen-assoziierten-Lebensmittelallergene Dau c 1 (Hauptallergen der Karotte) und Api g 1 (Hauptallergen des Selleries). Um das immunmodulatorische Potential dieses Konstrukts auszutesten, wurde ein Mausmodel für Mehrfachsensibilisierung mit oralem Allergiesyndrom etabliert. Wir konnten zeigen, dass nach intranasaler Applikation dieses Konstrukts die allergische Immunantwort in Richtung Th1 Antwort moduliert wurde und eine Suppression der allergischen Immunantworten durch Induktion von regulatorischen Immunmechansimen erreicht werden konnte.

(iii) Um die Effektivität von Toleranzinduktion mittels rekombinanter Allergene oder Allergenkonstrukte zu erhöhen, können mukosale Adjuvantien als sogenannte „mukosale Transportsysteme“ verwende werden. Eines der mukosalen Adjuvantien mit tolerogenem Potenzial ist Choleratoxin (CT) bzw. die B-Untereinheit von Choleratoxin (CTB). In der vorliegenden Arbeit beschreiben wir die gentechnologische Konstruktion eines Fusionsmoleküls von CTB und Bet v 1. Dieses Fusionsmolekül wurde intranasal in einem Mausmodel für Birkenpollenallergie appliziert um dessen immunmodulatorische Kapazität zu untersuchen. Die Allergie-reduzierende Wirkung des neuen Konstrukts beruhte auf der gleichzeitigen Modulierung der Th2 Immunantwort Richtung Th1 und der Induktion von lokalen, protektiven IgA Antikörpern.

Zusammenfassend, wir haben drei verschiedene Strategien (rekombinante Allergene, Multiallergenkonstrukte für mukosale Applikation und Verwendung neuer mukosaler Adjuvantien) zur Verbesserung der Prävention (und möglicherweise auch Therapie) von Typ I-Allergie entwickelt, deren Nutzen durch eine erfolgreiche Suppression der allergischen Immunantwort in Einfach- und Mehrfachsensibilisierungsmodele demonstriert wurde. Derartige experimentelle Studien sollen dazu beitragen, bestehende Behandlungsansätze zu verbessern, sowie auch neue Behandlungsstrategien für allergische Erkrankungen zu entwickeln.

ABSTRACT

Mucosal tolerance induction for treatment of type I allergy: Use of novel allergen constructs and adjuvants to improve treatment efficacy.

The prevalence of type I allergy is constantly increasing in industrialized countries: more than 25% of the population suffer from allergic disorders. With increasing age allergic individuals become co-sensitized to additional allergens, and become difficult to treat due to the multi-sensitization. Allergen-specific immunotherapy is state of the art treatment of allergic diseases. However, due to a low compliance of patients to frequent injections, and the reduced efficacy in multi-sensitized individuals, a change to a less invasive route of application via the mucosa offers a promising approach.

The aim of this thesis was to develop new treatment strategies for primary and secondary prevention of type I allergy, focusing on strategies being applied via the mucosal surfaces.

Therefore, three novel approaches were developed and tested that might lead to improvement of the current treatment strategies: (i) Use of recombinant allergens, (ii) development of multi-allergen constructs for mucosal tolerance induction, and (iii) use of mucosal adjuvants systems for improved mucosal treatment strategies. Furthermore, murine models of mono- and poly-sensitization were established, to investigate the immunological mechanisms of mucosal tolerance induction as well.

Ad i) Recombinant forms of allergens are promising tools for treatment of allergy, because of their defined structural and immunological properties as well as the standardized production procedures. Furthermore, identification and production of allergens as recombinant proteins are the essential steps to develop advanced forms of immunotherapeutica such as hypoallergenic variants, fusion molecules, or multi-allergen constructs. A novel allergen of Indianmeal moth, the thioredoxin Plo i 2 was identified and recombinantly produced. The allergenic potential of Plo i 2 was investigated by *in vivo* studies in mice, in comparison to the moth allergen Plo i 1. IgE-reactivity tested by blotting experiments with human sera, defined thioredoxin Plo i 2 as new relevant source in multi-sensitized indoor and seafood allergic patients.

Ad ii) In pollen allergic patients co-sensitization to homologue food allergens often occurs, termed as pollen-related food allergy and clinically manifested as “oral allergy syndrome” (OAS). With about 70% of birch pollen allergic patients, birch pollen-related food allergy is the

most common form of this multi-sensitivity. For a new experimental approach to treat birch pollen-related food allergy, a multi-allergen construct composed of the whole major birch pollen allergen Bet v 1 linked to the immunodominant T cell epitopes of the Bet v 1-related food allergens Dau c 1 (major allergen of carrot) and Api g 1 (major allergen of celery), were genetically engineered. In order to investigate immunomodulatory potential of this chimer, a murine model of poly-sensitization and OAS was established. Intranasal application of the chimer modulated allergic immune responses towards Th1 immune responses via regulatory mechanisms.

Ad iii) In order to enhance the effectiveness of mucosal tolerance induction with recombinant allergens or allergen constructs, mucosal adjuvants were used as mucosal delivery systems. The strong tolerogenic nontoxic B subunit of cholera toxin (CTB) served as mucosal adjuvant which was genetically conjugated to the birch pollen allergen Bet v 1. For improvement of mucosal tolerance induction the CTB-Bet v 1 fusion molecule was intranasally applied in a murine model of birch pollen allergy. The immunomodulatory properties of the fusion molecule were demonstrated by a modulation of Th2 immune responses towards Th1, accompanied by induction of protective IgA antibodies.

In summary, we have presented three different strategies - (i) production of recombinant allergens or allergen constructs, (ii) use of mucosal route of application, (iii) use of mucosal adjuvants - to improve prevention of type I allergy. The benefit of mucosal application of recombinant allergens conjugated to adjuvants as fusion protein or to other allergens as multi-allergen construct was demonstrated by successful suppression of allergic immune responses in mono- and poly-sensitized models. Such experimental studies will contribute to the improvement of new treatment approaches with increased effectiveness against the constantly increasing number of allergic diseases.

I. INTRODUCTION

1. Allergy

1.1. Overview of Hypersensitivity Reactions

Hypersensitivity reactions are caused by abnormal immune responses which lead to tissue injury and disease. “Hypersensitivity diseases” are initiated after the first contact with an antigen (e.g. usually harmless microbes, allergens, or self antigens) which “sensitize” the immune system after the second contact leading to overwhelming, uncontrolled strong immunological and pathophysiological reactions (1).
In most cases, the course of hypersensitivity diseases is chronic and progressive. Once the immune reaction is initiated it is a challenge to downregulate these inappropriate reactions (1).

Gell and Coombs classified hypersensitivity diseases into four different types, according to the type of immune response and effector mechanisms (Fig.1) (1-4).

Type I (immediate or IgE-mediated hypersensitivity, type I allergy): Type I is characterized by allergen-specific IgE binding to high affinity IgE-receptors (FcεRI) on the surface of basophils and mast cells (4). The cells are activated by cross-linking of bound IgE antibodies after reexposure to allergens leading to immediate release of inflammatory mediators (histamine, proteoglycans, leukotrienes, cytokines) (2). Within 5 to 10 minutes this release can lead to the induction of wheal, hives, redness, angioedema or even an anaphylactic shock. Two to 4 hours later a late-phase reaction mediated by recruited inflammatory cells (neutrophils, eosinophils, basophils) and T helper (Th) 2 cells occur (2-4).

Type II (antibody-mediated or cytotoxic hypersensitivity): Type II reactions are caused by cytotoxic antibodies, primarily IgG and IgM, which bind to host cells or tissues (2, 4). Cell-bound antibodies activate the classical pathway of the complement system and recruit inflammatory cells (macrophages, neutrophils and eosinophils) to interact with the antibody-coated host cells through their Fc receptors aiming to eliminate these cells (2). Type II immune reactions develop

within several hours (up to 24 hours) (2). Type II hypersensitivity disorders can be involved in autoimmune diseases, transplantations or drug reactions (1, 2, 4).

Type III (immune complex mediated hypersensitivity): Type III is characterized by binding of IgG and IgM antibodies to antigens located in tissues and form antigen-antibody-complexes in and around blood vessels (4). These immune complexes induce local inflammatory responses mediated by Fc receptors or complement activation similar as in type II hypersensitivity. Thus, these antigen-antibody-complexes and subsequently attracted effector cells (macrophages, neutrophils) cause damage to cells of adjacent tissues of lung, joints, kidney, skin and eyes (4). Systemic diseases such as serum sickness, systemic lupus erythematosus (SLE) or farmer lung belong to so called immune complex-mediated diseases. Type III reactions can take hours, days, or even weeks to develop (1, 2, 4).

Type IV (T cell mediated, delayed-type hypersensitivity DHT): Inflammation and tissue injury are caused by $CD4^+$ T cells activation (delayed-type hypersensitivity) or by $CD8^+$ CTL induced cell lysis (1). This delayed type reaction take typically 24 to 48 hours to develop (2). Common type IV disease are type I diabetes mellitus, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and contact dermatitis (3).

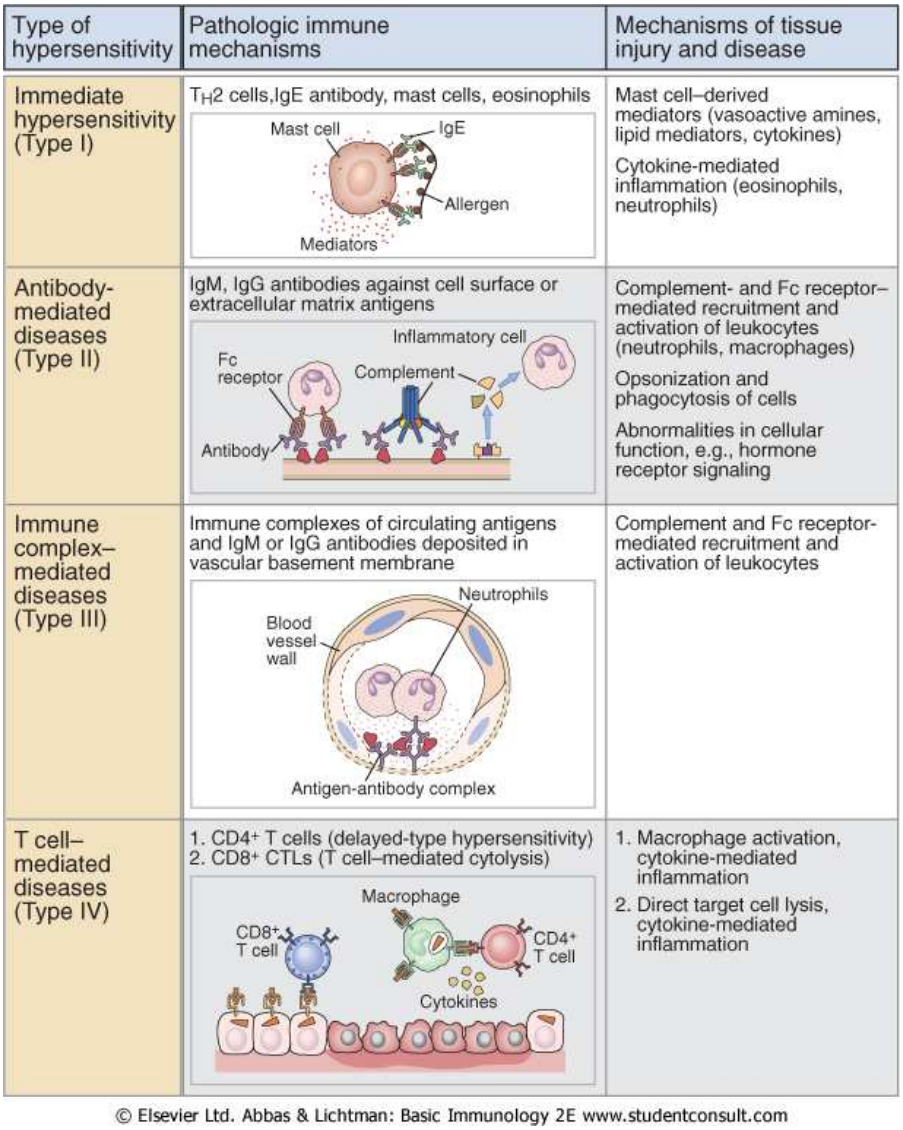

Fig.1: **Types of hypersensitivity diseases (3).**

1.2. Type I Allergy

IgE-mediated allergy is the classical form of a type I hypersensitivity reaction previously described.(1).

Prevalence of type I allergy

Type I allergy affects more than 25% of the population in industrialized countries, and is therefore one of the most common immunological disorder (1, 5). Since the last 50 years the prevalence rate has been growing exponentially, leading to the fact that atopic asthma has increased by as much as 400% in many regions (6). The reason for the constant increase of type I allergy is very complex and not fully elucidated. Apart from a genetic predisposition, certain factors such as changes in lifestyle (obesity, smoking, dietary habits), environmental influences (respirable dust, pesticides), or higher hygienic standards (hygiene hypothesis) have to be considered (7).

Hygiene hypothesis

The hygiene hypothesis - described by Strachan in 1989 – implies that decreased exposure to pathogens and microbial components during childhood leads to a modulation of the immune system towards a Th2 biased immune response (8, 9). The underlying mechanistic hypothesis implies that the lack of infection induced Th1 stimuli and the lack of parasite induced Treg stimuli in early life, may lead to an imbalance of Th1, Th2 and Treg responses with a dominant Th2 immune response (9, 10). Recent studies showed that exposure early in childhood – even during pregnancy – to certain microbial components, helminth infections or contact with pets can have a protective effect on the development of allergic diseases (11-17).

Gene environment

Many different genetic factors come together to increase the risk of developing allergic diseases.

There is evidence that the family history of atopy, including the maternal and/or paternal inheritance of genetic polymorphisms, has an important impact on IgE production and asthma development (18). For example, Leaves et al. showed that an allele at chromosome 7p, inherited from the father, is tightly linked to airway hyperresponsiveness (AHR) (18, 19), whereas a polymorphism of the high-affinity IgE receptor beta-chain (FcεRI-β) inherited from the mother has a strong association with a higher amount of allergen-specific IgE levels (18, 20).

Not only genetic predisposition but also so called epigenetic influences determine the outcome of immunological responses (21): Renz et al. described that maternal nutrition or maternal exposure to pollutants and tobacco smoke before birth along with genetic predisposition – can

induce changes in the morphogenesis of tissues and organs of the fetus (6). This prenatal exposure to harmful agents can lead to a disorder during the development of branching of the airways in the lung, and can thereby trigger the later susceptibility of development of asthma (6).

Mechanism of type I allergy

The immediate hypersensitivity reaction is induced by infiltration or invasion of an already encountered allergen. This allergen enters the epithelial barriers of the oral/nasal mucosa (22-24), conjunctival epithelium (25), respiratory tract (26), gastrointestinal tract (27) or the skin (5). Immature antigen presenting cells (APCs), mainly dendritic cells (DCs), take up these allergens and process them during activation and migration into the draining lymph nodes. There the activated DCs present allergens, which were processed to peptides, via MHC class II molecules to naïve $CD4^+$ T lymphocytes (1, 5). These T cells are activated by the interaction of peptide–MHC II-complexes with the allergen-specific T cell receptor (TCR) (signal 1) as well as costimulatory molecules such as CD40 - CD40 ligand (CD40L) and CD28 - CD80/CD86 (signal 2), and certain environmental cytokines (signal 3). Activated T cells differentiate into Th2 cells and start to proliferate and secrete the Th2 cytokines IL-4, IL-13 and IL-5 (1, 5). IL-4 induces B cell switching to IgE antibody producing B cells, while IL-5 activates and increases maturation of eosinophils. IL-13 stimulates epithelial cells to secrete enhanced amounts of mucus (1).
Allergen-specific IgE circulates in the plasma and tissues until it binds to high-affinity Fc receptors (FcεRI) on effector cells such as mast cells and basophils within tissues (1). These effector cells become activated by cross-linking of the allergen-specific IgE antibodies by the respective allergens, and start to secrete proinflammatory mediators (biogenic amines such as histamine; lipid mediators such as prostaglandins, leukotrienes and PAF; and cytokines such as TNF, IL-4, IL-5, IL-13). The late-phase reaction of type I allergy is described by an infiltration of activated neutrophils, eosinophils, basophils and Th2 cells to the site of inflammation by vasodilation, followed by a release of lipid mediators, cytokines and toxic proteins (e.g. ECP, MBP) leading to prolonged inflammatory reactions (1, 5).

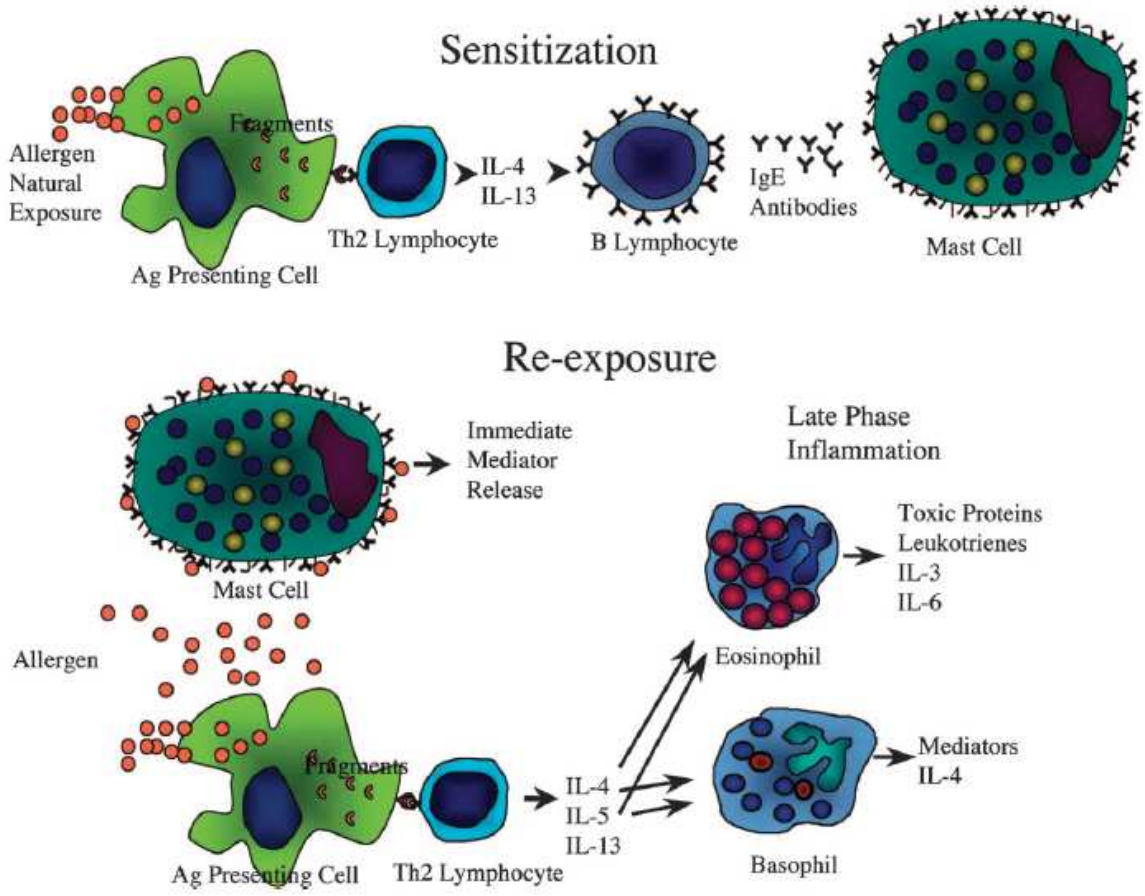

Fig.2: **Pathogenesis of type I allergy (28).**

Pathophysiology of type I allergy

The pathophysiological manifestation of type I allergy depends on the type of allergen (e.g. inhaled pollen allergen, food allergen) and the localisation of the allergic reaction (e.g. lung, gut, skin).

- Atopic dermatitis

Atopic dermatitis (AD) is a very common, chronically relapsing, inflammatory disorder of the skin. AD is a reaction of the skin to irritants, foods and environmental allergens, characterized by defined sealing and itching followed by erythema with oedema, vesicles and weeping in the acute stage, and skin thickening (lichenification) in the chronic stage (29). Fifteen to 30% of children and 2-10% of adults are affected by atopic dermatitis, and 30 to 35% of these children

are at risk of developing asthma or allergic rhinitis (29). The clinical history of AD often starts in infancy (in the first 6 months of life), but 70% of the atopic children will grow out this chronic disorder during adolescence. Although 50% of adults with an AD history have further episodes of these late-phase reactions. The high prevalence of AD is caused by genetic (parents with AD) as well as environmental factors (urban/industrial settings, higher socioeconomic status, smaller family size) (29).

- Allergic Rhinitis & Conjunctivitis

Allergic rhinitis (hay fever) - the most prevalent type I allergy - affects the upper respiratory tract and conjunctiva (allergic conjunctivitis), and is caused by allergic reactions to inhaled allergens (e.g. grass pollen allergens, birch pollen allergens, house dust mite (HDM) allergens). Leukocyte infiltration – mainly eosinophils – followed by mucus secretion, mucosal edema, coughing, sneezing, itching and dyspnoea are common manifestation (1, 30).

- Allergic Asthma

Bronchial asthma is a chronic inflammatory airway disease characterized by intermittent and initially reversible airway obstruction after repeated allergen exposure accompanied by the development of non-specific airway hyper-responsiveness (1, 31). About 8% of the adult population and up to 20% of children in Europe, North America and Australia suffer from asthma (32). Allergic asthma affects the lung tissue and the bronchial mucosa. Inhaled allergens, such as Der p 1 from house dust mite (33) or Bla g 1 and Per a 1 from cockroach (34, 35), trigger lymphocytes and eosinophils to extensive infiltration of the bronchial mucosa, to increased mucus production and submucosal edema, which leads to airway smooth muscle hyperplasia and airway remodelling (31).

- Systemic Anaphylaxis

Systemic anaphylaxis is the strongest immediate hypersensitivity reaction, which preferentially occurs in venom or food allergy, and is the most severe manifestation of type I allergy (1). The antigen enters the circulation via the skin or the gut mucosa. The systemic presence of the allergen activates mast cells in many different tissues and leads to the release of their mediators. The high amount of mediators distributed via blood vessels throughout the body, leads to a decrease of vascular tone and leakage of plasma, decrease of blood pressure and eventually an anaphylactic shock (1). Laryngeal edema, constriction of the upper and lower

airways, hives, and secretion of mucus in the respiratory and gastrointestinal tracts can precede the systemic shock symptomatic (1, 36).

- Primary Food Allergy

Food allergy is induced by ingested digestion-resistant allergens from commonly consumed foods (such as peanuts, tree nuts, fish, shellfish, milk) which trigger intestinal mucosal mast cells to release mediators. Increased peristalsis and fluid secretion accompanied by vomiting and diarrhea are the associated clinical symptoms (1), as well as urticaria (e.g. milk) (37) and systemic anaphylaxis (e.g. peanut) (38) caused by a systemic distribution of the allergen. Interestingly, in some cases, especially milk, egg and fish allergy, children can outgrow these sensitivities during adolescence (39). A Danish study showed that 97% of cow's milk allergic children become tolerant by the mid teen years (40, 41).

- Pollen-related Food Allergy

Pollen-related food allergy is induced by unstable food allergens (e.g. apple Mal d 1, carrot Dau c 1, celery Api g 1, hazelnut Cor a 1) which cross-react with IgE and T cells primed by homologous pollen allergens (e.g. birch Bet v 1, mugwort Art v 4, ragweed Amb a 8). The immediate allergic symptoms - termed "oral allergy syndrome" (OAS) - are restricted to the oral cavity with itching and swelling of the oral mucosa, lips, tongue, soft palate, pharynx or ear. As severe reactions atopic eczema, asthma or even anaphylaxis can occur in some patients (42).

1.3. Allergens

We are constantly exposed to different environmental antigens (such as food or airborne allergens), against some of which atopic individuals develop an allergic reaction after repeated exposure (43). The reason why an antigen can act as an allergen is still not fully understood.
There are, however, some characteristics common to many allergen, such as acid and proteolytic stability, low to medium molecular weight (5-70 kDa), enzymatic activity and high solubility in body fluids (1, 43).
An identification of the biologic function of these proteins is important for the improvement of new diagnostic and therapeutic strategies (44). Many allergens share similar structural and

functional properties (45); due to these common features they are classified in several protein families such as proteases, ligand-binding proteins, albumins, tropomyosins, or calcium-binding proteins; many pollen allergens are pathogenesis-related proteins, calcium-binding proteins and profilins; plant and animal food allergens are often lipid transfer proteins, profilins, or tropomyosins (44).

Cross-reactivity

Structural and amino acid sequence similarity between allergens is the major cause of cross-reactivity (46). An allergen-specific IgE recognizes structurally similar epitopes of allergens from close-related or unrelated species, leading to cross-reactivity and IgE-mediated hypersensitivity reaction (47, 48).

Cross-reactivity occurs not only at the B cell but also at T cell level. Bohle et al. demonstrated “cellular cross-reactivity” with the pollen allergen Bet v 1 and its homologous food allergen Api g 1 (from celery); these allergens share 73% amino acid sequence similarity in their major T cell activation region, therefore, Bet v 1-specifc Th2 cells can be activated perennially, even outside the pollen season by eating pollen-related food such as celery,. (49, 50).

So far cross-reactivity syndromes (such as birch-food-syndrome, mugwort–celery-spice-syndrome, ragweed-melon-banana-syndrome) were thought to be due to IgE-mediated reactions, but a role of T cell-mediated cross-reactivity should be also considered (50, 51).

Pan-allergens

There is also a “trans-phyla” or even “trans-species” cross-reactivity of so called pan-allergens, which are proteins with highly conserved amino acid sequence in species without close systemic relationship. Profilins are one of the first protein families described, as pan-allergens, with homologous allergens in many different heterogeneous allergen sources (52). Tropomyosins, such as Pen a 1 from shrimp, have been described as an important pan-allergen, which cross-reacts with homologous of species from other phyla such as the allergens of crustaceans, molluscs, house dust mites and cockroaches (53-56).

Another relevant pan-allergen is arginine kinase, firstly described by Binder et al. in Indianmeal moths (Plo i 1) with high IgE-cross-reactivity in seafood allergic patients (with king prawn, lobster, and/or mussel allergy) (57). Meanwhile arginine kinase was also described in crustacean such as the black tiger shrimp (Pen m 2), the white shrimp (Lit v 2) or the North Sea shrimp (Cra c 2) (58). Thioredoxin, another pan-allergen is described as ubiquitous protein with a highly conserved active site, already found in wheat, maize (59), fungi (60) or moths (61).

Allergens of particular clinical relevance

In industrialized countries the major allergens are derived from house dust mites *(Derntatophagoides pteronyssinus*) i.e. Der p 1 and Der p 2, cats (Fel d 1, Felis domesticus) as well as several trees and grasses such as Bet v 1 from birch tree (*Betula verrucosa*) and i.e. Phl p 1 and Phl p 5 from timothy grass (*Phleum pratense*) (62). Especially in North America, the ragweed allergens are relevant seasonal allergens (Amb a 1, Amb a 2, Amb a 3, Amb a 5, Amb a 6 from short ragweed (*Ambrosia artemisiifolia*), Amb t 5 from giant ragweed (*Ambrosia trifida*)) with constantly increasing prevalence of sensitization (62). The peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are highly allergenic, and the prevalence of allergic reactions to these allergens is constantly increasing worldwide (62).

1.4. Poly-Sensitization

The “career” of an atopic patient mainly starts with sensitization towards one class of allergens – so-called mono-sensitization. Over time these patients become sensitized to an increasing number of allergens and develop a so-called poly-sensitization (30). The sensitization process progressively develops from childhood to adulthood (63), and often reaches a peak in the third decade of life (64),

Poly-sensitization is a common feature of atopic patients and is clinically documented in several studies (63-65). Poly-sensitization as such is either a result of cross-sensitization to biologically similar allergens or occurs due to co-sensitization to allergens found in the same environment (63, 65, 66). Kang et al. showed a familial coincidence in poly-sensitization with a significantly higher frequency of poly-sensitized children born to poly-sensitized parents (67).

Silvestri et al. could show that 43,6% of a study population of asthmatic, mono-sensitized patients became poly-sensitized in the second survey (68). Moreover, they demonstrated that especially patients sensitized to the perennial allergens of house dust mite or (to a lower degree) seasonal pollen allergens seem to develop further sensitizations (68). In a recent study they hypothesize that the development of poly-sensitization - especially in children – is associated with a functional defect of T regulatory cells; a significant decrease of IL-10 and IFN-γ boost the onset of new sensitizations (30, 69).

Pollen-related food allergy

Pollen-related food allergy is very common among poly-sensitized patients. The patients are primarily sensitized to respiratory allergens (e.g. pollen of birch, mugwort, ragweed, grass ;) and develop classical symptoms of pollinosis; cross-reactive IgE recognizes food allergens homologous to pollen allergens due to their high structural similarity of major epitopes (40).
More than 70% of birch pollen allergic patients suffer from this cross-reactivity to pollen-related food allergens, clinically manifested as oral allergy syndrome (OAS) (see type I allergy – pathogenesis) (40). Examples are the birch pollen-associated plant food allergy, the mugwort–celery-spice-syndrome (Art v 4, Api g 4), the ragweed-melon-banana-syndrome (Amb a 8, Cuc m 2, Mus xp 1) and the mugwort-mustard-syndrome (Art v 4, Sin a 4) (70). In Europe a very common example of pollen-related food allergy is the birch pollen-related food allergy (BPRFA). In Austria more than 35,9% of pollen allergic patients react to Bet v 1, the major birch pollen allergen (71). Bet v 1 as well as many food allergens (e.g. Cor a 1 from hazelnut; Mal d 1 from apple, Pru a 1 from cherry, vegetables like Api g 1 from celery, Dau c 1 from carrot; Fabaceae seeds such as Ara h 1 from peanut, Gly m 4 from soybean) belongs to the pathogenesis-related-10 (PR-10) protein family (40). These Bet v 1–related food allergens share a high similarity with Bet v 1; they show similar molecular masses and isoelectric points, and a high

sequence identity between 38% (Dau c 1) and 67% (Cor a 1) (72). These similar properties are the main cause of cross-reactivity and consequently of birch pollen-related food allergy.

Patients with OAS are generally difficult to treat with conventional immunotherapy against birch pollen; even if the symptoms of the pollinosis improve, the oral allergy syndrome often remains unaffected by this treatment (73, 74).

Thus, one of the major challenges in allergy research is to develop new treatment approaches that will be also effective in poly-sensitized individuals.

2. Therapy

2.1. Allergen Specific Immunotherapy

More than 100 years ago, Noon and Freeman developed a causative treatment approach against allergic disease, referred to as “desensitization” or “hyposensitization” (75). Their approach of allergen-specific immunotherapy was the induction of tolerance via subcutaneous administration of increasing amounts of the relevant allergen extracts (75).

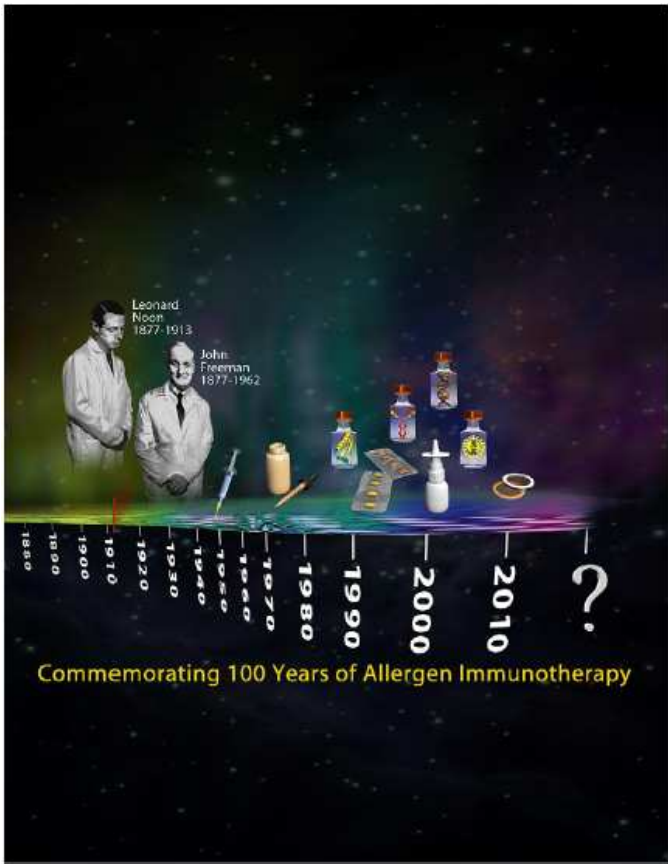

Fig.3 : **Cover image of Journal of Allergy and Clinical Immunology, January 2011, Volume 127, Number 1** (76).

Nowadays, the common therapeutic approaches against allergy are - apart from the recommendation of avoidance of the allergen (which is not always possible, e.g. house dust mite allergens) – the combination of a symptomatic treatment with e.g. anti-histamines (77, 78),

corticosteroids (79), or epinephrine (80), with allergen-specific immunotherapy (SIT) as a causative treatment.

Immunotherapy is the gold standard for treatment of a broad range of IgE-mediated allergic diseases (75, 81), but seems to have considerable limitations especially in poly-sensitized individuals, such as patients with birch pollen related food allergy (82). A successful birch pollen therapy does not consequently lead to a mitigation of the oral allergy syndrome (73, 74).

In order to improve the compliance to immunotherapy the change to a less invasive route of application via the mucosa (e.g. oral, nasal, bronchial, sublingual) has been investigated in several studies (83-91). These sublingual immunotherapy (SLIT) seems to be the most promising alternative strategy to conventional SIT.

Subcutaneous Immunotherapy (SCIT)

Subcutaneous immunotherapy (SCIT) is a well-established form of immunotherapy, whose clinical safety and efficacy in rhinitis, asthma and especially in venom allergy are confirmed by several studies (92). SCIT starts with weekly injections of low doses of the allergen over 8-16 weeks. Subsequently, the allergen dose is gradually increased, until a maintenance dose is reached (5-20 µg of the major allergen), which is administrated once a month for a period of 3 to 5 years (93). To reduce or avoid local side-effects coapplication of antihistamine a few hours before injections is recommended. After a successfully completed SCIT the patient can expect a protection of about 3 to 5 years.

Many reviews of describe the effects of successful application of SCIT in different allergic diseases (94, 95). The latest meta-analysis update in 2010 on SCIT, in which 88 randomized controlled trials were included, showed the efficacy of SCIT by reduced asthma symptoms, a decreased necessity of asthma medications, a rare occurrence of anaphylactic reactions as well as improved bronchial hyper-responsiveness (81).

Although SCIT is a well established and highly effective therapy for many treated individuals, not everyone benefits from this treatment: Severe side-effects can occur with the risk of developing anaphylactic shocks (96). During the long-lasting treatment many injections need to be applied,, which diminishes the compliance of the patients, particularly of children.

Sublingual Immunotherapy (SLIT)

A less invasive form of immunotherapy is sublingual immunotherapy (SLIT), during which an allergen extract in a liquid or pill formulation is placed under the tongue for 1 to 2 minutes followed by swallowing (83, 97, 98). The commonly used protocol of SLIT is a daily treatment starting pre-seasonally and continuing perennially for 3-5 years (99). The optimum dosage, a perfect duration of the treatment and the ideal frequency of administration are still under investigation. In currently available SLIT vaccines for grass pollen, tree pollen, house dust mite and cat allergy, the applied doses of allergen are 30 to 50 times greater than in conventional SCIT (99).

An updated meta-analysis of 2010, that included 49 trials, confirmed significant reductions of symptoms and medication after SLIT. The side effects are quite rare, apart from local reactions in the oral cavity or the gut (81).

The oral mucosa is supposed to be an “immune-privileged site”; because of its high vascularization and permeability, as well as its tolerizing properties towards commensal bacteria and the anti-inflammatory properties of the local cells (100).

Mechanism of allergen-specific immunotherapy

For many years, the mechanisms of effective SIT were unknown, but within the last decades much light has been shed on the underlying cellular and molecular events of successful SIT.

Akdis described the induction of long-lasting immune tolerance by generation of allergen-specific Treg cells accompanied by release of the regulatory cytokines IL-10 and transforming growth factor β (TGF-β) as well as an early decrease of mast cell and basophil degranulation (101). IL-10 and TGF-β induce suppression of T cell proliferation along with a reduction of Th1 (IFN-γ) and Th2 (IL-5 and IL-13) cytokines (102). One important aspect in tolerance induction via SIT is an early and rapid increased production of antigen-specific IgG4 and IgA antibodies. This is supported by IL-10 as potential IgE suppressor and TGF-β as IgA switching factor (101, 103). Additionally IgG4 antibodies block the cross-linking of IgE on the Fcε receptor of mast cells and basophils by competing with IgE for binding allergens (101).

In recent studies it has been speculated that also IL-10-secreting type I T regulatory (Tr1) cells play a major regulatory role in tolerance induction and long-term effects of immunotherapy

(101, 104). In the delayed phase of SIT a shift from an allergen-specific Th2 towards a Th1 immune response has been observed as important factor for successful desensitization (105).

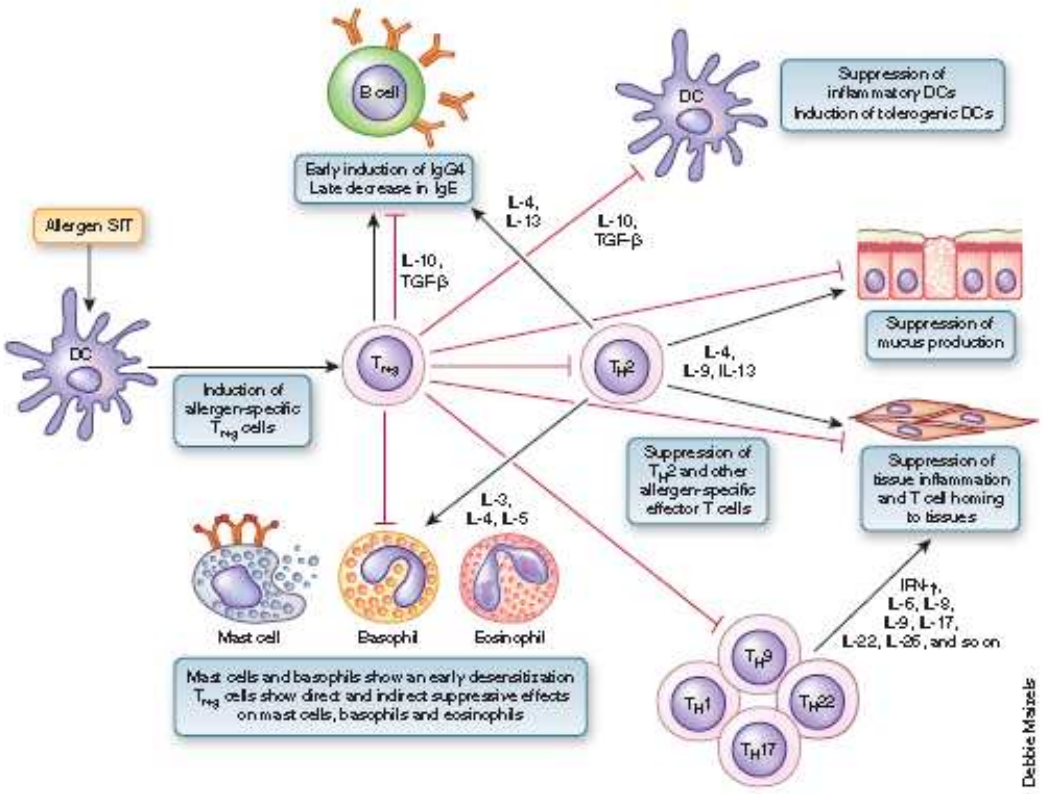

Fig.4: **Mechanisms of allergen-specific immunotherapy (101).**

The main mechanism of SLIT is supposed to be the same as at conventional SIT with a focus on IL-10 induced Treg cells and IgG4 blocking antibodies. Bohle et al. described mechanistic differences in the early and the later phases of sublingual therapy, initially showing a Treg dominance and later on an upregulation of the expression of the Th1 cytokine IFN-γ (106). Ciprandi et al. showed an increased production of IgA antibodies and TGF-β during sublingual tolerance induction in mono-sensitized birch pollen allergic patients (107).

Latest studies referred to a newly characterized local APC population, the oral Langerhans cells (oLC). It has been speculated that oLCs may play a major role in allergen uptake and T cell activation in the oral cavity during SLIT (108).

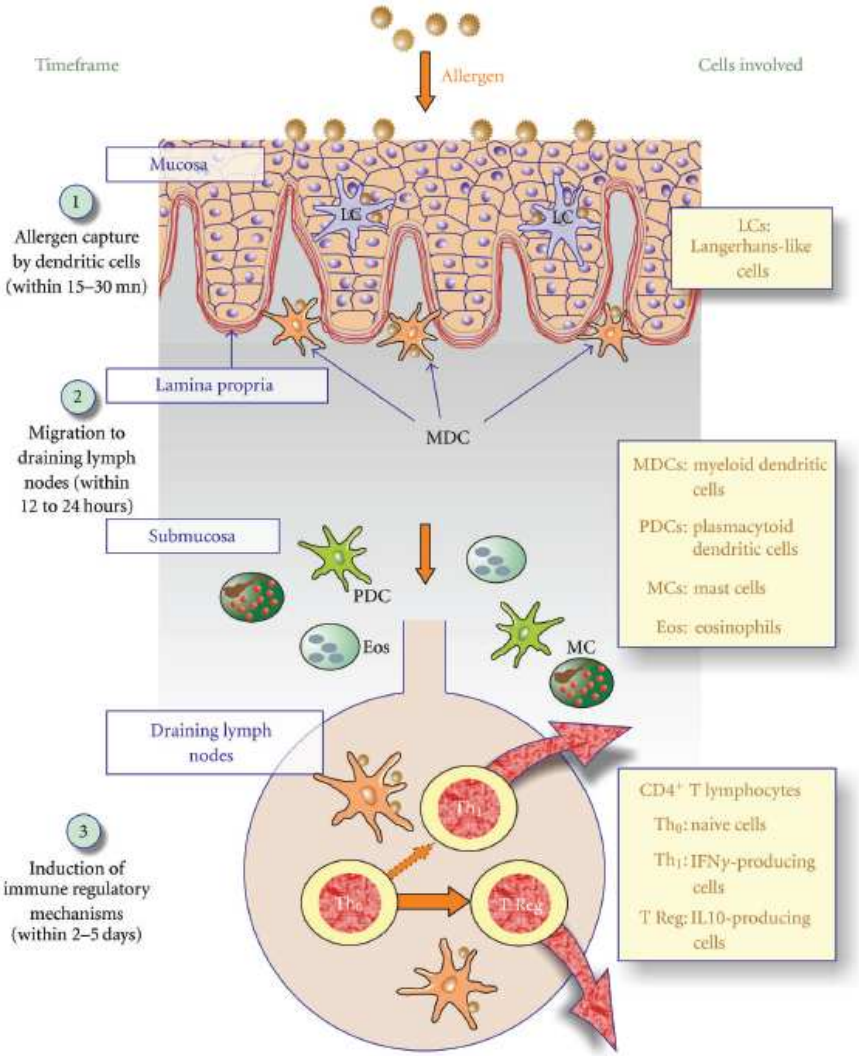

Fig.5: **Sublingual administration of allergens during SLIT (109).**

2.2. New Treatment Strategies

Conventional and experimental immunotherapeutic

The common preparations used for specific immunotherapy are crude **allergen extracts.** Even if these extracts show a high efficacy with long-lasting clinical effects in mono-sensitized individuals in several studies (110-112), there are still some limiting factors for effectiveness. Especially the use of extracts, which include a variety of different allergens and antigens

towards which the patient is not sensitized to, poses a risk of de novo sensitization to other antigens in the extract. Another drawback can be the presence of bioactive substances, which could be potentially proinflammatory leading to Th2 responses (75, 83).

Therefore, new immunotherapeutic agents and treatment strategies are under investigation in several studies worldwide, in order to improve the efficacy and safety of allergen-specific immunotherapy (28, 75, 101).

One possibility for improvement is the use of **recombinant allergens**. These are biotechnologically produced proteins with defined structure and of standardized quality. A big advantage of these products is that the molecules can be genetically modified by mutagenesis, deletion of epitopes or a reassembling of fragments leading to molecules with lower allergenic potential. The application of single recombinant proteins or a mixture of allergens, have been studied in many clinical trials and animal studies (28, 75, 101, 113-115). Such studies with recombinant wild-typ Bet v 1 or Bet v 1 fragments showed the immunogenic potential of these constructs in specific immunotherapy for mono-sensitized patients (116-119).

Another approach is **peptides immunotherapy**. These peptides consist of short, linear aligned amino acids representing the immunodominant T cell epitopes of relevant allergens. Due to their short length, they lack IgE binding activity and consequently do not induce IgE-mediated side effects, which was already shown in several clinical and experimental studies (101, 120-123).

An additional approach for efficient immunotherapy is the genetically engineering of novel **constructs** composed of allergens, peptides and/or adjuvants. The main reason of this co-application in the form of allergen constructs is to overcome the less successful therapy of poly-sensitized individuals by similar administration of multiple allergens. Several studies in mouse models or human cell cultures showed promising effects in suppressing Th2 immune responses and inducing Treg activation (122, 124-128).

Further improvement for the efficacy of immunotherapies is the use of certain **adjuvants**. The most commonly used adjuvants is aluminium hydroxide salts, which however is known to induce Th2 responses and might therefore not resemble to the adjuvant for immunotherapy. A variety of studies identified several other adjuvants with promising immunogenic properties to

modulate allergic immune responses; CpG oligonucleotide, which induce strong Th1 polarization (28, 75, 101, 129), monophosphoryl lipid A (MPL) (130, 131); carbohydrate-based particle (CBP) (132, 133), virus-like particle (134), the nontoxic cholera toxin B subunit (CTB) (135) and *E. coli* heat labile Endotoxin B subunit (LTB) (136, 137), as well as lactic acid bacteria have been tested as immunomodulators (75, 101, 138).

A recently established strategy is genetic vaccination with naked allergen-encoding nucleic acids. These **DNA or RNA vaccines** should facilitate the development of a personalized vaccine corresponding to the patient's current sensitization profile. An important step in the development of these vaccines is a rapid degradation of nucleic acids which avoids side-effects (75, 139-144).

Prophylactic vaccination instead of therapy

Prophylactic treatment for prevention of allergic sensitization could be a new approach to stop the allergic pandemic (145). The difficulties of treating an already established allergy are obvious when considering the unsatisfactory efficacy of conventional immunotherapy (146). A prophylactic vaccination against allergy could prevent the development of allergy before initial sensitization has occurred and strong IgE responses and T cell priming have been established.
Especially children with a genetic predisposition for developing allergy might be are target candidates for prophylactic vaccination. Population studies indicate that allergic sensitization mainly occurs shortly after birth; as demonstrated by correlation of birth during pollen season and an increased development of pollinosis (147). Therefore, the optimal time window for prophylactic intervention seems to be prenatal period and/or shortly after birth (145).
Such a prenatal or postnatal applied "allergy vaccine" could induce "normal", non-IgE mediated, allergen-specific immune responses with IgG antibody production like common vaccinations against infectious diseases (146). It has been already shown that allergen-specific IgG induced via SIT in pregnant mothers is transmitted through the placenta or breast milk to the child (148). IgG antibody induction with hypoallergens or early tolerance induction via mucosal routes (e.g. oral, nasal) with T cell epitope-containing allergen peptides or recombinant hypoallergens, have been suggested as promising prophylactic tool in animal studies (145).

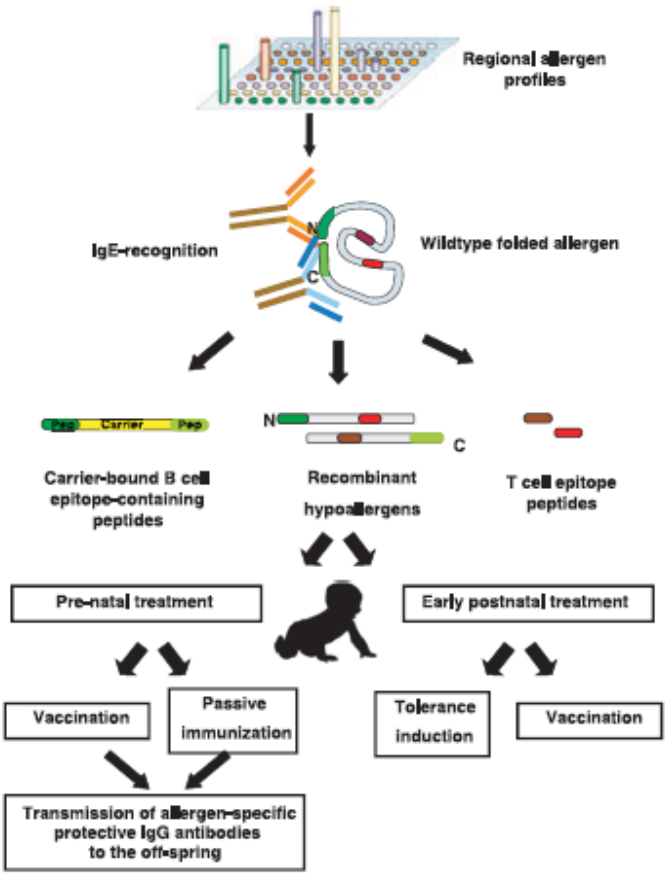

Fig.6: **Possible strategies for prophylactic SIT (145).**

3. Mucosal Immunity

3.1. Mucosal Immune System

The mucosal surfaces, covering the oral, respiratory, gastrointestinal and urogenital tracts, extend to an area of more than 400 m^2 in the human body (149), thereby harbouring 80% of all immunologically active cells within the mucus membrane (150).
The mucosal immune system is responsible for three main functions (151):

- to protect the mucosal surface against invasion and colonization by potentially dangerous microbes (151).
- to prevent the uptake of undegraded antigens including foreign proteins derived from ingested food, airborne material and commensal microorganisms (151).
- to prevent the development of potentially harmful immune responses to these antigens if they reach the interior of the body (151).

The mucosal immune response is based on the recirculation of immunocytes from the inductive sites to the effector sites within the whole mucosal lymphoid organ system has termed the system as “common mucosal immune system” (151, 152).

The **inductive sites** of the mucosal immune system consist of an organized mucosa-associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes (LN) (153). In more detail it includes the gut-associated lymphoid tissue (GALT) with the Peyer´s patches (PP), isolated lymphoid follicles (ILF), the mesenteric LNs, and the appendix; the genital-associated lymphoid tissue (GENALT), the eye-associated lymphoid tissue (EALT) composed of the conjunctiva-associated lymphoid tissue (CALT), the lacrimal duct-associated tissue (LDALT), and the eustachian tube-associated lymphoid tissue (TALT); the salivary gland- and duct-associated lymphoid tissue (SALT/SDALT), the larynx-associated lymphoid tissue (LALT), the bronchus-associated lymphoid tissue (BALT, which is only present in 40% of healthy individuals), and the nose-or nasopharynx-associated lymphoid tissue (NALT), as well as other mucosa-associated follicles (153-155). The latter lymphoid tissue of the nose is described as lymphoid tissue of the Waldeyer´s pharyngeal ring in humans, and comprises the pharyngeal lymphoid tissues such as the nasopharyngeal, tubal, palatine, and lingual tonsils

(153, 154). In rodents the equivalent of the Waldeyer´s ring are paired NALT structures located dorsally at the floor of the nasal cavity (153).

The mucosal **effector sites** on the other hand, are nonorganized lymphoid tissues including the lamina propria of various mucosae of the upper respiratory tract or the reproductive tract, secretory glandular tissues and surface epithelia (152, 153).

Mucosal immune responses

The immune responses at the mucosal surfaces depend on the nature of the antigen, the type of APCs which are involved, and the local microenvironment (151).
One of the first lines of defence of the mucosal immune system is mediated by epithelia and their associated glands (e.g. salivary glands); they defend the epithelial barrier by nonspecific or innate immune responses with production of mucins and antimicrobial proteins (156). The foreign antigens and microorganisms, which breach this barrier, are caught by dendritic cells (DCs) with different strategies adapted to the respective mucosal location (Fig.7). Either intra- or subepithelial DCs at the inductive site migrate into the narrow spaces between epithelial cells to “fish” antigens directly from the lumen. This might be the case at mucosal surfaces without lymphoid follicles and microfold (M) cells such as the bronchial or vaginal mucosae. The follicle-associated epithelium of the gut, NALT (tonsils, adenoids), EALT and SALT are equipped with M cells, which are specialized cells for endocytosis and transepithelial transport (155-157). APCs such as DCs, macrophages, B cells, and follicular DCs, are located in the pockets of these M cells to capture delivered antigens.

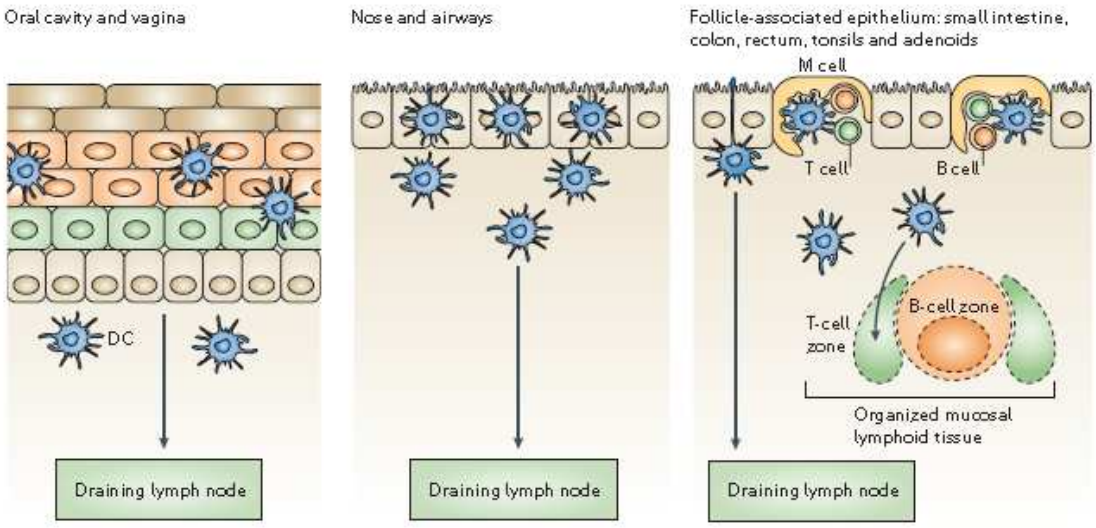

Fig.7: **Antigen capture at the mucosal surface** (156).

These loaded DCs migrate to the draining lymph nodes of the MALT, mature and present the antigen to naïve T cells (Fig.8). The activated T cells differentiate into helper T cells or Tregs, and interact with already antigen-primed B cells via CD40L/CD40 and secrete TGF-β to induce B cell class switching to IgA (1). These activated memory/effector T and B cells upregulate the expression of "homing receptors", which are tissue-specific adhesions molecules (e.g. selectins, integrins) and chemokine receptors that guide these lymphocytes back to the mucosal effector sites by interaction with endothelial counter receptors in the mucosal vasculature (156). These homing receptors can be expressed by epithelial cells throughout the whole mucosal immune system, and thus activated lymphocytes can be simultaneously attracted to all relevant MALTs (156). An example is the enteromammary axis, where cells secreting intestinal antigen-specific IgA migrate from the intestine to the mammary glands (150).
At the mucosal effector sites effector T cells and IgA secreting plasma cells leave the blood vessel and migrate to the mucosa (157).

The mucosal adaptive immune response is associated with a local production and secretion of dimeric and multimeric IgA by plasma cells at the mucosal surface (156). **Secretory IgA** (sIgA) is protease-resistant because of its dimerization, the high degree of glycosylation and its association with a glycosylated fragment called "secretory component" (J chain). This resistance is necessary to avoid its degradation during the transport through the protease-rich

external environments of the mucosa (156) (Fig.8). The dimeric IgA, is actively transported across the epithelial cells to the lumen which is mediated via the epithelial polymeric immunoglobulin receptor (pIgR) interacting with the secretory component (156). To prevent mucosal invasion sIgA promotes the occlusion of antigens in the mucus, induces immune exclusion by prevention of direct contact of pathogens with the mucosal surface, and block incoming pathogens within epithelial-cell vesicular compartments during pIgR-mediated transport (156). Additionally, sIgM and blocking IgG antibodies are produced locally and secreted in the lumen as well (157).

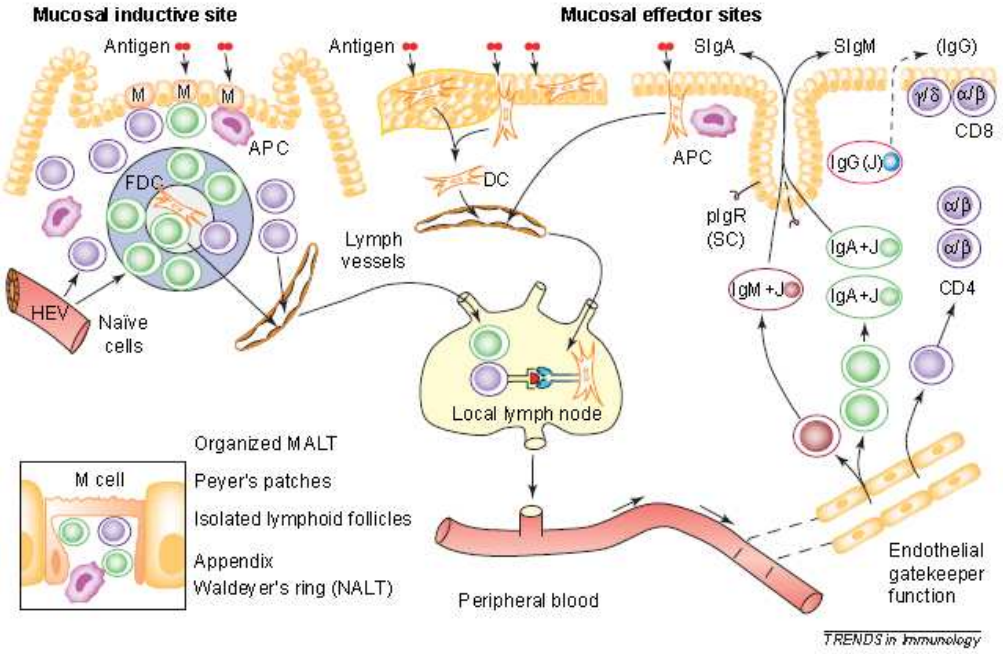

Fig.8: **Human mucosal immune system** (153).

3.2. Mucosal Tolerance

In 1911 Wells and Osborne defined the specific suppression of systemic cellular and/or humoral immune responses to an antigen that was first administered by the oral route, as “oral tolerance” (158-160). Due to the fact that this antigen-specific unresponsiveness can be induced by other mucosal routes such as nasal, sublingual, inhalative, rectal or genital as well, it is now usually referred to as mucosal tolerance (150).

Mucosal tolerance can be described as a form of peripheral tolerance, where mature lymphocytes in the peripheral lymphoid tissue are rendered incapable of responding to a foreign antigen (1, 161). Two major mechanisms responsible for tolerance induction are active suppression of immune responses mediated by induced regulatory T cells, and the initiation of clonal anergy (functional unresponsiveness) or deletion (cell death) of antigen-specific T cells (1, 160).

Based on these physiological mechanisms, mucosal tolerance induction has been suggested as a potent therapeutic strategy for immunologic diseases.

Key players of mucosal tolerance

The induction of regulatory cells via mucosal delivery of antigens is essential for the development of tolerance (151, 162). In mice, four main types of regulatory T cells have been distinguished.

The **antigen-induced $CD4^+$ Th2-like cells** counteract the differentiation of Th1 effector cells via IL-4 and IL-10 cytokine production. These Th2-like cells are mostly effective in suppressing Th1 cytokines IL-2 and IFN-γ thereby inhibiting inflammatory reactions, such as airway inflammation or diabetes (160).

$CD4^+$ $CD45RB^{low}$ $CD25^-$ $Foxp3^-$ type 1 regulatory T cells (**Tr1**) have a high suppressive potential by secretion the potent immunosuppressive, regulatory cytokine IL-10 (163). Tr1 cells are solely defined on the basis of their cytokine production profile - $IL\text{-}10^+$ $IL\text{-}4^-$ $IFN\text{-}\gamma^{low}$ - because of the currently unknown linage-defining transcription factor (164). Especially tolerance induction in the small intestine is mainly regulated by Tr1 cells as predominant producers of IL-10. It has been shown that deficiency of IL-10 in the intestinal mucosa leads to loss of tolerance (163).

The **$CD4^+$ $CD25^-$ Th3 cell** subset predominantly produces TGF-β, an immunoregulatory cytokine and important mediator of immunosuppression, cell differentiation and IgA class switching (160).

$CD4^+CD25^+$ $Foxp3^+$ regulatory T cells (**Treg**) can be subdivided into natural Tregs (*nTreg*) and inducible Tregs (*iTreg*). nTregs are produced during CD4 T cell differentiation in the thymus as a consequence of high-affinity interactions of the TCR with self-antigens (163). iTregs differentiate from naïve T cells to regulatory T cells in the secondary lymphoid tissues induced by antigen presentation and in presence of TGF-β. Treg cells modulate tolerance by cell-cell interaction via the APC-inactivating surface molecule CTLA-4 and/or secretion of IL-10 and TGF-β (162, 163).

Recent studies showed that B cells besides antibody production can play a regulatory role in tolerance induction as well. The two major effector functions of **regulatory B** cells are first the inhibition of antigen-specific proliferation and cytokine production of Th1 and Th2 cells, and second the induction and/or recruitment of $Foxp3^+$ Treg cells (165).
It has been shown that **$CD19^+$ $CD5^+$ TGF-β producing regulatory B cells (Br3)** appear to be involved in immune tolerance induction similarly to Th3 cells (166, 167). Moreover a negative regulator of tolerance was described with **$CD19^+$ $CD5^+$ Foxp3+ regulatory B cells (Breg**) (166, 168). A role of both, **$CD19^+$ $CD5^+$ IL-10 producing Br1** and Br3 cells in modulation of tolerance induction to allergens in non-IgE mediated food allergy was discussed recently (166, 167, 169).

Immunomodulation of the mucosal immune response

Mucosal application of antigens can have different effects on the immune response such as induction of systemic tolerance, systemic priming and induction of local secretory IgA production.
The main factors that determine which form of mucosal tolerance is developed, are the nature of the antigen, the antigen dose and the frequency of application (115).

Structure/nature of the antigen

Wiedermann et al. showed that the nature of the antigen influences the form of immune response. They compared mucosal immune responses of the inhalant allergen Bet v 1 (major birch pollen allergen) with the dietary antigen ovalbumin (OVA), both chemically coupled to the

mucosal adjuvant CTB (non-toxic B subunit of cholera toxin). They showed that the suppressive capacity of CTB depends on the nature of the antigen (170). Subsequent studies identified the type of linkage (chemically versus genetically conjugated) of the adjuvants as a relevant factor for modulation of mucosal immune responses.

Further studies of our lab showed successful induction of tolerance via mucosal application with whole allergen molecules (Bet v 1,from birch Hev b 1/3 from latex, Ves v 5 from wasp venom) (170-172), allergen-derived peptides (Bet v 1, Phl p 1/5 from grass) (122), fragments of allergens (Bet v 1) (119, 173) or genetically engineered allergen chimers (Bet v 1–Phl p 1–Phl p 5) (127).

We confirmed that the form of tolerance differs depending on the structure of the molecule. Mucosal tolerance induction with the Bet v 1 molecule that carries conformational IgE epitopes, (170-172) leads to a total suppression of Th2 as well as Th1 immune responses, accompanied by an upregulation of the regulatory markers TGF-β, IL-10 and Foxp3, mucosal application of the non-native hypoallergenic Bet v 1-fragment (half of the whole Bet v 1 protein including the immunodominant T cell epitope) induced tolerance regulated via Foxp3, but not TGF-β and IL-10 (173). Wild et al. described the phenomenon of *split tolerance* with mucosal IgA induction associated with TGF-β and IL-10-regulated systemic immunosuppression. Split tolerance was induced by mucosal application of a genetically engineered chimer composed of the whole Bet v 1 molecule and 2 immunodominant peptides of grass pollen allergens (127).

Dose and frequency of antigen application

It has been shown that the mechanisms of mucosal tolerance induction depend on the dose of antigen (174). This dose-dependent induction of systemic tolerance is termed *high-dose tolerance* and *low-dose tolerance*.

High-dose tolerance occurs after single application of a high dose of antigen (> 0,5 mg/g body weight in mice), which leads to anergy and/or deletion of antigen-specific T cells (174). Whereas anergy takes place in the absence of costimulation via CD80/CD86 and CD28 during APC-T cell interaction deletion occurs through Fas/Fas ligand-dependent inhibition leading to apoptosis (Fig.9).

Low-dose tolerance is achieved by multiple applications of low doses of antigen (< 0,1 mg/g body weight) which supports the induction of regulatory T cells and immunosuppressive cytokines (115, 150, 174, 175) (Fig.9). Interestingly, oral application of very

low doses of antigen (< 0,005mg/g body weight in mice) can lead to sensitization towards this antigen (150).

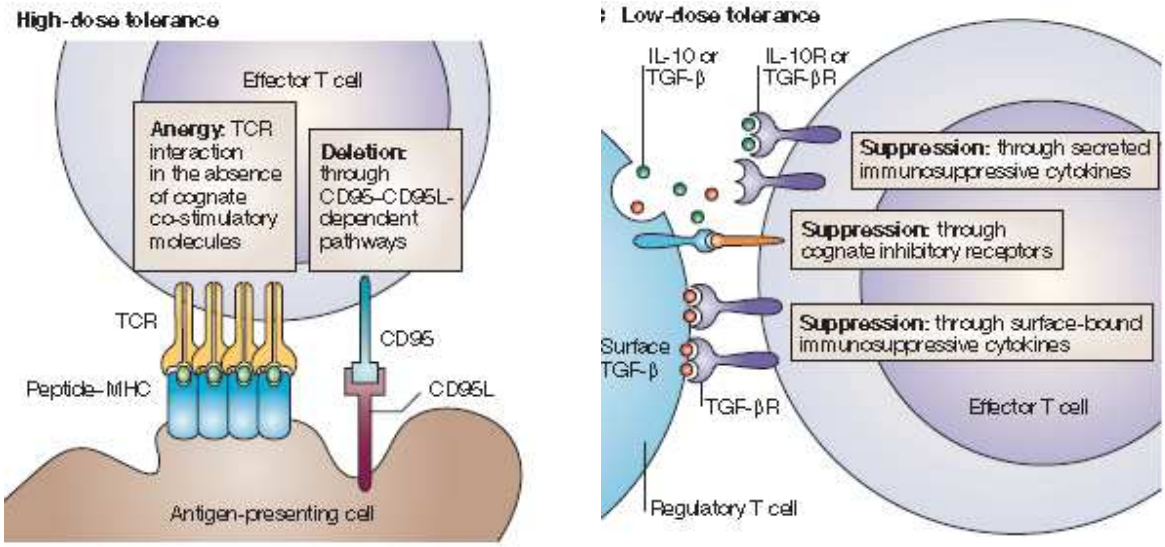

Fig.9: **Mechanisms of oral tolerance; high-dose and low-dose tolerance (174).**

Mucosal delivery systems

To improve tolerance induction via the mucosal route several mucosal delivery systems have been studied as promising candidates for mucosal vaccination; vectors based on attenuated pathogens such as *Bordetella pertussis*, *Salmonella typhi* or *S. paratyphi*; commensal bacteria such as lactobacilli, streptococci or staphylococci; bacterial proteins such as cell-binding subunit components of *E. coli* heat-labile enterotoxin and cholera toxin; and virus-like particles have been used (151).

These delivery systems implicate promise advantages:

- enhance the immunogenicity and uptake of antigens (138).
- protect the antigen from physical elimination or enzymatic digestion.
- target antigens to mucosal inductive sites (e.g. M cells).
- stimulate and modulate the antigen-induced immune response (151).

One of the best studied and most potent mucosal delivery systems is the recombinantly produced **nontoxic B subunit of cholera toxin** (**CTB**) The mucosal adjuvant CTB linked to allergens promotes mucosal immunity mainly by SIgA induction, and modulates anti-inflammatory tolerance to self-antigens or allergens (176). Interestingly, the immunomodulatory capacity of CTB depends on the nature of the antigen as well as on the type of coupling (170). The potent effectiveness of CTB as a mucosal adjuvant was confirmed in several animal studies (176-178). Clinical studies in individuals with Behcet disease revealed promising results for human use (151, 179).

We and others demonstrated the great potency of **lactic acid bacteria (LABs**) as mucosal delivery vehicles and adjuvant systems (138, 180, 181). LABs are known to modulate both mucosal and systemic immune responses by induction of secretory IgA as well as proinflammatory (IFN- γ) or regulatory (IL-10) cytokines inducing a shift towards a Th1 response (138, 180, 182). Genetically modified LABs represent an attractive vaccination approach. These LABs could transport expressed antigens directly to the inductive sites to induce mucosal tolerance (138, 180).

II. AIM OF THE THESIS

The aim of this thesis is to investigate new treatment strategies for primary and secondary prevention of type I allergy, with the main focus on mucosal tolerance induction. Therefore novel tools and approaches should be tested in murine models of mono- and poly-sensitization, to study the different immunological mechanisms of tolerance induction.

In order to characterize, prevent, or treat allergic disease, the allergens were planned to be identified, produced in recombinant form and tested in a suited animal model.
Indianmeal moths present a relevant source of indoor allergies. However, so far only one allergen, the arginine kinase Plo i 1, has been characterized and produced in recombinant form. The first aim of this thesis thus was to characterize the allergenicity of a novel Indianmeal moth allergen, the thioredoxin Plo i 2.
For this purpose, immunization studies with the **recombinant protein** Plo i 2 in a murine model of type 1 allergy should be performed according to published literature (171, 172, 183). Both humoral and cellular antigen-specific responses will be investigated *in vitro* and *in vivo*. The human IgE-reactivity should be tested by immunoblot analysis with sera of indoor allergic patients.

In order to improve the efficacy of treatment strategies for multi-sensitized patients, the second part in the thesis should be dedicated to the production of a **multi-allergen construct** for the prevention of birch pollen-related food allergy.
The immunodominant T cell epitopes of the Bet v 1 related food allergens Api g 1 (major celery allergen) and Dau c 1 (major carrot allergen) were identified by epitope mapping. A novel multi-allergen construct composed of the whole molecule of Bet v 1 (major birch pollen allergen) and the immunodominant T cell epitopes of Api g 1 and Dau c 1 should be genetically engineered. In order to test the tolerogenic potential of this construct, a model of birch pollen-related food allergy (BPRFA) was planned to be established. In this model, mice should be intranasally tolerized with the multi-allergen chimer or with a mixture of the recombinant allergens Bet v 1, Api g 1 and Dau c 1 in a prophylactic approach, followed by poly-sensitization with all 3 allergens and a sublingual challenge with birch pollen, celery and carrot extracts. Thereafter, the antigens-specific immune responses at the humoral and cellular level should be evaluated,

with a special focus on the mucosal immune response in the lymphoid tissues such as NALT, SLT and buccal mucosa.

Combined application of **mucosal adjuvants** with allergens during specific immunotherapy might increase the induction of mucosal tolerance. Thus, in the third part of the thesis, the recombinant birch pollen allergen Bet v 1 will be genetically linked to the mucosal adjuvant CTB, the nontoxic subunit of cholera toxin. This fusion protein CTB-Bet v 1 should be used for tolerance induction in a murine model of birch pollen allergy. The CTB-Bet v 1 construct was planned to be intranasally applied in Bet v 1-sensitized mice, to evaluate the parameters of systemic and mucosal immune responses by measuring of antibody production in sera as well as cellular immune responses. Real time RT-PCR studies should provide mechanistic insights.

The main focus of this work was to present different promising tools for treatment and prevention of allergic diseases.

III. PUBLICATIONS

Publication I

Arginine kinase and thioredoxin: Comparison of two allergens from the Indianmeal moth *Plodia interpunctella*

Elisabeth Hoflehner*, Marina Binder*, Wolfgang Hemmer, Raphael Panzani, Vera Mahler, Reinhart Jarisch, Ursula Wiedermann, Michael Duchêne

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* equally contributed

Contribution to publication I:
Performing of all in vivo animal studies; immunoblots inhibition experiments of human sera; analysis of western blot data and all in vivo data; writing of the manuscript in collaboration with Michael Duchêne.
70 percent.

Publication II

Prevention of Birch Pollen-related Food Allergy by Mucosal Treatment with Multi-Allergen-Chimers in Mice

Elisabeth Hoflehner, Karin Hufnagl, Irma Schabussova, Joanna Jasinska, Karin Hoffmann-Sommergruber, Barbara Bohle, Rick M. Maizels, and Ursula Wiedermann. *PLoS One*. 2012;7(6):e39409. Epub 2012 Jun 29.

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Construction, expression and purification of the multi-allergen chimer; performing of all experiments; analysis of data; writing of the manuscript.
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Publication III

Use of a genetic cholera toxin B subunit/allergen fusion molecule as mucosal delivery system with immunosuppressive activity against Th2 immune responses

Merima Bublin*, Elisabeth Hoflehner*, Birgit Wagner, Christian Radauer , Stefan Wagner , Karin Hufnagl , Dorothee Allwardt , Michael Kundi , Otto Scheiner , Ursula Wiedermann, Heimo Breiteneder.

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Performing of all in vivo animal studies; analysis of all in vivo data; writing of the manuscript in collaboration with Merima Bublin.
50 percent.

III.I PUBLICATION I

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Thioredoxin from the Indianmeal Moth *Plodia interpunctella*: Cloning and Test of the Allergenic Potential in Mice

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Abstract

Background/Objective: The Indianmeal moth Plodia interpunctella is a highly prevalent food pest in human dwellings, and has been shown to contain a number of allergens. So far, only one of these, the arginine kinase (Plo i 1) has been identified.

Objective: The aim of this study was to identify further allergens and characterise these in comparison to Plo i 1.

Method: A cDNA library from whole adult P. interpunctella was screened with the serum of a patient with indoor allergy and IgE to moths, and thioredoxin was identified as an IgE-binding protein. Recombinant thioredoxin was generated in E. coli, and tested together with Plo i 1 and whole moth extracts in IgE immunoblots against a large panel of indoor allergic patients' sera. BALB/c mice were immunised with recombinant thioredoxin and Plo i 1, and antibody production, mediator release from RBL cells, T-cell proliferation and cytokine production were measured.

Result: For the first time a thioredoxin from an animal species was identified as allergen. About 8% of the sera from patients with IgE against moth extracts reacted with recombinant P. interpunctella thioredoxin, compared to 25% reacting with recombinant Plo i 1. In immunised BALB/c mice, the recombinant allergens both induced classical Th2-biased immune responses such as induction IgE and IgG1 antibodies, upregulation of IL-5 and IL-4 and basophil degranulation.

Conclusion: Thioredoxin from moths like Plo i 1 acts like a classical Type I allergen as do the thioredoxins from wheat or corn. This clearly supports the pan-allergen nature of thioredoxin. The designation Plo i 2 is suggested for the new P. interpunctella allergen.

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Introduction

Arthropods represent more than three quarters of all animal species, and get into contact with humans in multiple ways. Arthropod antigens can cause Type I allergies with various manifestations, such as the acute and life-threatening insect venom allergies [1], perennial rhinitis, dermatitis and bronchial asthma caused by house dust mites [2] or cockroaches [3,4], as well as oral symptoms caused by seafood [5].

Although moths like cockroaches are typical and widespread household pests, there is much less research on Type I allergy against moth proteins. Historically, that moths cause inhalant allergies had been reported as early as 1928 by Vaughan [6]. In the following decades, there have been occasional case reports on bronchial asthma caused by moths, such as by the clothes moth Tineola bisselliella in an infested home [7] or the wax moth Galleria mellonella in a company producing fish bait [8]. More recently, IgE immunoblots of clothes moth [9] or silkworm moth [10] demonstrated specific IgE against moth antigens.

The Indianmeal moth Plodia interpunctella is a common household and stored product pest. Its larvae feed on dry foodstuffs such as grains, nuts, dried fruit, or chocolate. This pest had been suspected early to be a possible source of allergens in mills [11]. The first P. interpunctella allergen to be identified on the molecular level (Plo i 1) was an arginine kinase [12]. Arginine kinase (EC 2.7.3.3) phosphorylates arginine, whereas the related creatine kinase (EC 2.7.3.2) acts on creatine. Both enzymes thus help to store metabolic energy, where creatine kinase is typically found in vertebrates and arginine kinase is found in lower species

such as arthropods and some protozoa [13]. The arthropod arginine kinases exhibit IgE cross-reactivity [12], and several further arginine kinases have been identified as allergens, e.g. from shrimp [14,15] and mite [16].

IgE immunoblots of P. interpunctella whole larval extracts were positive in 51% when probed with sera from indoor allergic patients [12]. In this study we extend this work using extracts from adult moths. IgE screening of a cDNA library of the imago stage of P. interpunctella revealed a further allergen, a thioredoxin.

Thioredoxins are ubiquitous redox-active proteins found in eukaryotes and prokaryotes. They activate proteins by reduction of cysteine disulphide bonds [17,18]. Thioredoxins have been identified as allergens in wheat and maize [19] as well as fungi such as Malassezia spp. [20]. In human allergic bronchopulmonary aspergillosis, IgE cross-reactivity of fungal and human thioredoxin was found to result in IgE autoreactivity [21]. Recently, wheat flour thioredoxin was identified as an allergen in patients with baker's asthma [22].

In this study, the allergenic potential of thioredoxin as a new arthropod allergen was examined in comparison to Plo i 1 by IgE immunoblotting and in a mouse immunisation model.

Materials and Methods

Ethics statement and patient characteristics

The study was approved by the ethics committee of the Medical University of Vienna and the Vienna General Hospital (application EK Nr. 322/2008). The anonymised sera to be tested in immunoblots (n = 156, of which n = 154 were allergic patients' sera and n = 2 nonallergic control sera) are displayed in detail in Table 1.

The diagnosis of Type I allergy was based on case history and symptoms (rhinitis/conjunctivitis, allergic asthma), skin prick testing as well as CAP-RAST (Pharmacia, Uppsala, Sweden) testing using a panel of extracts from indoor (house dust mite, cat dander) and outdoor (birch pollen, grass pollen) allergen sources. The cDNA library from adult P. interpunctella was screened with the serum from an indoor-allergic patient with IgE reactivity to Indianmeal moths.

Construction of a P. interpunctella cDNA library from imagines

P. interpunctella larvae were left to hide for metamorphosis in a roll of cardboard with vertical holes. When the moths emerged, they were immediately frozen in liquid nitrogen. Four hundred moths (around 3.2 g) were homogenised in 30 ml of TRIzol reagent (Life Technologies, Frederick, MD), and about 10 mg of total RNA were extracted. Poly(A)$^+$ RNA was prepared using the Poly(A)Ttract system (Promega, Madison, WI, USA) with a yield of 4.9 mg from 4 mg of total RNA. The cDNA library prepared with the Uni-ZAP system (Stratagene, La Jolla, CA, USA) [23] contained 1.25×10^6 clones.

IgE screening of the lZAP cDNA library

lZAP phages (2.8×10^5) from the primary library were plated on Escherichia coli XL1-Blue (Stratagene). Synthesis of recombinant proteins was induced by adding nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) soaked in 10 mM isopropylthio b-D-galactoside (IPTG). The filters were blocked, and probed with a 1/10 dilution of a serum which contained IgE against several moth proteins. IgE bound to recombinant moth proteins was detected by ^{125}I-labeled anti-IgE antibodies (Pharmacia) followed by autoradiography. Further rounds of screening were then performed to obtain single phage clones.

The cDNA-containing plasmids were obtained from the isolated phages by in vivo excision [23]. Twenty two cDNAs were sequenced using Thermosequenase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and IRD800-labeled primers on a LI-COR sequencer (LI-COR, Lincoln, NE, USA). The deduced protein sequences were compared with the SwissProt database using the FastA program [24]. Nucleotide sequences were aligned with the ClustalW program [25].

Expression of P. interpunctella thioredoxin in E. coli

The oligodeoxynucleotide primers ExTrxF 59-GGG ACT TCC ATA TGT CGA TCC ACA TCA AAG ACG TTG AGG AC-39 and ExTrxR 59-TCC GCT CGA GTT AAT GAT GGT GAT GAT GAT GCT TGA GTT TCA AAA TGG TGG ACC G-39 were used for PCR to amplify the P. interpunctella thioredoxin coding sequence from the cDNA template (clone 1). The primers contain NdeI and XhoI restriction sites (bold) for cloning into the expression plasmid pET-17b (Novagen, Merck, Darmstadt, Germany) plus a hexahistidine-encoding sequence (underlined) for recombinant protein purification.

The recombinant pET-17b plasmid was transformed into E. coli BL21-CodonPlus (DE3) (Stratagene). The cultures were grown to an OD_{600} of 0.75, recombinant protein production was induced by addition of 1 mM IPTG and shaking overnight at 18uC.

Table 1. Demographics and symptoms of the groups of patients' sera examined in this study.

Symptomatic Type I allergy against	Abbre-viation in Fig. 1	Number of patients	Age range	Mean age	Female: male ratio	RC [%]	Asthma [%]	AD [%]
House dust mites but not animal dander	M	69	1–64	21.3	31 : 38	77	38	6
House dust mites and animal dander	M+D	43	9–61	30.0	16 : 27	74	49	12
Animal dander but not house dust mites	D2M	20	3–56	27.7	10 : 10	60	65	0
Seafood	SF	22	17–76	38.5	7 : 15	50	36	0
Nonallergic controls	NC	2	37–49	44.0	1 : 1	0	0	0
Total allergy patients		154	1–76	27.0	64 : 90	70	44	6
Total individuals		156	1–76	27.2	65 : 91			

The sera were from Floridsdorfer Allergiezentrum, Vienna, Austria. The last three colums give the percentage of patients reporting rhinitis and/or conjuctivitis (RC), asthma or asthma-like symptoms ("asthma"), or atopic dermatitis (AD).

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Harvesting of cells, lysis and purification of recombinant thioredoxin via metal chelate affinity chromatography under native conditions were performed according to the supplier's protocol (Qiagen, Hilden, Germany). Recombinant P. interpunctella arginine kinase (Plo i 1) was produced as described [12].

IgE-immunoblots of moth extracts and the recombinant allergens

Whole extracts from imagines of the Mediterranean flour moth (Ephestia kuehniella), a species closely related to P. interpunctella but considerably larger, were obtained by grinding of 150 moths with liquid nitrogen in a mortar. Fifteen ml of 1× reducing gel loading buffer were added, samples were denatured for 10 min at 95°C, and debris was removed by centrifugation. The whole extracts or, respectively, the purified recombinant allergens (arginine kinase (Plo i 1) and moth thioredoxin) were separated on preparative 12.5% SDS - polyacrylamide gels with an approximate protein concentration of 20 mg/cm (extracts) or 10 mg/cm (of each purified recombinant protein) as estimated by Coomassie blue-stained test gels. Proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell), and 5 mm strips were cut after the transfer. The strips were blocked 2×5 min and 1×30 min at room temperature with buffer G (42 mM Na_2HPO_4, 6.4 mM NaH_2PO_4, 0.5% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin, 0.05% (w/v) NaN_3, pH 7.5), and incubated with a 1/10 dilution of patients' sera in buffer G overnight at 4°C. After washing 2×5 min and 1×30 min in buffer G, bound IgE was detected by overnight incubation at room temperature with ^{125}I-labeled anti-IgE antibodies (IBL, Hamburg, Germany), washing as above, and autoradiography at −80°C.

Ethics statement on animal treatment and sampling

The animal study was performed according to institutional guidelines for animal use and care. The study was approved under the number GZ BMBWK-66.009/0246-BrGT/2005 by the Austrian Federal Ministry of Science and Research.

Female BALB/c mice (n = 5 per group, aged 7 weeks) were purchased from the Research Institute for Laboratory Animal Breeding (Himberg, Austria). Sensitisation was performed by four subcutaneous (s.c.) injections (day 0, 14, 28, 42) of 1 mg, 5 mg or 25 mg of Plo i 1 (arginine kinase) or Plo i 2 (thioredoxin) adsorbed to aluminium hydroxide ($Al(OH)_3$; Serva, Heidelberg, Germany). Control mice were sham-treated with PBS. Blood samples were taken before treatment (day 0), and 7 days after the last immunisation (day 49) by tail bleeding. At sacrifice cell suspensions from spleens were prepared as described [26].

Allergen-specific antibody levels in mouse sera

Microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 mg/ml of the recombinant allergens Plo i 1 or Plo i 2, respectively, and incubated with sera. Serum samples were diluted 1/500 for IgG2a, 1/1000 for IgG1 and 1/10 for IgE. Rat anti-mouse IgG2a, IgG1 and IgE antibodies (1/500, Pharmingen, San Diego, CA) were used, followed by peroxidase-conjugated mouse anti-rat IgG antibodies (1/2000, Jackson Immuno Lab, West Grove, PA) [26]. Data are shown as optical density (OD) after subtraction of baseline levels from pre-immune sera.

Rat basophil leukaemia cell mediator release assay (RBL assay)

Sera were incubated with RBL-2H3 cells (cell line from rat basophilic leukemia, available from ATCC, No. CRL-2256) at dilutions of 1/10. IgE-dependent degranulation of RBL cells was induced by adding 0.03 mg of each allergen (Plo i 1 or Plo i 2) diluted in 100 ml Tyrode's buffer. Supernatants were analysed for ß-hexosaminidase activity as described previously [27]. Data are reported as percen-tages of total ß-hexosaminidase released after addition of 1% Triton X-100 and are shown after subtraction of baseline release levels obtained with pre-immune sera.

Lymphocyte proliferation and cytokine production

Proliferation of splenocytes (2×10^5 cells/well) after stimulation with the recombinant allergens Plo i 1 or, respectively, Plo i 2 (2 mg/well) was measured as described previously [27]. The ratio of the mean proliferation after antigen stimulation (counts per minute [cpm]) and medium control values [cpm], i.e. the stimulation index [SI], was calculated.

Levels of IL-4, IL-5 and IFN-c were measured in spleen cell cultures (5×10^6 cells/well) incubated for 48 hours with Plo i 1 or Plo i 2 (10 mg/well) [26]. Cytokine levels were measured with mouse ELISA kits (Endogen, Woburn, MA) and were shown in pg/ml after subtraction of baseline levels of unstimulated cultures.

Statistics

Data are expressed as means ± SEMs. For statistical analysis p values > 0.05 were defined significant. Pair-wise comparison of allergen- sensitised versus sham-treated control groups was performed by using the non-parametric Kruskal–Wallis test, followed by ad hoc post analysis.

Results

Prevalence of IgE binding to moth extracts in sera from indoor-allergic patients

Previously we had shown that 51% of unselected patients with indoor allergies had serum IgE antibodies against P. interpunctella larval extracts. In the present study we tested 154 anonymised type I allergic patients' sera and two non-allergic control sera from Vienna (see Table 1). These comprised the following groups of patients: patients with indoor allergies to house dust mite (n = 69), to house dust mite and animal dander (n = 43), to animal dander but not house dust mite (n = 20), in addition seafood allergic patients (n = 22), and two non-allergic control sera.

Extracts from whole E. kuehniella moths, flour moths closely related to P. interpunctella, but yielding significantly more extract per animal, were used to prepare immunoblot strips. These strips were used to probe all the sera for IgE antibodies against moth antigens. The results are shown in Fig. 1 (top parts). A low IgE reactivity, which we interpret as weak binding of the moth antigens to the secondary antibody, was observed in the sera from nonallergic individuals (NC) and the buffer controls (BC) and is always taken into account. In the indoor allergic patient groups, 48% the sera reacted significantly with moth allergens contained in the whole extract, similar to the 51% found previously for P. interpunctella larval antigens [12]. This varied from 43% in the group of indoor allergic patients with mite allergy only (M) to 53% in the patients with allergies to mites and animal dander (M+D). In the group with indoor allergy to animal dander but not to mites (D≥M), 50% reacted with moth allergens. Although the frequency of IgE recognition did not vary so much between the groups, a striking difference was observed in the quantity of moth specific IgE. In a number of the mite allergic and moth IgE-positive patients, and even more in the mite and dander allergic and moth IgE-positive patients, very high moth specific IgE levels were observed. None of the 20 patients tested negative for mites (D≥M) showed such a high level of reactivity. The binding of IgE to moth antigens was highest, however, in the group of seafood allergic patients (SF), in

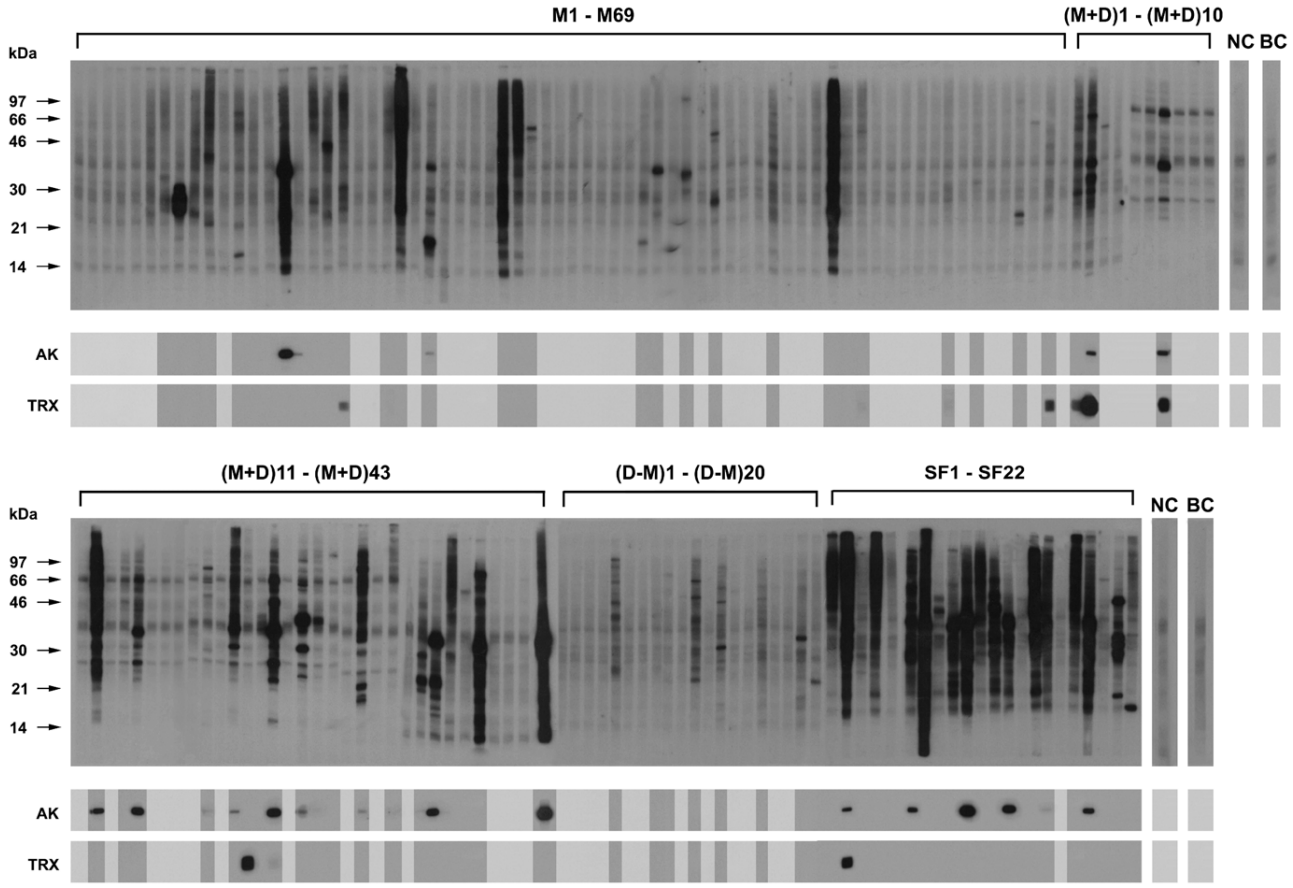

Figure 1. IgE immunoblots with patients' sera. Top panels: extracts from whole mediterranean flour moths (E. kuehniella) were separated by polyacrylamide electrophoresis, blotted onto nitrocellulose, and probed with the sera from various groups of patients with indoor allergy or seafood allergy as well as from control individuals. The indoor allergic patient groups were: M, housedust mite allergic patients (n = 69), M+D, patients allergic to mites and animal dander (n = 43), D2M, patients with allergy to animal dander but not to mites (n = 20). Patients SF, seafood allergic patients (n = 22), NC, nonallergic control individuals (n = 2), BC, buffer control without serum, only secondary anti-IgE antibody. Molecular weights [kDa] are indicated at the left side. Bottom panels, the recombinant P. interpunctella 40 kDa arginine kinase (AK) and 12 kDa thioredoxin (TRX) were probed with those patients' sera containing IgE to E. kuehniella, and only the parts of the strips containing the two recombinant allergens are shown.
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relative numbers, 95% of the sera were positive, and 55% of these sera contained very high levels of levels of IgE binding to moth antigens.

Taken together, immunoreactive bands of various molecular weights were observed on the blots and in order to identify more moth allergens, a moth cDNA library was constructed and screened.

Cloning of P. interpunctella thioredoxin as an IgE-binding protein from adult moths

As described above, a λZAP cDNA library from P. interpunctella imagines was probed with the serum from a patient with indoor allergies and IgE binding to several moth antigens. The primary screen gave 40 strong and 10 weak positive signals. After reprobing, 22 single cDNA clones were obtained and sequenced. Twenty of these encoded a P. interpunctella protein with significant sequence similarity to thioredoxins. Three of the 20 clones contained single base insertions and deletions resulting in truncation of the open reading frame. The other 17 clones comprised the identical open reading frame encoding a protein of 11.7 kDa. The 39-untranslated region of 689 bp between the stop codon and the poly-A tail was rather long. Minor differences were obtained in this region, but these did not allow to group the cDNAs into possible isoforms. The complete sequence of clone 1 was submitted to the EMBL/GenBank/DDBJ databases (accession number FR681573).

The BlastP search clearly identified the protein as thioredoxin. Fig. 2 shows P. interpunctella thioredoxin (Pi) aligned with selected other thioredoxins. The most closely related sequence with 83% amino acid identity was from the domestic silkworm Bombyx mori (Bm). The thioredoxin from the more distantly related arthropod Litopenaeus vannamei (Lv) and human thioredoxin (Hs) were 58% and 46% identical, respectively, to P. interpunctella thioredoxin. The allergenic fungal thioredoxin (2J23_A) Mala s 13 from Malassezia sympodialis (Ms) and wheat (Triticum aestivum) thioredoxin Tri a 25 (Ta) (CAB96931) were 46% and 41% identical. All the sequences contained the active site consensus WCGPC with the two redox-active cysteines.

We obtained only two other IgE-positive cDNAs from the same screen not coding for thioredoxin. The delicate extraction of RNA from adult moth material, a possible advantage of λZAP phage expressing thioredoxin leading to a preponderance in the composition of the plated library, or a peculiarity of the patient's serum used for screening may contribute to this finding. We did

```
Pi                  MSIHIKDVEDLTARLTEA  GDKLVVIDFMATWCGPCKIIGPKL 42
Bm                  -------SD--KT--A--  G-----------------M----- 42
Lv                  -VYQV--Q--S-KQ-N--  GN-------Y--------M-A--- 42
Hs                  -VKQ-ESKTAFQEA-DA-  G-----V--S--------M-K-FF 42
Ms    MRGSHHHHHHLVPRG-VQVISSY-QFKQV-G   G--V-----W--------M---VF 55
Ta  MAASAATATAAAVGAGEV-SVHSL-QW-MQIE--NAAK-------T-S-----R-MA-IF 60

Pi  DEIA AEMADSIVVVKVDVDECEDIATEYSINTMPTFVFVKNGKPVEQFSGANVEKLRST 101
Bm  ---- ---S-----------------S--N--S-----------KLDE------D--KT- 101
Lv  E-LS QS-S- V-FL-----------QDNQ-AC----L-M---QKLDSL-----D--LEL 100
Hs  HSLS EKYSN VIFLE----D-Q-V-S-CEVKC----Q-F-K-QK-GE-----K---EA- 100
Ms  EK-SDTPAG-KVGFY------QSQ--Q-VG-RA------F---QKIDTVV--DPS--QAA 115
Ta  ADL-  KKFPAA-FL------LKS--EQF-VEA----L-M-E-DVKDRVV--IK-E-TNK 118

Pi  ILKLK   106
Bm  ---H-   106
Lv  VE-N-   105
Hs  -NE-V   105
Ms  -TQHSA  121
Ta  VGLHAAQ 125
```

Figure 2. Comparison of thioredoxin sequences. The deduced amino acid sequence of P. interpunctella thioredoxin Plo i 2 (Pi) was aligned with Bombyx mori thioredoxin (ABM92269) (Bm) with 83% identity. The other arthropod thioredoxin from the shrimp Litopenaeus vannamei (ACA60746) (Lv) was 58% identical, and human thioredoxin (NP_003320) (Hs) shared 47% amino acid identity with Pi. The allergenic fungal thioredoxin (2J23_A) Mala s 13 from Malassezia sympodialis (Ms) and wheat (Triticum aestivum) thioredoxin Tri a 25 (Ta) (CAB96931) were 46% and 41% identical, respectively with Plo i 2. The residues equal to Plo i 2 are marked with dashes. The important thioredoxin consensus sequence WCGPC with the two active site cysteines was found in all the sequences (bold type).
doi:10.1371/journal.pone.0042026.g002

not investigate this matter in more detail and focused on the characterisation of thioredoxin as a novel allergen from moths.

Prevalence of IgE binding to recombinant P. interpunctella thioredoxin and arginine kinase (Plo i 1) in sera from indoor-allergic patients

Recombinant thioredoxin (11.7 kDa) and arginine kinase, Plo i 1, (39.9 kDa) [12] from P. interpunctella were separated together under denaturing and reducing conditions in SDS-PAGE, blotted onto nitrocellulose, and strips were probed with the sera that had shown positive reactivity with the whole moth extracts. The signals from the recombinant allergens arginine kinase Plo i 1 and thioredoxin are displayed in Fig. 1 as two blocks AK (40 kDa) and TRX (12 kDa) mounted below the whole moth extract strips.

Counting only the clearly positive signals, seven patients' sera had IgE towards P. interpunctella thioredoxin, six in the various indoor allergic groups and one in the seafood allergic group, but none in mite negative group. This corresponds to 8% of all moth extract positive patients, 5% of all allergic patients of this study. Again the strongest signals were observed in patients with concomitant mite allergy or concomitant mite and dander allergy, and in the seafood allergic patients. Due to this small but significant number of sera with IgE to moth thioredoxin, we proposed to name this allergen Plo i 2, and it is now available in the IUIS allergen nomenclature database as Plo i 2.0101.

In contrast to the Plo i 1 homolog, we noted the absence of 12 kDa bands of the natural thioredoxin in the blots from the whole moth extracts. This could have several causes, for example, the concentration of thioredoxin could simply be low, or as the immunoblots for whole moth extracts were not optimised for small proteins, some thioredoxin might have been lost during blotting. Alternatively, thioredoxin still bound covalently to its interaction partners [17,18] could migrate at higher molecular weights. Finally, the patients' IgE could possibly recognise P. interpunctella thioredoxin better than the E. kuehniella homolog. To investigate this cross-reactivity, we performed an inhibition experiment as

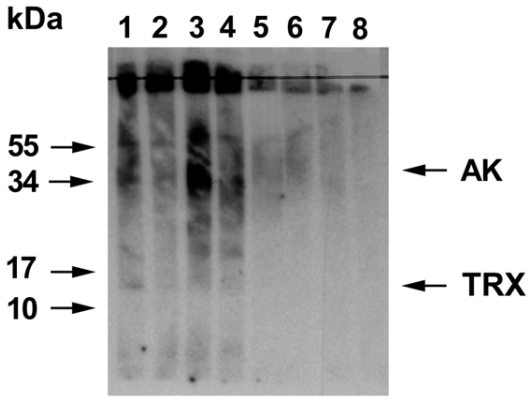

Figure 3. Immunoblot inhibition experiment. Extracts from E. kuehniella were separated by polyacrylamide electrophoresis, blotted onto nitrocellulose, and strips were probed with serum samples preincubated with or without 10 mg of recombinant allergen overnight. Lane 1, thioredoxin (Plo i 2) positive serum; lane 2, same serum preincubated with recombinant P. interpunctella thioredoxin; lane 3, arginine kinase (Plo i 1) positive serum; lane 4, same serum preincubated with recombinant Plo i 1; lane 5, nonallergic control serum, lane 6, same serum preincubated with Plo i 2; lane 7, same serum preincubated with Plo i 1; lane 8, buffer control. Molecular weights [kDa] are indicated at the left side, the positions of thioredoxin (TRX) and arginine kinase (AK) at the right side.
doi:10.1371/journal.pone.0042026.g003

done previously [12] (Fig. 3). Two 1 ml samples of a 1:10 dilution of a thioredoxin positive serum in buffer G (see Materials and Methods) were preincubated with or without 10 mg of recombinant P. interpunctella thioredoxin overnight at 4uC, and then used to probe strips of blotted E. kuehniella antigens as described above (Fig. 3, lanes 1, 2). Similarly an inhibition was carried out with a Plo i 1 positive serum and recombinant Plo i 1 (Fig. 3, lanes 3, 4) or a nonallergic control serum (Fig. 3, lanes 5–7), here lane 5 shows the uninhibited serum, lane 6 was preincubated with recombinant thioredoxin and lane 7 with recombinant arginine kinase. Lane 8 represents the buffer control. One important result of the experiment was that the 12 kDa thioredoxin band only appeared after the blot was exposed for 14 days instead of two days. This shows that the extracts contained only a low concentration of thioredoxin and explain the absence of signals in the previous blots. With the help of specific antibodies against recombinant Plo i 2, it should be possible to better quantify the allergen in the extracts and to check if it forms covalent adducts of larger molecular weight with its interaction partners.

The main result of the inhibition experiment was that recombinant Plo i 2 was able to block the binding of the patient's IgE to the E. kuehniella thioredoxin (Fig. 3, lane 2) as well as recombinant Plo i 1 blocked the binding to E. kuehiella arginine kinase (Fig. 3, lane 4). So the allergens from the closely related moths are cross-reactive.

The immunoblot experiment with the recombinant arginine kinase (Fig. 1, lower panels) showed that 21 of all the patients with IgE to moth extracts (25%) had specific IgE to Plo i 1, and again

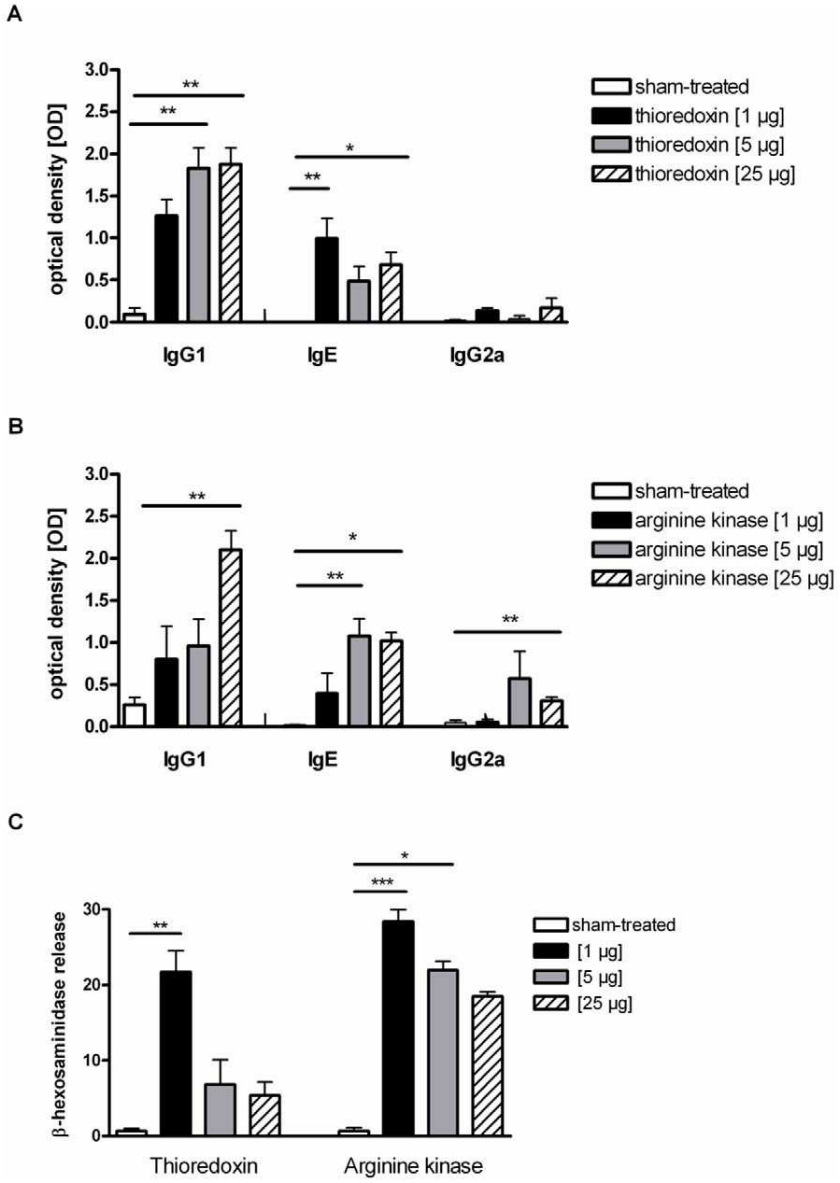

Figure 4. Antibody levels in mouse sera. (A), thioredoxin, (B), arginine kinase-specific antibody responses in sera after immunisation with three different antigen concentrations (1 mg, 5 mg, 25 mg) measured by ELISA; (C), IgE-mediated basophil degranulation in sera, b- hexosaminidase release from thioredoxin- and arginine kinase-sensitised mice, treated with 1 mg (black bars), 5 mg (grey bars) or 25 mg (striped bars), compared to sham-treated controls (white bars). *p ¸ 0.05, **p ¸ 0.01, ***p ¸ 0.005.
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the groups with mite, mite and dander, and seafood allergies had the highest prevalence and displayed the strongest signals, no Plo i 1 positive reactions were detected in any mite negative patient. The prevalence of IgE to Plo i 1 is the same as the 25% frequency of recognition of Plo i 1 in moth larval extract positive patients observed previously [12].

Antibody production in sensitised mice

To investigate the immunogenicity of thioredoxin (Plo i 2) and arginine kinase (Plo i 1) we sensitised mice four times subcutaneously with three different concentrations of the recombinant allergens (1 mg, 5 mg, 25 mg). Treatment with thioredoxin induced strong Th2 responses with increased IgG1 levels for all three concentrations, whereas treatment with 5 mg and 25 mg showed significantly increased antibody levels in comparison to sham-treated mice. IgE antibody production in thioredoxin-treated mice was significantly upregulated after sensitisation with the lowest concentration of 1 mg and with the highest concentration of 25 mg allergen, respectively, compared to controls. The typical Th1-associated IgG2a was only enhanced in mice, treated with 1 mg and 25 mg thioredoxin (Fig. 4A).

Arginine kinase-specific IgG1 was induced with all three concentrations, but only treatment with 25 mg showed a significantly increased antibody production, compared to controls. Whereas arginine kinase-specific IgE was significantly induced with a concentration of 5 mg as well as 25 mg, in comparison to sham-treated mice, IgG2a production was significantly increased in 25 mg- arginine kinase-treated mice (Fig. 4B).

For thioredoxin, IgE-dependent basophil degranulation was induced with all three concentrations, significantly increased only in 1 mg-sensitised group in comparison to controls. Similarly, immunisation with 1 mg and 5 mg arginine kinase induced significantly high IgE-depended basophil degranulation compared to controls (Fig. 4C).

Cytokine production in sensitised mice

Both arginine kinase and thioredoxin induced strong cellular responses with high IL-5 and IL-4 levels in restimulated spleen cell cultures, while IFN-c was induced only by arginine kinase. Th2 cytokine production in splenocytes of thioredoxin-treated mice showed significantly induced IL-5 levels in 1 mg-sensitised groups, compared to sham-treated mice. Thioredoxin-specific IL-4 was increased in all treated groups, in comparison to controls. Thioredoxin did not induce IFN-c (Fig. 5A). Arginine kinase showed similar high IL-5 levels for all three sensitisation concentrations like thioredoxin. In particular, application of 5 mg arginine kinase showed the highest IL-5 and IL-4 production. Sensitisation with 5 mg or 25 mg arginine kinase induced significantly increased IL-4 levels compared to sham-treated group. Moreover, a low production of IFN-c was measured in arginine kinase-treated mice, which reached significant IFN-c levels in 1 mg and 5 mg treated mice compared to controls (Fig. 5B).

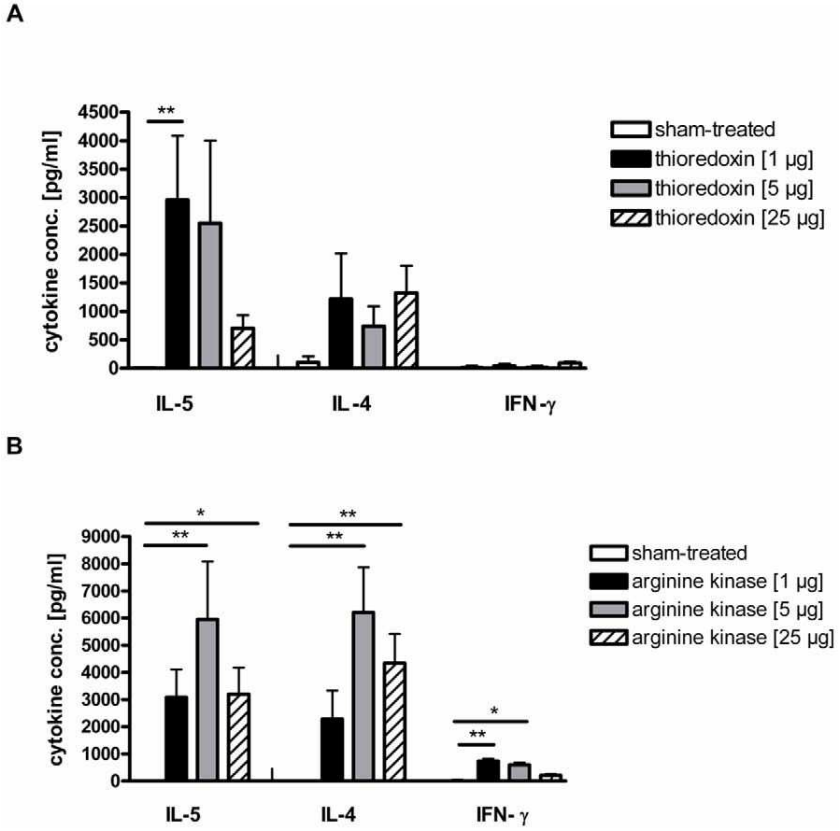

Figure 5. Cellular immune responses. IL-5, IL-4 and IFN-c levels in supernatants of restimulated spleen cell cultures of mice, treated with 1 mg (black bars), 5 mg (grey bars) or 25 mg (striped bars) of (A), thioredoxin or (B), arginine kinase, or sham-treated (white bars). Groups were compared to sham-treated controls. *p ¸ 0.05, **p ¸ 0.01.
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Proliferative response of stimulated lymphocytes

Thioredoxin restimulated splenocytes showed an increased proliferation response (SI: 4) for all 3 treatment groups. Immunisation with 25 mg thioredoxin provoked a significantly induced proliferation compared to sham-treated mice.

Arginine kinase-sensitised mice showed cellular responses only in the group with 1 mg treatment concentration, not with 5 mg or 25 mg, in comparison to controls (Fig. 6).

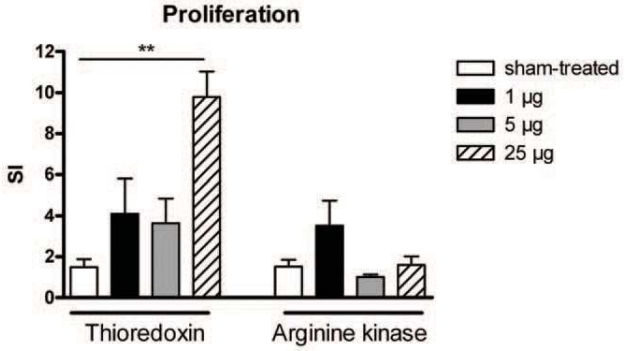

Figure 6. In vitro splenocyte stimulation. Stimulation index [SI] of thioredoxin or arginine kinase restimulated spleen cell cultures of mice, treated with 1 mg (black bars), 5 mg (grey bars) or 25 mg (striped bars) antigen, compared to sham-treated controls (white bars). **p , 0.01.
doi:10.1371/journal.pone.0042026.g006

Discussion

The IgE immunoblot experiments probing indoor allergic patients' sera with extracts from adult moths confirmed previous results on larval extracts [12] and showed IgE positivity in 48% of the sera of indoor allergic patients, therefore moths have to be considered a relevant source of allergens.

The complex and only partially resolved immunoblot band patterns show that there are numerous moth IgE antigens. Although many patients without mite allergy have IgE against moth components, the in vitro reactivity is weaker than in mite positive patients. As some of these individuals may bind to cross-reactive carbohydrate determinants (CCDs) in the moth extracts, we do not know at this time how much mite-independent moth allergy could exist. Certainly, the allergens cross-reactive between moth and mite may contribute to this enhanced IgE reactivity. An additional phenomenon is that the mite and animal dander allergic patients together with the seafood allergic patients show the highest levels of IgE binding to moths. It appears as if these polysensitised patients show a stronger atopic predisposition and higher IgE levels. So far we do not know what is the contribution of moth allergens in the sensitisation of these patients.

IgE screening of a P. interpunctella cDNA library produced a number of immunopositive clones mostly encoding thioredoxin. Testing the moth positive sera against recombinant thioredoxin gave a positive reaction in 5% of all examined allergic patients, 8% of moth positive patients, whereas recombinant Plo i 1 tested positive in 14% of all sera, 25% of moth positive sera. The data show that thioredoxin (now named Plo i 2) is a minor allergen, however, the majority of those with positive reactivity showed a high level of thioredoxin-specific IgE. The prevalence of thioredoxin reactivity was highest in the group of indoor allergic patients reacting with mites and animal dander.

Recombinant thioredoxin like arginine kinase induced a Th2-biased immune response with significantly enhanced IgE and IgG1 antibody levels at the humoral site as well as a marked upregulation of IL-5 and IL-4 cytokines on the cellular site. Moreover, significantly enhanced IgE-mediated basophil degranulation with even 1 mg allergen confirmed the clinical relevant Th2-biased immune response of thioredoxin as well.

Thioredoxin is a truly ubiquitous protein with pleiotropic activities found both in eukaryotes and prokaryotes. It had been identified as an allergen in wheat and maize [19], in fungi [20,21], and in this study for the first time in a representative from the animal kingdom strengthening its denomination as a member of a novel pan-allergen family [20] like the profilins [28].

Finally, we have noted that under certain conditions thioredoxins may also diminish the allergic response [29]. Treatment of commercial wheat allergen extract with reduced wheat thioredoxin, thioredoxin reductase, and NADPH reduced its allergenic potency, measured in a dog skin test model [30]. Similarly, thioredoxin reduced the allergenicity of b-lactoglobulin, the major bovine milk allergen [31]. In comparison to wild-type mice, transgenic mice overexpressing human thioredoxin showed a diminished mast cell histamine release in response to 2,4-dinitrophenylated bovine serum albumin [32]. Apoptosis can be induced in human bronchial epithelial cells by house dust mite-stimulated eosinophils. This effect is weaker when the epithelial cells overexpress human thioredoxin [33]. Further studies will have to show whether moth thioredoxin might also have any moderating activity on allergic responses similar to those reported for wheat and human thioredoxins.

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Author Contributions

Conceived and designed the experiments: EH MB UW MD. Performed the experiments: EH MB. Analyzed the data: EH MB WH VM RP RJ UW MD. Contributed reagents/materials/analysis tools: WH VM RP RJ. Wrote the paper: EH MD. Wrote applications for the ethics committees: UW MD.

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III.II PUBLIKATION II

Prevention of birch pollen-related food allergy by mucosal treatment with multi-allergen-chimers in mice

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Prevention of Birch Pollen-Related Food Allergy by Mucosal Treatment with Multi-Allergen-Chimers in Mice

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Abstract

Background: Among birch pollen allergic patients up to 70% develop allergic reactions to Bet v 1-homologue food allergens such as Api g 1 (celery) or Dau c 1 (carrot), termed as birch pollen-related food allergy. In most cases, specific immunotherapy with birch pollen extracts does not reduce allergic symptoms to the homologue food allergens. We therefore genetically engineered a multi-allergen chimer and tested if mucosal treatment with this construct could represent a novel approach for prevention of birch pollen-related food allergy.

Methodology: BALB/c mice were poly-sensitized with a mixture of Bet v 1, Api g 1 and Dau c 1 followed by a sublingual challenge with carrot, celery and birch pollen extracts. For prevention of allergy sensitization an allergen chimer composed of immunodominant T cell epitopes of Api g 1 and Dau c 1 linked to the whole Bet v 1 allergen, was intranasally applied prior to sensitization.

Results: Intranasal pretreatment with the allergen chimer led to significantly decreased antigen-specific IgE-dependent b-hexosaminidase release, but enhanced allergen-specific IgG2a and IgA antibodies. Accordingly, IL-4 levels in spleen cell cultures and IL-5 levels in restimulated spleen and cervical lymph node cell cultures were markedly reduced, while IFN-c levels were increased. Immunomodulation was associated with increased IL-10, TGF-b and Foxp3 mRNA levels in NALT and Foxp3 in oral mucosal tissues. Treatment with anti-TGF-b, anti-IL10R or anti-CD25 antibodies abrogated the suppression of allergic responses induced by the chimer.

Conclusion: Our results indicate that mucosal application of the allergen chimer led to decreased Th2 immune responses against Bet v 1 and its homologue food allergens Api g 1 and Dau c 1 by regulatory and Th1-biased immune responses. These data suggest that mucosal treatment with a multi-allergen vaccine could be a promising treatment strategy to prevent birch pollen-related food allergy.

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Introduction

One of the most common type I pollionosis is caused by the airborne allergens of birch pollen (BP). In Europe, more than 70% of BP-allergic patients develop an immediate hypersensitivity reaction against pollen-related food allergens, termed as birch pollen-related food allergy (BPRFA) and clinically manifested as oral allergy syndrome (OAS). IgE antibodies specific for Bet v 1, the major BP allergen, cross-react with epitopes of homologous food allergens such as Mald1 (apple), Cora1 (hazelnut), Api g 1 (celery), or Dau c 1 (carrot) [1,2]. Due to this cross-reactivity, Bet v 1-specific IgE can induce hypersensitivity reactions towards these food allergens. The symptoms of the BPRFA are usually restricted to the oral cavity and can range from swelling and itching of lips, tongue, soft palate and pharynx to systemic reactions such as urticaria, asthma or even anaphylaxis [3,4]. Most of these patients also display food induced symptoms outside the BP season, indicating that homologous food allergens provide a perennial boost of BP-specific immune responses [5].

For BP mono-sensitized individuals common specific immunotherapy (SIT) is well established and is regarded as a successful therapy. However, for treatment of patients with multiple sensitivities or BPRFA, SIT has low efficacy and is associated with an increased risk of anaphylactic side-effects [1,6,7]. Improving this treatment could either be achieved by the application of well defined recombinant single allergens or a mixture thereof, or allergen peptides according to the patient T cell recognition pattern. Additionally, exploiting different routes of vaccination, e.g. changing the subcutaneous to a less invasive administration via the mucosa (i.e. oral, nasal, sublingual) could improve the efficacy of this treatment [8].

We previously demonstrated that mucosal administration of recombinant allergens prevented allergic sensitization in mono-sensitized mice [9]. In poly-sensitized mice, however, application of a mixture of recombinant antigens did not efficiently elicit protective effects [8,10]. More recently, we demonstrated that mucosal application of either a multi-peptide construct, covering the immunodominant T cell epitopes of the major birch and grass pollen allergens, or a multi-allergen chimer, consisting of the scaffold allergen Bet v 1 in its native conformation anchoring two or more immunodominant peptides from major grass pollen allergens, prevented multi-sensitization against these allergens [8,10].

In the current study we established a model of BPRFA in poly-sensitized mice to validate the protective effects of mucosal treatment with a respective chimer. For this purpose we designed a pollen-food-allergen chimer consisting of Bet v 1, acting as a potent tolerogen, fused with additional immunodominant peptides of its homologous food allergens Api g 1 from celery and Dau c 1 from carrot. Our data provide evidence for the efficacy and underlying mechanisms of mucosal treatment with this chimer in preventing local and systemic Th2 immune responses in poly-sensitized mice.

Methods

Animals

Female 7-week-old BALB/c mice (n = 12 per group) were obtained from Charles River (Sulzfeld, Germany). All experiments were repeated 3 times.

Ethics Statement

The animal studies were performed according to institutional guidelines for animal use and care. The study was approved by the Animal Experimentation Ethics Committee of the Medical University of Vienna and the Ministry of Science and Research (GZ 66.009/229-BrGT/2005; GZ 66.009/35-II/10b/2010).

Antigens and Antibodies

Recombinant Bet v 1.010, Api g 1.0101 and Dau c 1.0103 were obtained from Biomay AG (Vienna, Austria). Birch pollen (BP) from Betula verrucosa was purchased from Allergon (Välinge, Sweden), and protein extracts of BP, celery (Apium graveolens) and carrot (Daucus carota) were prepared as previously described [11,12].

Anti-TGF-b, anti-IL-10R and anti-CD25 blocking antibodies were produced in house at the University of Edinburgh, UK (and provided by R. Maizels). Rat IgG isotype control antibody was used (Sigma-Aldrich).

Epitope Mapping Studies

For T cell epitope mapping a panel of 48 peptides of Api g 1 and 48 peptides of Dau c 1 were used (provided by B. Bohle). Spleen cell suspensions from Api g 1 or Dau c 1 immunized mice were incubated with 5 mg/well of each of the peptides, which overlapped for three amino acids (neighbours sharing nine residues) spanning the whole amino acid sequence of the respective antigens. Proliferative responses were measured according to previous description [12].

Construction of the Birch Pollen–food–chimer Expression Plasmid

Complementary templates from Api g 1 (Genbank Access number: Z48967) and Dau c 1 (Genbank Access number: Z84376) respectively, were used to amplify the identified immunodominant encoding regions by PCR (cDNA templates were provided by K. Hoffmann-Sommergruber). Api g 1-T cell epitope specific primers were designed including NcoI and EcoRI restriction sites (Api g 1 fwd 59-CATGCCATGGATGGAGTTAACAAGGAG-39, Api g 1 rev 59-ATGAATTCAACATGGTTTTCAATGGA-39; restriction sites underlined); Dau c 1-T cell epitope specific primers were designed including HindIII and XhoI restriction sites (Dau c 1 fwd 59-ACCAAGCTTGCCGTGGTTCCTGAAGAG-39, Dau c 1 rev 59-CCG CTCGAGTTAATTAGCAATGAGGTAGGC-39).

For construction of the Api g 1-Bet v 1-Dau c 1-chimer we ligated the PCR amplicons of Api g 1 and Dau c 1 into the respective restriction sites on the 59- and 39-end of a pHis Parallel 2 - Bet v 1 plasmid, equipped with a hexahistidyl (6×His) affinity tag as previously described [8]. Subsequent DNA sequence analysis (GATC Biotech, Konstanz, Germany) verified correct sequences and the integrity of open reading frame.

Expression, Purification and Refolding of Recombinant Api g 1-Bet v 1-Dau c 1-chimer

The Api g 1-Bet v 1-Dau c 1-expression plasmid was transformed into electrocompetent BL 21(DE3)plysS E. coli cells (Invitrogen, NV Leek, Netherlands) and grown in LB/1 mM ampicillin medium at 37uC under vigorous shaking until an optical density (OD_{600}) of 0.7. Expression was induced by adding 0.001 mol/L isopropyl b-D-thiogalactopyranoside (IPTG) and incubation continued for additional 3 hours before harvesting.

6×His-tagged r Api g 1-Bet v 1-Dau c 1-chimeric protein was produced in inclusion bodies and therefore purified from E. coli lysate under denaturing conditions. Cells were solubilized in denaturating lysis buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 0.01 mol/L tris, pH 8.0) and purified by nickel nitrilotriacetic acid affinity column (GE Healthcare, Uppsala, Sweden).

Purified protein fractions were monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled and refolded by stepwise dialysis against 0.02 mol/L $NaPO_4$, pH 8.0 while gradually reducing urea concentration from 6 mol/L to 0 mol/L. After a final dialysis against 0.01 mol/L $NaPO_4$, pH 7.2, Api g 1-Bet v 1-Dau c 1-chimer was lyophilized and stored at 220uC.

Concentration of bacterial endotoxins was determined using the Limulus amebocyte lysate assay (QCL-1000, Cambrex, Walkersville, MD, USA) according to the users manual. Endotoxins were removed using the EndoTrap Blue affinity column (Profos, Regensburg, Germany) according to the manufacture's instructions. Protein concentration was determined using the bicinchronic acid protein assay (Pierce, Rockford, IL, USA).

Protein Analysis

Physiochemical identification and characterization of the recombinant construct was done by preparative HPLC and mass spectrometry (piCHEM, Graz, Austria), and secondary structure by far UV light circular dichroism spectroscopy (CD), as previously described [8].

Immunological characterization was done by immunoblot analysis using a monoclonal mouse anti-Bet v 1 IgG antibody (BIP1, 1/10), sera from 3 BP allergic patients with concomitant BPRFA containing Api g 1-, Dau c 1-, and Bet v 1-specific IgE (1/5) or mouse sera from a chimer-tolerized, poly-sensitized mouse (1/5). For detection secondary rat anti-mouse IgG1 antibody (1/500; BD Pharmingen, San Diego, CA, USA) followed by an alkaline phosphatise-conjugated goat anti-rat IgG (1/2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or alkaline phosphatase-conjugated mouse anti-human IgE (1/1000; Phar-

mingen) antibodies and NBT/BCIP substrate mixture were used. Negative controls with sera from untreated mice and non-allergic patients, or buffer control were run in parallel.

Animal Treatment

Allergy prevention in mono-sensitized mice with either rBet v 1, rApi g 1 or rDau c 1. Mono-sensitization was performed by 3 intraperitoneal (i.p.) injections (day 22, 36, 50) of 5 mg per Bet v 1, Api g 1 or Dau c 1 adsorbed to aluminium hydroxide ($Al(OH)_3$; Serva, Heidelberg, Germany) in 14 day intervals, adapted from Hufnagl et al. [13]. For prevention of allergic sensitization 10 mg of either of the allergen Bet v 1, Api g 1 or Dau c 1 were intranasally (i.n.) applied in 30 ml of 0.9% NaCl 3 times in 7 day intervals (day 0, 7, 14), prior to mono-sensitization, adapted from Wiedermann et al. [12]. Samples were taken one week after the last treatment (day 57).

Allergy prevention in poly-sensitized mice with a mixture of all three allergens. Poly-sensitization was performed by applying a mixture of Bet v 1, Api g 1 and Dau c 1, 5 mg each adsorbed to $Al(OH)_3$ as described above. For prevention of poly-sensitization mice were i.n. pretreated with a mixture of 10 mg each of Bet v 1, Api g 1 and Dau c 1, in 30 ml of 0.9% NaCl. Allergy prevention and poly-sensitization protocols were adapted from Hufnagl et al. [10].

Allergy prevention with the birch pollen-food chimer in poly-sensitized mice. Intranasal pretreatment with the BP-food chimer was performed by using 15 mg of the chimer in 30 ml of 0.9% NaCl per application (adapted from Wild et al. [8]) as described above, prior to poly-sensitization. Control mice were i.n. sham-treated with 30 ml of 0.9% NaCl prior to poly-sensitization. One week after the last i.p. immunization, mice were sublingually (s.l.) challenged with a mixture of BP extract, carrot extract and celery extract, by applying 100 mg each in 15 ml of 0.9% NaCl with a pipette under the tongue of the mice. To prevent swallowing of the extracts mice were fixed in the scruff during and until 20 seconds after treatment. Mice were challenged on 3 consecutive days in 24 hour intervals (day 57, 58, 59). 24 hours after the last treatment mice were sacrificed (day 60) (Fig. 1).

In vivo application of neutralizing antibodies. In some experiments, i.n. chimer-pretreated and poly-sensitized mice were i.p. injected with 0.5 mg of blocking antibodies. Anti-CD25 was applied prior poly-sensitization (day 15) (adapted from Leech et al. and Wilson et al.) [14,15], anti-TGF-b before s.l. challenge (day 56) (adapted from Taher et al. [16]), and anti-IL-10R was injected before poly-sensitization (day 19) and before s.l. challenge (day 56) (adapted from Taher et al., Leech et al. and Wilson et al. [14,15,16]). Pretreated and poly-sensitized control mice were sham-treated with 0.5 mg isotype control antibodies.

Sampling

Blood samples were taken before treatment and on the day of sacrifice by tail bleeding. Sera were collected and stored at 220uC until analysis. On the day of sacrifice cell suspensions from spleen, cervical lymph nodes (CLN) and NALT were prepared as described [12,17,18]. Additionally, sublingual tissues (SLT) and buccal mucosa (BM) were prepared by excising the whole mandible from the head and dissecting the cheek skin and the tongue with the floor of the mouth (SLT). The cheek skin was stretched and mucosal tissue was scraped off with a scalpel and collected in RNAlater buffer (Qiagen, Valencia, CA) for RNA isolation. SLT was separated from the tongue and stored in RNAlater buffer as well [19].

Allergen-specific Antibody Levels and Total IgA in Serum

Microtiter plates (Nunc, Roskilde, Denmark) were coated with each of the recombinant allergens Bet v 1, Api g 1 or Dau c 1 (5 mg/mL) prior to incubation with sera in dilutions of 1/500 for antigen-specific IgG2a and 1/10 for antigen-specific IgA detection. Rat anti-mouse IgG2a or IgA antibodies (1/500, Pharmingen) were used, followed by peroxidase-conjugated mouse anti-rat IgG antibody (1/2000, Jackson Immuno Lab, West Grove, PA) [12]. Results show the OD values after subtraction of baseline levels from pre-immune sera.

For determination of total IgA levels in sera, microtiter plates (Nunc) were coated with rat anti-mouse IgA (1/250, clone C10-3, Pharmingen) and incubated with 1/100 diluted sera. For detection biotinylated anti-mouse IgA (1/1000, clone C 10-1, Pharmingen) antibody, streptavidin (1/10000) and ABTS substrate were used.

Results are shown in ng/mL after subtraction of baseline levels of pre-immune sera.

Rat Basophil Leukemia Cell Mediator Release Assay (RBL Assay)

For measurement of functional allergen-specific IgE, rat basophil leukemia (RBL) cells (RBL-2H3 cell line, ATCC, No. CRL-2256) were incubated with sera obtained from pretreated and poly-sensitized mice at dilutions of 1/10, 1/100 and 1/300. Degranulation of RBL cells was induced by adding 0.03 mg of Bet v 1, Api g 1 or Dau c 1 diluted in 100 ml Tyrodes buffer.

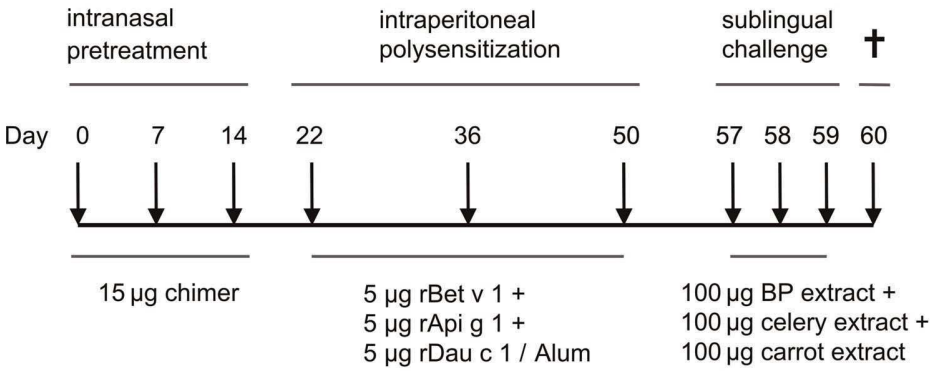

Figure 1. Experimental design. Mice were intranasally pretreated with either the chimer (chimer-treat) or sham-treated (poly-sens) 3 times in 7 days intervals followed by 3 intraperitoneal sensitizations with a mixture of rBet v 1, rApi g 1 and rDau c 1 in 2 weeks intervals. Thereafter, mice were sublingually challenged with a mixture of BP, celery and carrot extracts on 3 consecutive days.
doi:10.1371/journal.pone.0039409.g001

Supernatants were analyzed for antigen-specific IgE-dependent b-hexosaminidase activity as previously described [13].

Cytokine Production

IL-4, IL-5, IFN-c and TGF-b production was measured in spleen (5×10^6 cells/well), CLN (5×10^5 cells/well) and NALT suspensions (5×10^5 cells/well) incubated for 48 hours with each allergen (15 mg/well) as described [17]. Levels of IL-4, IL-5 and TGF-b were measured with ELISA kits (eBioscience, San Diego, CA, USA), IFN-c levels were measured as previously described [20]. All cytokine levels are shown in pg/mL after subtraction of baseline levels of unstimulated cultures.

Magnetic Sorting of CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25$^-$ T Effector Cells

CD4$^+$CD25$^-$ T effector cells (Teff) and CD4$^+$CD25$^+$ Treg cells were isolated from pooled spleen cells of chimer-pretreated or control poly-sensitized mice, with the MACS CD4$^+$CD25$^+$ Treg isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. The purity of sorted cells was acquired by flow cytometry using a BD FACSCalibur (BD Biosciences Pharmingen) and analysed by FlowJo software.

Treg Suppression Assay

CD4$^+$CD25$^-$ Teff cells (5×10^4) were cocultured in U-bottom 96-well plates with CD4$^+$CD25$^+$ Treg cells in various ratios (1/2, 1/8, 1/16). The cells were stimulated with 1 mg/mL anti-mouse CD3 (eBioscience) and 5×10^4 irradiated (3000 rad) splenocytes at 37uC for 3 days. For the last 16 hours of culture, cells were pulsed with 0.5 mCi ^{3}H-thymidine/well (Perkin-Elmer, Wellesley, MA, USA), harvested and proliferative responses were measured by scintillation counting (1450 Microbeta Liquid Scintillation and Luminescence counter, Perkin-Elmer). Results are expressed as absolute counts per minute (cpm). The percent suppression mediated by Treg cells was calculated by the following formula: [(cpm of Teff alone – cpm of Teff treated with Treg)/cpm of Teff cells alone]*100.

Quantification of mRNA Expression by Real-time RT-PCR

Total RNA from NALT was isolated from equal pooled cell suspensions. Total RNA extracted from RNAlater-stabilized, pooled SLT and BM was homogenized in liquid nitrogen prior to purification by using RNeasy Minikit combined with DNase digestion (RNase-free DNase Set, Qiagen). The concentration of extracted RNA was measured by NanoDrop ND-1000 spectrophotometer (PeqLab, Erlangen, Germany), RNA probes were standardized and then reverse-transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Gene expression was determined by quantitative real-time RT-PCR using LightCyclerH FastStart kit with TaqManH or CYBRGreen according to the manufacturer's instructions (Roche, Mannheim, Germany) on a LightCyclerH instrument 1.2 (Roche). For quantification of TGF-b, IL-10 and Foxp3 mRNA, predesigned TaqManH assays were used. Data are presented as the relative ratio of the target genes to the housekeeping gene 5-aminolevulinic acid synthase 1 (Alas1) (Universal ProbeLibrary (UPL) probe #64, Roche) [21].

Statistics

Data are expressed as means ± SEMs from 3 independent experiments. For statistical analysis p values > 0.05 were defined significant. Pair-wise comparison of sham-treated sensitized versus pretreated groups was performed by using the Mann-Whitney U-test and one-way ANOVA-test.

Results

Intranasal Pretreatment with Bet v 1, Api g 1 and Dau c 1 Alone or as a Mixture does not Suppress Immune Responses Against All 3 Allergens

Mucosal application of Bet v 1 prior to intraperitoneal polysensitization significantly reduced serum IgE responses as measured by IgE-induced basophil degranulation to Bet v 1, but not to Api g 1 or Dau c 1 (Fig. 2A). Similarly, mucosal application of Api g 1 or Dau c 1 prior to poly-sensitization showed significantly decreased IgE-induced b-hexosaminidase release only for the respective allergen (Fig. 2A).

In poly-sensitized mice, mucosal pretreatment with a mixture of all 3 allergens significantly reduced basophil degranulation to Bet v 1, but not to the homologous food allergens (Fig. 2B).

Construction and Characterization of the Birch Pollen-food Allergen Chimer

Based on previous T cell epitope mapping experiments with Api g 1 and Dau c 1 the immunodominant regions of these allergens were selected for designing the Bet v 1-food allergen chimer. In the case of Dau c 1 the immunodominant peptide detected in mice is also a major T cell epitope in allergic patients [22]. The immunodominant T cell epitope from Api g 1 (DGVNKEALTF-DYSVIDGDILLGFIESIENHV, peptide 27) including a 6×His-tag was inserted at the N-terminus of the Bet v 1.0101 encoding sequence. At the C-terminus the immunodominant T cell epitope of Dau c 1 (AVVPEENIKFADAQNTALFKAIEAYLIAN, peptide 47) was added (Fig.3A). Correct insertion of the templates was checked by sequence analysis.

After purification and refolding of the chimer with a theoretical mass of 27,6 kD, endotoxin levels of < 0,05 EU/mg of purified protein were measured, which corresponds to baseline levels of commercially available proteins [9].

Secondary structure elements of the chimer, analyzed by CD spectra, were in good agreement with the spectra obtained from rBet v 1 [8]. Only minimal variations due to the added peptides (data not shown) were observed. Immunoblot analysis confirmed that the conformation of Bet v 1 remained unchanged after linkage of the peptides. The chimer was recognized by Bet v 1-specific monoclonal antibody (BIP1, lane 3) and by IgE from sera of 3 BP allergic patients with a BPRFA (lane 5, 6, 7) as well as IgE from sera of a chimer-treated poly-sensitized mouse (lane 1). Negative controls did not elicit any IgE binding to the chimer (lane 2, 8, 4, 9) (Fig. 3B).

Intranasal Pretreatment with the BP-food-chimer Suppressed Humoral and Cellular Immune Responses in Poly-sensitized Mice

Antibody responses. Intranasal pretreatment with the chimer significantly reduced IgE-mediated basophil degranulation to all three allergens in comparison to the untreated poly-sensitized group (Fig. 4A). Moreover, Api g 1-, Dau c 1- and Bet v 1-specific IgG2a antibody production was enhanced, indicating a shift towards Th1 responses (Fig. 4B). Additionally, pretreatment with the chimer increased serum levels of total and allergen-specific IgA antibodies in comparison to poly-sensitized controls (Fig. 4C).

Cytokine production. Pretreatment with the chimer significantly decreased IL-5 levels in supernatants of allergen restimulated spleen and CLN cell cultures (Fig. 5A). Additionally, the IgE

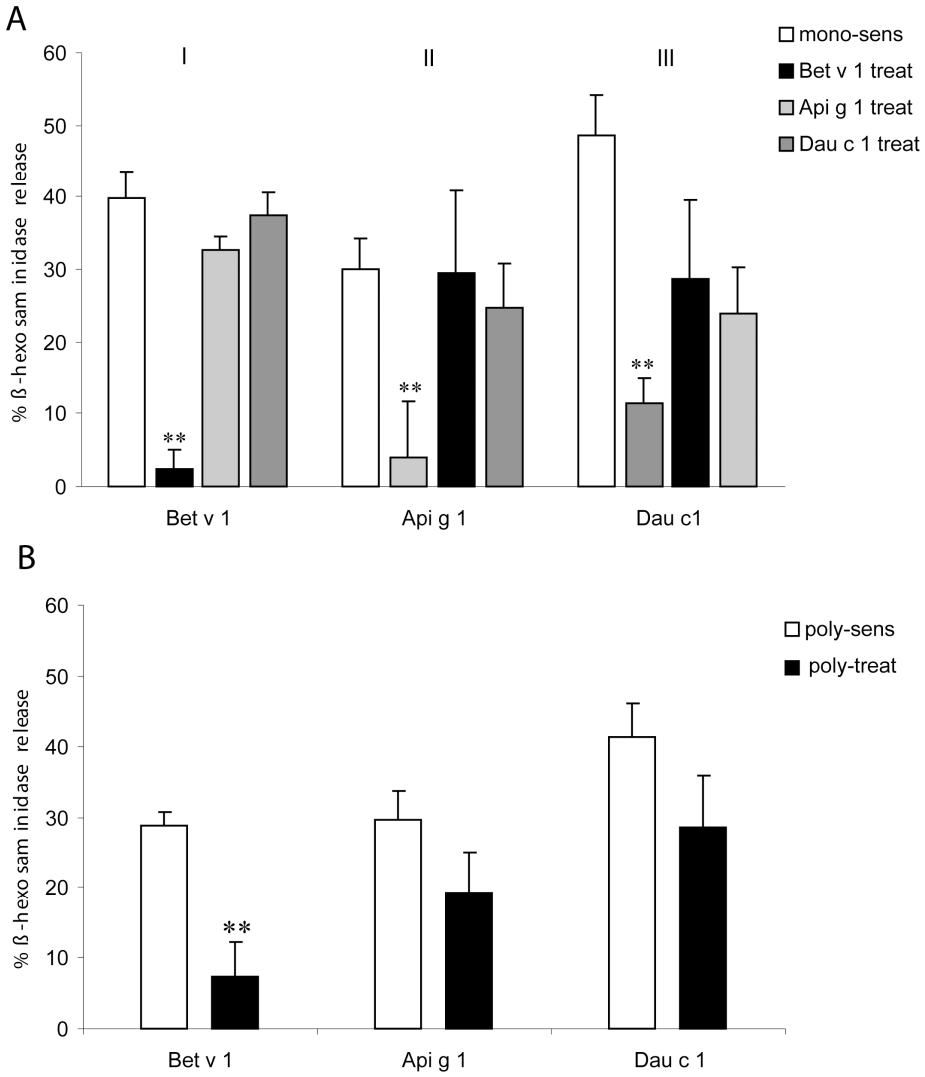

Figure 2. IgE-dependent allergen-specific basophil degranulation by sera. (A) ß-hexosaminidase release from (I.) rBet v 1-, (II.) Api g 1- or (III.) Dau c 1-sensitized mice; each group was i.n. pretreated with rBet v 1 (black bars), rApi g 1 (light-grey bars), rDau c 1 (dark-grey bars) or sham-treated (white bars). (B) ß-hexosaminidase release from mice i.n. pretreated with a mixture of rBet v 1/rApi g 1/rDau c 1 (black bars) compared with poly-sensitized controls (white bars). **p > 0.01.
doi:10.1371/journal.pone.0039409.g002

switching factor IL-4 was markedly reduced for all 3 allergens in spleen cell cultures of chimer treated mice compared to poly-sensitized controls: IL-4 (pg/mL): polysens: Bet v 1-restimulated: 14,35G22,60; Api g 1-restimulated: 43,92G53,84; Dau c 1-restimulated: 58,87G64,83; chimer-treat: Bet v 1-restimulated: 5,06G7,45; Api g 1-restimulated: 7,61G8,14**; Dau c 1-restimulated: 11,89G13,70**; **p > 0,01.

IFN-c production in spleen cell cultures was significantly enhanced after Bet v 1 - but not after Api g 1 or Dau c 1 - stimulations compared to poly-sensitized controls (Fig. 5B). However, in the CLN (Fig. 5B), and also in the NALT (data not shown) cultures, IFN-c production was significantly increased in the pretreated mice after re-stimulation with all three allergens.

Prevention of Allergic Polysensitization with the Chimer is Associated with Regulatory Mechanisms

The mRNA expression of TGF-b, IL-10 and Foxp3 in NALT was enhanced in chimer-pretreated mice compared to poly-sensitized controls (Fig. 6A). Increased Foxp3 expression was detected in SLT and BM of chimer-pretreated mice (Fig. 6B).

Application of anti-TGF-b, anti-IL10R or anti-CD25 blocking antibodies significantly abrogated the suppression of IL-5 and IL-4 production in spleen cell cultures of chimer-treated mice (Fig. 7A). Furthermore anti-TGF-b treatment of chimer-treated mice led to diminished antigen-specific and total IgA levels in sera (Fig. 7B).

Treg cells isolated from chimer-treated mice exhibited stronger suppressive potential as Treg cells from poly-sensitized mice (Fig. 8). The percent suppression mediated by Treg cells isolated from chimer-treated mice was 4.8-fold higher (ratio 1/2) than Tregs derived from poly-sensitized mice.

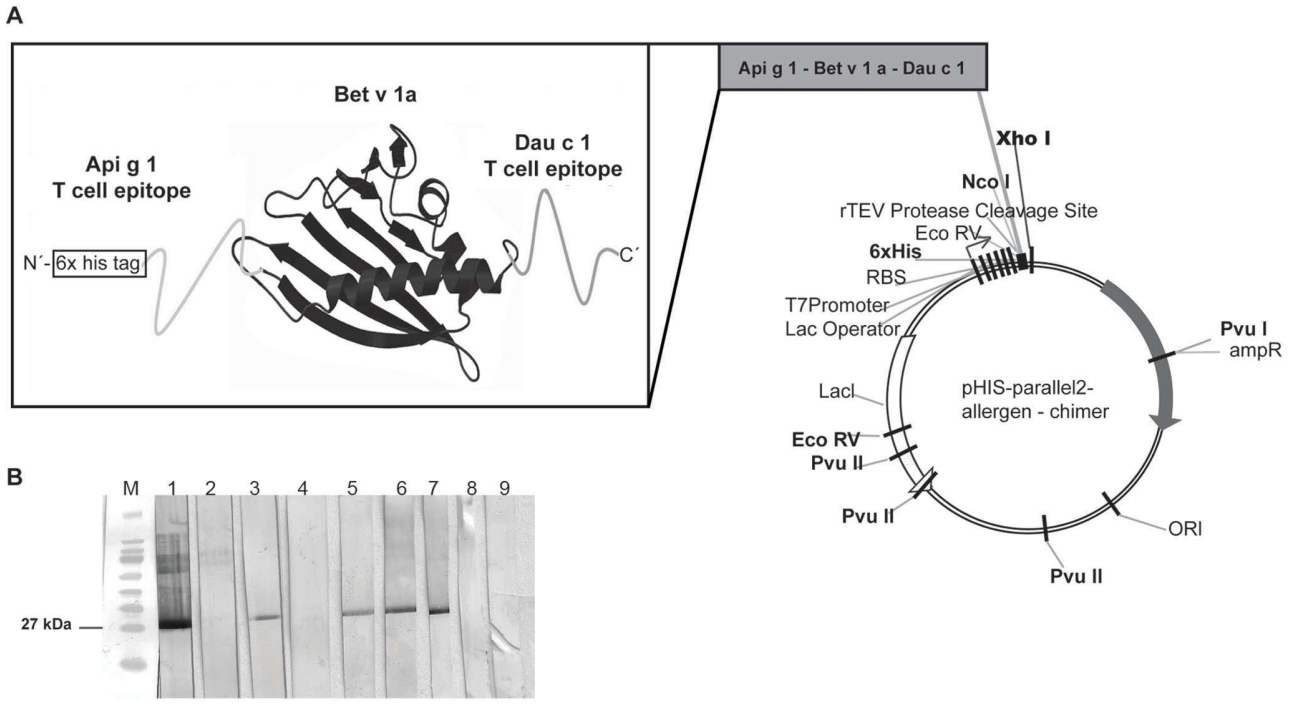

Figure 3. Construction and characterization of the pollen-food-chimer. (A) Design of the pHis-parallel 2-chimer composed of Bet v 1 protein, flanked by peptides from Api g 1 and Dau c 1. (B) Immunoblot: IgE binding to the chimer of sera from chimer-pretreated/poly-sensitized mice (lane 1) and of BP allergic patients with BPRFA (lane 5, 6, 7), and of Bet v 1 monoclonal antibody (lane 3). Negative controls sera from untreated mouse and non-allergic patient, or buffer control were run in parallel (lane 2, 4, 8. 9).
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Discussion

Clinical data regarding the efficacy of specific immunotherapy (SIT) with BP against pollen-related food allergies provide controversial results: While it has been shown that SIT with BP extract could achieve good effects in mono-sensitized patients [23], SIT in multi-sensitized patients with BPRFA often provided only limited success on the food-related symptoms [6,7,24]. Bucher et al. attributed this observation to the reason that BPRFA is not only caused by Bet v 1-cross reactive allergens, but also due to other less well-defined cross-reactive food allergens, which are not included in BP extracts used for SIT [2]. Along these lines, Bohle et al. described the existence of exclusive food-specific T lymphocytes, additionally to BP-reactive T cells in pollen-food multi-sensitized patients and suggested this finding to be one of the reasons why allergic symptoms of pollen-related food allergens are not modulated by BP SIT [1].

Here we describe a murine model for BPRFA which enables us to study the role of both IgE and T cells responses in sensitization to Bet v 1 and Bet v 1-related food allergens as well as the efficacy of prevention of multi-sensitization using a novel allergen chimer covering the major T cell epitopes of Bet v 1, Dau c 1 and Api g 1 (Fig. 1, Fig. 3A).

In order to mimic the clinical situation of BPRFA with OAS, mice were challenged sublingually with pollen and food extracts. Our data show that the sensitization protocol led to systemic Th2-biased immune responses as well as local immune responses to Bet v 1, Api g 1 and Dau c 1. Similarly as seen in humans, we observed that mucosal application of the single allergens, in particular with Bet v 1, did not reduce the allergic responses to the related food allergens indicating that other than Bet v 1 epitopes are necessary for successful prevention of BPRFA (Fig. 2A). Furthermore, a mixture of the allergens also did not suppress the immune responses to all allergens (Fig. 2B). A similar negative interference between several protein allergens was previously described by us, when intranasally applying a mixture of birch and grass pollen allergens aiming to prevent allergic poly-sensitization to these allergens [8]. The failure to prevent allergic poly-sensitization with the allergen mixture pointed out the necessity for creating multi-allergen constructs.

Therefore, we engineered a pollen-food chimer, composed of the Bet v 1 protein as scaffold for linkage of the immunodominant T cell epitopes of Api g 1 and Dau c 1 (Fig. 3A). Of notice, these immunodominant T cell epitopes in mice are located in regions of the dominant T cell sequences of humans with BPRFA [22,25].

Mucosal application of the chimer led to a marked immuno-modulation characterized by a shift towards Th1 immune responses (increase in IgG2a) accompanied by a significant down-regulation of allergen-specific IgE to all three allergens (Fig. 4A/B). In accordance, reduced antigen-specific IL-4 and IL-5 levels versus significantly enhanced antigen-specific IFN-c levels were observed in restimulated spleens and CLNs after chimer pretreatment (Fig. 5). These findings are in line with our previous studies in poly-sensitized mice using either poly-peptides, hybrid peptides or allergen chimers for prevention of poly-sensitization [8,10]. Additionally, intranasal pretreatment with the chimer increased IgA antibody levels primarily in sera (Fig. 4C) and less at the mucosal sites (data not shown). This might be explained by a matter of increased systemic circulation due to the highly vascularized tissue of the oral cavity, as it has been suggested in humans [26]. Studies by Pilette et al. showed that successful SIT positively correlates with increased serum IgA antibodies [27]. There is evidence that TGF-b plays a major role in IgA

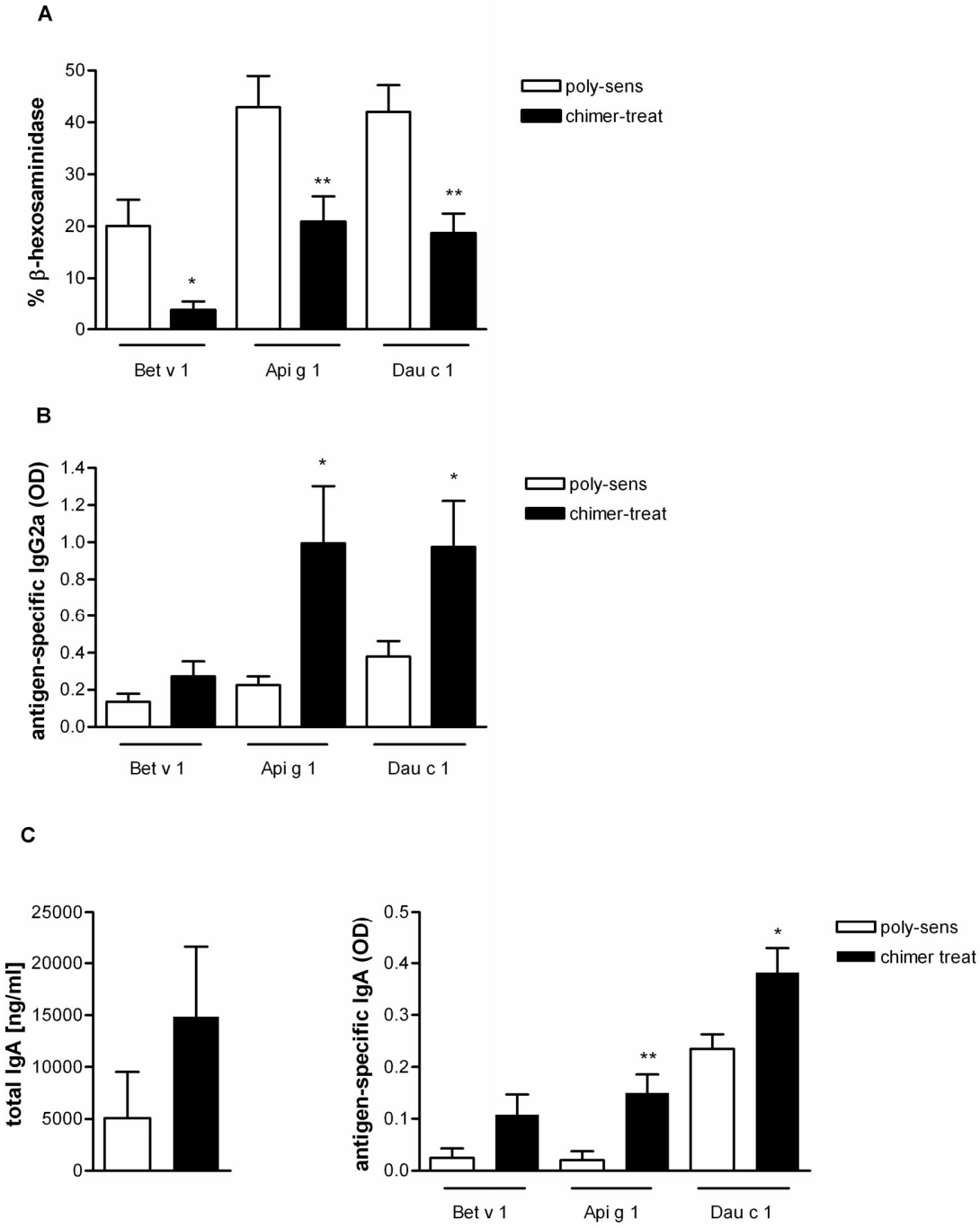

Figure 4. Antigen-specific cellular and humoral responses in mice pretreated with the chimer and poly-sensitized control mice. (A) IgE-mediated basophil degranulation. (B) Allergen-specific IgG2a antibodies in sera. (C) Total and antigen-specific IgA antibodies in sera. Chimer-pretreated mice (black bars); poly-sensitized control mice (white bars). *p > 0.05, **p > 0.01.
doi:10.1371/journal.pone.0039409.g004

production [28,29]. Indeed, we have shown that the application of anti-TGF-b antibody to chimer-treated mice reduced IgA levels in sera (Fig. 7B).

Regarding the mechanisms of chimer-induced immunomodulation, we detected an upregulation of TGF-b, IL-10 and Foxp3 mRNA levels in the NALT (Fig. 6A). Additionally, increased Foxp3 mRNA expression occurred in sublingual tissue and the buccal mucosa (Fig. 6B). This is in line with our former data showing that immunomodulation with a grass-birch pollen chimer but also with Bet v 1 alone is mediated by Treg cells [8,9]. The clinical relevance of this finding is supported by human studies showing an increase of regulatory Foxp3-positive T cells in the nasal mucosa of patients after successful systemic as well as sublingual grass pollen SIT [30,31]. In the latter study it was suggested that regulatory T cells either migrate from draining lymph nodes to the sublingual tissue during SLIT or may be

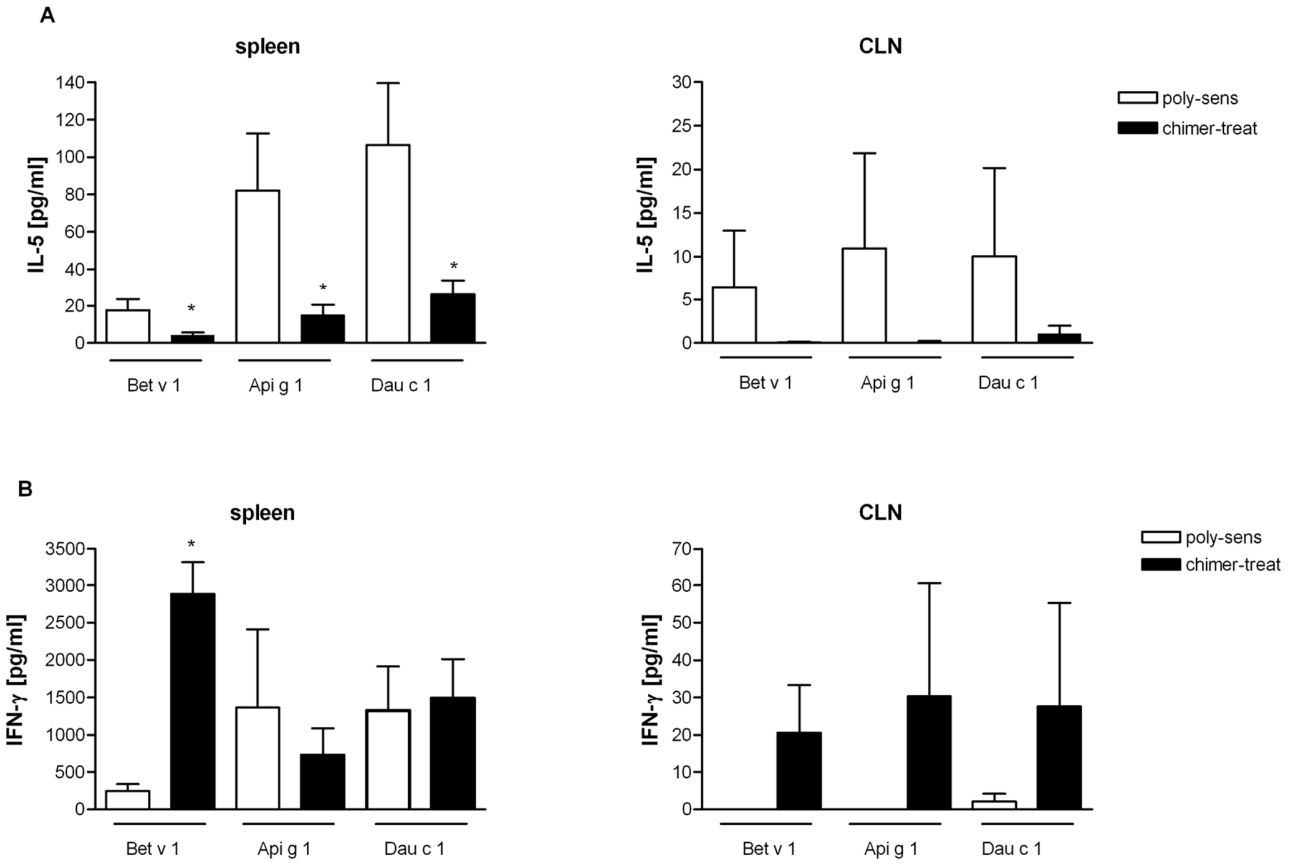

Figure 5. Antigen-specific cytokine production in mice pretreated with the chimer and poly-sensitized mice. (A) IL-5 levels and (B) IFN-c levels in supernatants of spleen and cervical lymph nodes (CLN) cell cultures after antigen stimulation. Chimer-pretreated mice (black bars); poly-sensitized control mice (white bars). *p > 0.05, **p > 0.01.
doi:10.1371/journal.pone.0039409.g005

induced by local dendritic cell-T cell interactions after repeated allergen exposure via the oral mucosa [31]. Furthermore, it was shown that sublingual treatment with allergens led to induction of IL-10 and TGF-b-releasing cells within the oral and nasal mucosa [31].

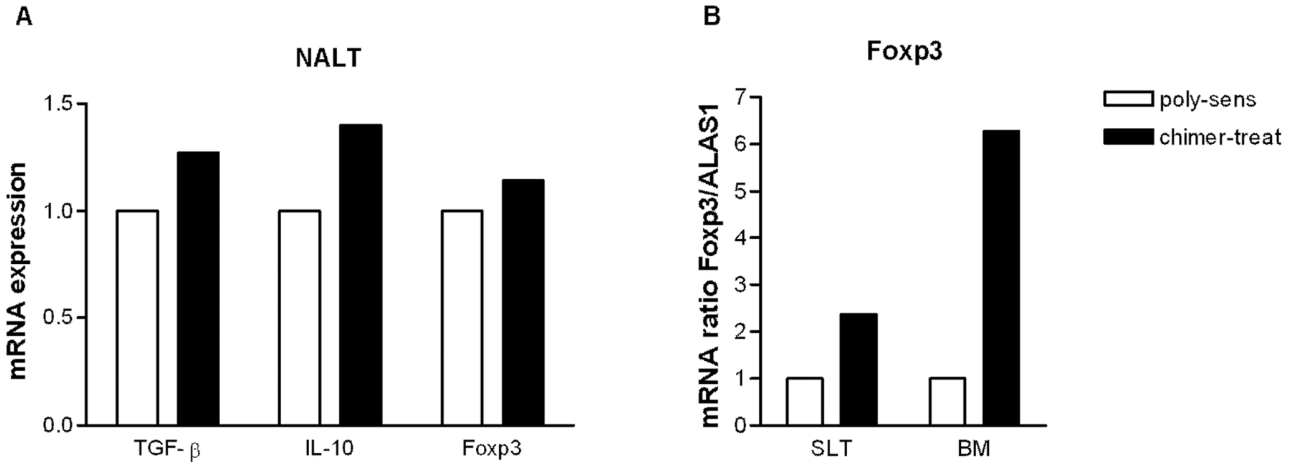

Figure 6. mRNA expression levels of regulatory markers on inductive and local effector sites. (A) TGF-b, IL-10 and Foxp3 mRNA expression in NALT, and (B) Foxp3 mRNA expression in SLT and BM of chimer-pretreated mice (black bars), shown as relative values in comparison with poly-sensitized controls (white bars). Data are presented as relative ratio of the target genes to the housekeeping gene Alas1.
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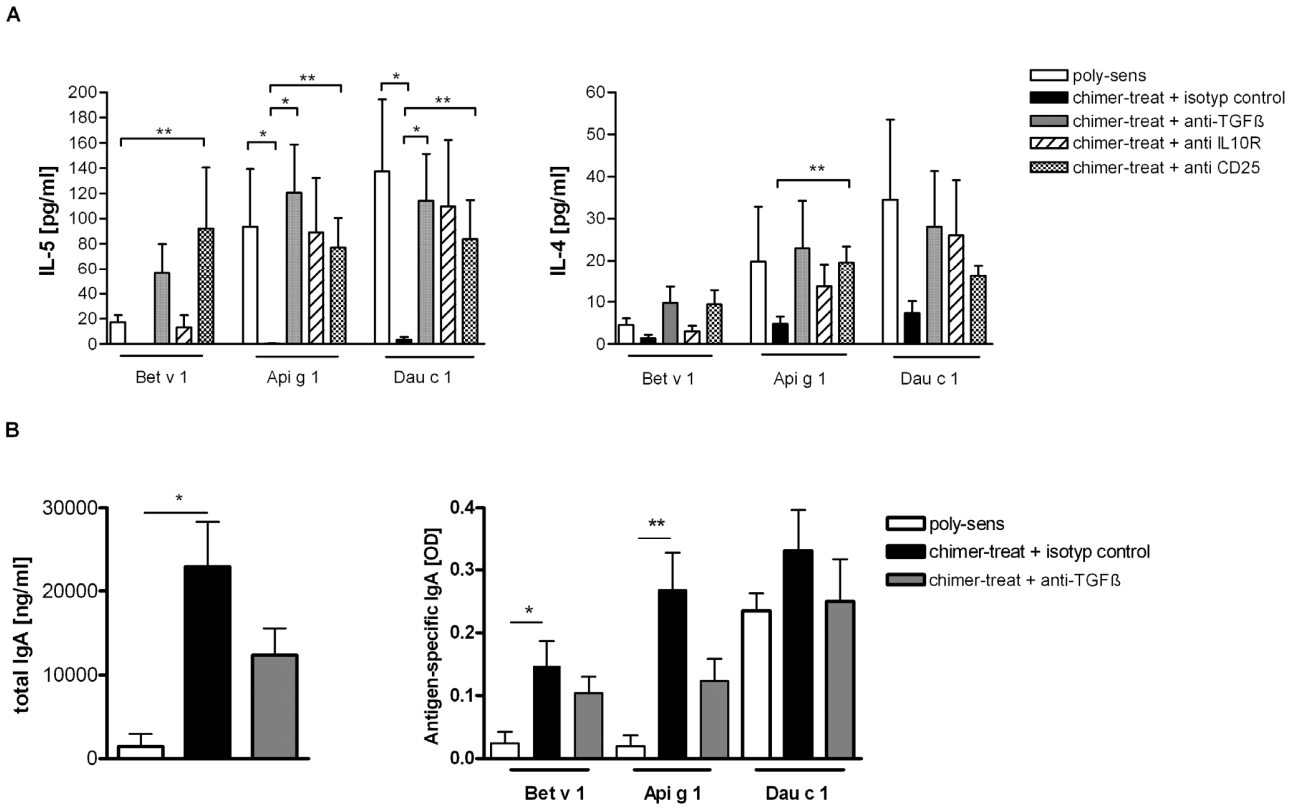

Figure 7. Effects of blocking antibodies. (A) Effects of anti-TGF-b, anti-IL-10R and anti-CD25 on levels of IL-5 and IL-4 in supernatants of antigen-stimulated spleen cell cultures. (B) Effects of anti-TGF-b on total and antigen-specific IgA production in sera. Chimer-pretreated mice treated with isotype control antibody (black bars) in comparison with chimer-pretreated and anti-TGF-b treated mice (grey bars), anti-IL10R treated mice (striped bars) and anti-CD25 treated mice (dotted bars). Poly-sensitized control group (white bars). *p > 0.05, **p > 0.01.
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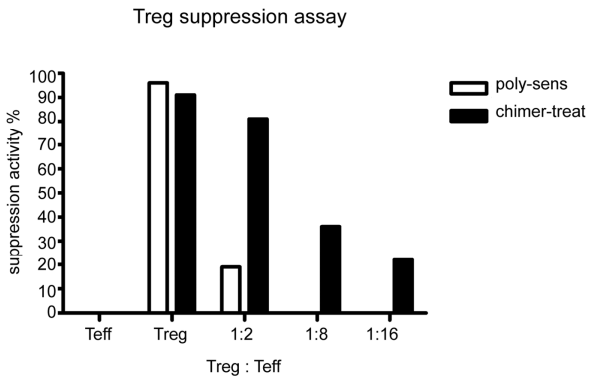
In order to investigate the immunosuppressive capacity of chimer-induced regulatory T cells in our BPRFA model, neutralizing antibodies against $CD25^+$ T cells, TGF-b and IL-10R were injected after mucosal application of the chimer and/or poly-sensitization in mice (Fig. 7A). Indeed, each of these antibodies significantly abrogated the suppressive effect of the chimer pretreatment, indicating that $CD25^+$-T cells, TGF-b as well as IL-10 play a potential role in immunomodulation for the prevention of BPRFA. Consistent with this, a study in house dust mite allergic patients showed that application of IL-10 and TGF-b blocking antibodies in vitro abrogated the immunosuppressive effects of $CD4^+CD25^+$-T cells induced by SIT [32].



Proliferation [cpm]	Teff: Treg		
	1:2	1:8	1:16
poly-sens	6741 ± 407,32	9391 ± 810,86	9472 ± 1614,86
chimer-treat	392 ± 259,94***	1308 ± 645,97***	1595 ± 765,07***

*** *p* values < 0,001; using One-way-Anova test.

Figure 8. Characterization of Treg cells by Treg suppression assay. Suppressive activity of Treg cells derived from chimer-treated mice (black bars) and poly-sensitized controls (white bars). Purified $CD4^+CD25^-$ T effector cells (Teff) and $CD4^+CD25^+$ T regulatory cells (Treg) were obtained by cell sorting (MACS), then cultured alone or cocultured in three different ratios (Treg:Teff: 1:2, 1:8, 1:16) in combination with irradiated splenocytes and stimulated by anti-CD3 antibody, before pulsing with [^{3}H] thymidine. The percent suppression mediated by Treg cells is calculated by the following formula: [(cpm of Teff alone – cpm of Teff treated with Treg)/cpm of Teff cells alone]*100.
doi:10.1371/journal.pone.0039409.g008

Allergic diseases have been linked with deficiency in function of Tregs [33]. Indeed we have shown that chimer-induced Tregs have higher suppression potential in comparison to Tregs derived from poly-sensitized mice (Fig. 8).

Taken together, we constructed an allergen-chimer for prevention of multiple sensitizations to pollen and pollen-related food allergens. We demonstrated that mucosally applied chimers covering important pollen and food-related epitopes can be used to down-regulate/prevent systemic and local allergic immune responses to all allergens, most likely by a combined induction of regulatory pathways and Th1-biased immune responses. Such mucosal allergen constructs might therefore provide promising new tools for mucosal intervention against different levels of multi-sensitization, including the birch pollen-related food allergy.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: UW. Performed the experiments: EH KH JJ IS. Analyzed the data: EH KH IS UW. Contributed reagents/materials/analysis tools: KHS BB RMM UW. Wrote the paper: EH UW.

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III.III PUBLICATION III

Use of a genetic cholera toxin B subunit/allergen fusion molecule as mucosal delivery system with immunosuppressive activity against Th2 immune responses

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Use of a genetic cholera toxin B subunit/allergen fusion molecule as mucosal delivery system with immunosuppressive activity against Th2 immune responses

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Abstract

Induction of peripheral tolerance can be facilitated when the antigen is linked to the B subunit of cholera toxin (CTB), an efficient mucosal carrier. In the present study, a genetic fusion molecule of Bet v 1 and CTB was produced to test whether mucosal application of this construct would lead to suppression of Th2 responses. Intranasal pretreatment of BALB/c mice with rCTB-Bet v 1 prior to allergic sensitisation with the allergen significantly decreased IgE but markedly increased allergen-specific IgG2a levels in sera as well as IFN-)' production of splenocytes. This Th1 shift was supported by an increased T-bet/GATA3 mRNA ratio. IL-5 production within the airways was suppressed after the pretreatment with rCTB-Bet v 1, while local allergen-specific IgA antibodies were markedly enhanced by pretreatment with the construct. Upregulation of Foxp3, IL-10 and TGF-[3 mRNA expression was detected in splenocytes after pretreatment with unconjugated allergen but not with the fusion molecule, indicating that antigen conjugation to a mucosal carrier modifies the immunomodulating properties of an antigen/allergen.

Keywords: Bet v 1; Conjugated allergen; CTB

1. Introduction

Mucosally induced immunological tolerance has become an attractive strategy for prevention and treatment of autoimmune and allergic diseases [1,2]. Several studies using animal models demonstrated that nasal or oral administration of allergens could be used for prevention and treatment of allergic immune responses [3–6]. Meta-analysis of 22 trials including 979 patients suffering from seasonal or perennial allergic rhinitis showed that sublingual administration of allergen extracts resulted in a significant reduction of both symptoms and medication requirements [7]. Although oral/sublingual or nasal administration offers an effective and safe method for induction of immunological tolerance, the doses of allergen used in these trials and the frequency of allergen administration have been rather high presumably due to failure of absorption of the allergens and their degradation before entering a mucosal tissue [1,8].

The non-toxic B subunit of cholera toxin (CTB) and the closely related *E. coli* heat-labile enterotoxin B subunit (LTB) were used to overcome such limitations by serving as mucosal

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carrier molecules of chemically or genetically conjugated antigens for the induction of oral tolerance [9–13]. When used as a carrier and given by various mucosal routes in the absence of cholera toxin (CT) as an adjuvant, CTB induced a strong mucosal IgA immune response and antigen-specific tolerance to itself and to the conjugated antigen [14,15]. Conjugation with CTB may greatly facilitate antigen delivery and presentation to the mucosa-associated lymphoid tissues due to its affinity for the cell surface receptor GM1-ganglioside which is located on most mammalian cells including dendritic cells, B cells and macrophages [16].

It has been shown that a single oral or nasal administration of microgram amounts of ovalbumin (OVA) chemically coupled to CTB or LTB could suppress an allergen-specific IgE response in naive [12] as well as in sensitised mice [11]. However, in previous studies we demonstrated that the suppressive activity depended on the allergen used when chemically coupled to CTB [13]. Intranasal administration of CTB-OVA prior to sensitisation led to suppression of Th2 immune responses to OVA. In contrast, intranasal administration of chemically coupled CTB-rBet v 1 prior to sensitisation was shown to enhance the production of Th2-promoting cytokines such as IL-4, IL-5, as well as IL-10 and Bet v 1-specific antibodies of all isotypes. It was suggested that, apart from the nature of the antigen, the method of conjugation might influence the effects of CTB/CTB-like molecules [12,17]. The present study was performed to investigate whether recombinant production of a fusion molecule, rather than chemically coupled products, could improve the immunomodulatory capacity of the mucosal delivery system. Therefore, a fusion molecule of CTB and the major birch pollen allergen Bet v 1a was genetically engineered to test whether mucosal application of this rCTB-Bet v 1a could suppress the Th2 response. Using a mouse model of birch pollen allergy we demonstrated that, in contrast to Bet v 1 chemically coupled to CTB, Bet v 1 genetically fused to CTB promoted suppression of systemic and local allergic immune responses.

2. Methods

2.1. Construction of the CTB-Bet v 1a expression vector

The mature CTB coding sequence was amplified by PCR from the CVD 103-HgR live oral cholera vaccine (Orochol, Berna, Switzerland). Prior to construction of the CTB-Bet v 1a fusion gene, the oligonucleotide sequence encoding a flexible tetrapeptide GPGP (hinge) [18] was fused to the 3′ end of the CTB gene. At the 5′ end of CTB the restriction site NcoI was added to the nucleotide sequence. The Bet v 1a encoding cDNA was amplified by PCR from its original cloning vector with DNA primers containing the oligonucleotide sequence encoding a hinge tetrapeptide at the 5′ end and SalI at 3′ end. Both PCR products were gel purified and fused by PCR to obtain a DNA coding for the CTB-Bet v 1a fusion protein. Following cloning into *E. coli* XL-1 Blue cells and confirmation of the DNA, the CTB-Bet v 1a fusion construct was inserted into the expression vector pelBpHis-Parallel 2 [19].

2.2. Expression, purification and refolding of recombinant CTB-Bet v 1 fusion protein

Competent BL21 [DE3] *E. coli* cells were transformed with the CTB-Bet v 1a expression vector and grown in LB medium supplemented with 1 mM ampicillin at 37 °C until an OD_{600} of 0.6–0.8 was reached. Protein expression was then induced by the addition of isopropyl β-d-thiogalactopyranoside to a final concentration of 1 mM. Cells were harvested 12 h post-induction. 6×His-tagged rCTB-Bet v 1a protein was produced in inclusion bodies and therefore, purified under denaturing conditions. The cells were solubilised in buffer A containing 10 mM Na-phosphate, pH 8, 8 M urea and 20 mM β-mercaptoethanol. The solubilised rCTB-Bet v 1a was purified using a Ni^{2+}-NTA affinity column (Qiagen, Valencia, CA, USA) and subjected to a refolding procedure. Gradual removal of the denaturant agent (8 M urea) was performed by stepwise dialysis. The urea concentration was reduced from 8 to 1 M in refolding buffer containing 20 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 20% glycerol and reduced and oxidized glutathione at a molar ratio of 3:1. After refolding, the sample was dialyzed against the refolding buffer in the absence of the redox pair components and glycerol. The protein was stored at 4 °C or lyophilised and stored at −20 °C. Concentration of bacterial endotoxins was determined using the LAL Endochrome test (Charles River Endosafe, Charleston, MA, USA) according to the user manual. Removal of endotoxins from the sample was achieved using the EndoTrap Blue affinity matrix (Profos, Regensburg, Germany) according to the manufacture's instructions. Protein concentration was determined according to the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

2.3. SDS-PAGE and immunoblot analysis

Samples containing 5–10 J.g of protein were subjected to 8% SDS-PAGE. Samples were either boiled for 5 min prior to electrophoresis or loaded directly onto the gel without heat treatment. Immunoblot analysis of rCTB-Bet v 1a was performed using a monoclonal mouse anti-Bet v 1 IgG antibody (BIP1) or a serum pool of birch pollen allergic patients containing Bet v 1-specific IgE [4].

2.4. N-terminal sequencing

The protein monomer was blotted onto a polyvinylidene difluoride membrane, Coomassie stained, excised and sequenced using a Procise 491 sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Enzyme-linked immunosorbent assays (ELISAs)

rCTB-Bet v 1a was checked for biological activity by GM1-binding capacity as described elsewhere [20]. Briefly, microtiter plates were coated with 0.3 J.g of GM1 per well. After blocking, GM1 was incubated with 10 ng of purified rCTB-Bet v 1a or bacterial CTB (Sigma–Aldrich, Germany) as positive control. As controls for non-specific binding, BSA and rBet v 1a were coated. Bound rCTB-Bet v 1a was detected with a 1:8000 diluted alkaline phosphatase (AP)-conjugated rabbit anti-CTx antibody (Sigma). For human IgE ELISA experiments microtiter plates were coated with 0.1 J.g of rCTB-Bet v 1a per well. After blocking, a 1:4 diluted pool of Bet v 1a-specific IgE positive birch pollen allergic patients sera was applied. Bound IgE was detected with a 1:1000 diluted AP-conjugated mouse anti-human IgE antibody (BD Pharmingen, CA, USA). Three sera of non-allergic subjects were used as negative controls. OD values were counted positive when they exceeded the mean OD of the negative controls by more than three standard deviations. For IgE ELISA inhibition experiments, the diluted serum pool was pre-incubated with 0, 10, 20, 40 and 60 J.g/ml of rCTB-Bet v 1a and equivalent molar amount of rBet v 1 (0, 6, 12, 24 and 36 J.g/ml) as positive controls or CTB as a negative control before proceeding with the immunoassay as described above. Inhibition values are given as percent reduction of bound IgE compared to the controls where no inhibitor protein has been added.

2.6. Generation of a tentative model of rCTB-Bet v 1a

A model of the three-dimensional structure of rCTB-Bet v 1a was generated using the Swiss-PDB Viewer [21] with the previously published structures of CTB (pdb.1fgb) and the structure of Bet v 1a (pdb.1bv10) as templates.

2.7. Animals

Female 7-week-old BALB/c mice were obtained from the Forschungsinstitut fuer Versuchstierzucht (Himberg, Austria). All experiments were approved by the Animal Experimentation Ethics Committee of the Medical University of Vienna and the Ministry of Science and Research (GZ 66.009/229-BrGT/2005).

2.8. Antigens

Recombinant Bet v 1a was obtained from Biomay AG (Vienna, Austria). Pollen from *Betula verrucosa* (BP) was purchased from Allergon (Välinge, Sweden), and a protein extract was prepared as previously described [5]. CTB was purchased from Sigma–Aldrich (Germany).

2.9. Experimental design

Intranasal (i.n.) treatment was performed by applying 3 times 20 J.g of the rCTB-Bet v 1a fusion molecule in a 30 J.l volume to anesthetized mice ($n = 5$/group) in 7-day intervals (day 0, 7 and 14). As controls, mice were i.n. treated with 10 J.g rBet v 1a or 10 J.g CTB or i.n. sham-treated with 30 J.l buffer (50 mM Tris–HCl, pH 7,4, 150 mM NaCl). Nine days after the last pretreatment mice were 3 times immunized intraperitoneally (i.p.) with 1 J.g rBet v 1a adsorbed to aluminium hydroxide ($Al(OH)_3$), Serva, Heidelberg, Germany) in 14-day intervals (days 23, 37 and 51). One week after the immunization, an aerosol challenge with 1% wt/vol BP extract was performed on 2 consecutive days (days 58, 59), as previously described [3].

2.10. Sampling

Blood samples were taken before treatment and 2 days after aerosol challenge (day 61) by tail bleeding. After sacrifice (day 62) spleen cell and lung cell cultures were prepared and bronchioalveolar lavages (BAL) were collected as previously described [5].

2.11. Airway inflammation

BAL fluids were spun onto microscope slides and stained with haemacolor staining solution (Hemacolor, Merck Darmstadt, Germany). The number of eosinophils was expressed as percentage of the total counted cell number as previously described. Supernatants of BAL were used for measurement of IL-5 levels [3].

2.12. Allergen-specific antibody levels in mouse serum and BAL

Microtiter plates (Nunc, Roskilde, Denmark) were coated with rBet v 1a (2 J.g/ml) prior to incubation with sera or BAL. Serum samples were diluted 1:500 for IgG2a and BAL samples were applied undiluted for IgA antibody detection. Rat anti-mouse IgG2a and IgA antibodies (1/500; Pharmingen, San Diego, Calif) were used, followed by peroxidase-conjugated mouse anti-rat IgG antibodies (1/2000; Jackson Immuno Lab, West Grove, Pa). Color development was performed as previously described [5]. Antibody levels were expressed in optical density (OD) units. Results were reported after subtraction of baseline levels obtained with preimmune sera.

IgE antibody levels in sera were measured by rat basophil leukemia (RBL) cell mediator release assay, performed as previously described [22]. RBL-2H3 cells were incubated with sera obtained from pretreated and sham-treated sensitised mice at dilutions of 1/30 to 1/3000. Degranulation of RBL cells was induced by adding 0.03 J.g of rBet v 1a, diluted in 100 J.l Tyrode's buffer. Supernatants were analyzed for [3-hexosaminidase activity. Results were reported as percentages of total [3-hexosaminidase released after addition of 1% Triton X-100 and are shown after subtraction of baseline release levels obtained with preimmune sera.

2.13. Lymphocyte proliferation and cytokine production in spleen and lung cell cultures

Proliferation of splenocytes (2×10^5 cells/well) stimulated with rBet v 1a (2 J.g/well) and BP extract (5 J.g/well) was measured after 4 days following ^{3}H thymidine incorporations as previously described [5]. For determination of cytokine production, spleen and lung cell cultures were incubated with BP extract (25 J.g/well) for 48 h. Supernatants were taken for measurements of IL-4, IL-5 and IL-10 by mouse ELISA kits (Endogene, Cambridge, MA) and IFN-)' by ELISA as previously described [3]. Cytokine levels were shown in pg/ml after subtraction of baseline levels of unstimulated cultures.

2.14. Quantification of mRNA expression by real-time RT-PCR

Total RNA was isolated from pooled spleen and lung cell suspensions at the day of sacrifice using RNeasy Minikit (Quiagen, Valencia, CA), treated with DNase (Quiagen) and then reverse-transcribed into cDNA using random hexamers (GeneAmp RT-PCR kit, PE, Boston, MA). Gene expression was determined by quantitative real-time PCR on a LightCycler® instrument 1.2 using LightCycler® FastStart kits without (TaqMan) or with SYBRGreen according to the manufacturer's instructions (Roche, Mannheim, Germany). Amplification of the housekeeping gene 5-aminolevulinate synthase (ALAS1) (Universal ProbeLibrary probe #26, Roche) was performed in order to standardize the amount of sample cDNA added. Data are presented as the relative ratio of the target genes to the housekeeping gene ALAS1. The efficiency of all target and reference genes was determined by serial cDNA dilution. For quantification of TGF-[3, IL-10 and Foxp3 mRNA predesigned TaqMan® assays were used. An initial activation step for 10 min at 95 °C was followed by 50 cycles of 10 s at 95 °C, 30 s at 60 °C and 1 s at 72 °C. Primers for T-bet, GATA-3 and CTLA4 were designed using the Primer3 software from the Whitehead Institute for Biomedical Research. The amplification was performed as described [23] by an activation step for 10 min at 95 °C which was followed by 50 cycles of 5 s at 95 °C, 5 s at 65 °C and 15 s at 72 °C and melting curve analysis (SYBR-Green).

2.15. Statistics

For statistical analysis p-values <0.05 were defined significant. Comparisons of sensitised group versus pretreated groups were performed by ANOVA and linear contrasts. Prior to analysis data were log-transformed in order the achieve homogeneity of variances. Residuals were tested for deviations from the normal distribution by Kolmogorov–Smirnov tests.

3. Results

3.1. Production of the rCTB-Bet v 1a fusion protein

The sequence coding for mature CTB was obtained by PCR of a genomic DNA preparation of an oral live attenuated *Vibrio cholerae* vaccine (CVD 103-HgR) [24]. The CTB sequence was modified at the 3' end by PCR to include the oligonucleotide sequence encoding a flexible hinge tetrapeptide (GPGP). The Bet v 1a encoding sequence was inserted at the 3' end of the CTB-hinge sequence (Fig. 1A). The model generated with the Swiss-PDB Viewer gave a hypothetical

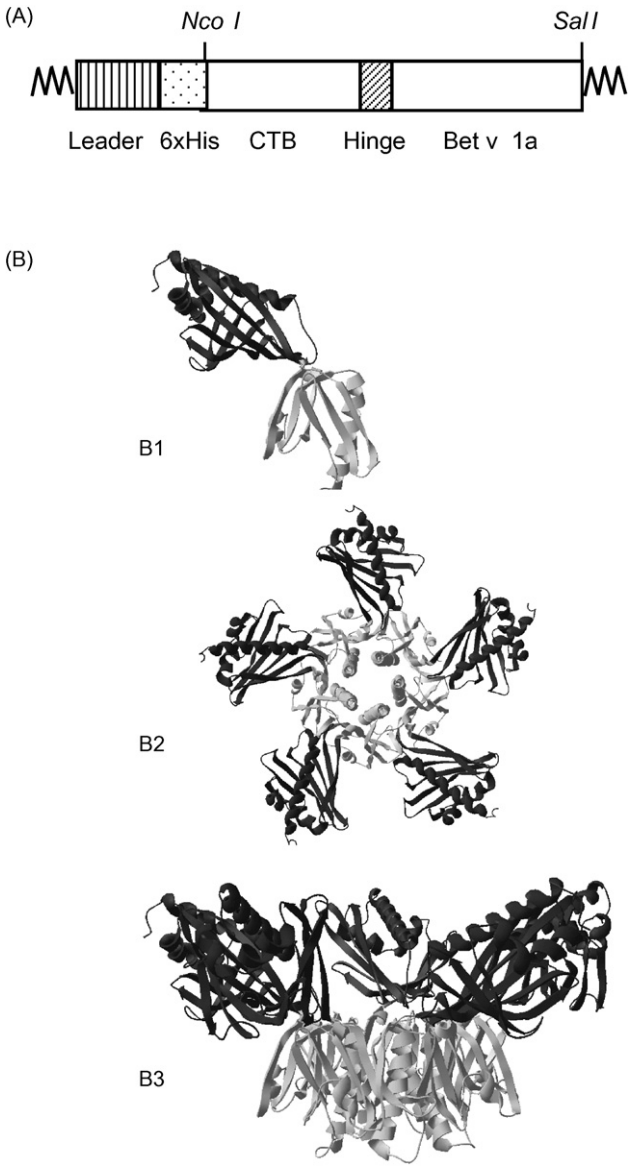

Fig. 1. (A) Composition of the rCTB-Bet v 1a construct. (B) Visualization of the tentative three-dimensional structure of rCTB-Bet v 1a. The B subunits are represented in gray shades. Bet v 1 molecules fused to CTB are shown in black. (B1) rCTB-Bet v 1a monomer; (B2) top view and (B3) side view of rCTB-Bet v 1a pentamer.

structure of the rCTB-Bet v 1a fusion as shown in Fig. 1B. The resulting DNA construct coding for a 29 kDa fusion protein was inserted into an expression plasmid and expressed at high amounts in *E. coli* in its monomeric form. The protein was purified under denaturing conditions via Ni-chelate affinity chromatography and refolded by stepwise dialysis against decreasing concentration of the denaturant in the presence of oxidized and reduced form of glutathione. After refolding, Coomassie stain and immunoblot analysis using BIP 1, a monoclonal mouse anti-Bet v 1 IgG antibody showed the presence of the pentameric (approximately 175 kDa) and monomeric (approximately 30 kDa) forms of the rCTB-Bet v 1a fusion protein (data not shown). The pentameric rCTB-Bet v 1 dissociated into monomers by heat treatment confirming its pentamer formation. To test whether CTB-Bet v 1 was correctly processed in *E. coli*, the N-terminal amino acid residues of the monomers were determined, revealing the peptide sequence MSYYH. This sequence was in accordance with the predicted N-terminus of the mature protein confirming the correct cleavage of the bacterial leader sequence in *E. coli*.

3.2. Biological and immunological activity of rCTB-Bet v 1 in vitro

Purified rCTB-Bet v 1a was tested for its biological activity by measuring its ability to bind to GM1 ganglioside in ELISA experiments. rCTB-Bet v 1a revealed GM1 ganglioside binding capacity comparable to that of commercially available CTB (Fig. 2A). Heated rCTB-Bet v 1a or CTB showed minimal binding to GM1 ganglioside confirming the specificity of the rCTB-Bet v 1a-GM1 ganglioside binding.

To study whether fusion of Bet v 1a to CTB affected the allergenic potency of Bet v 1a, the IgE antibody reactivity to rCTB-Bet v 1a was tested by ELISA and ELISA inhibition assay using a pool of birch pollen allergic patients sera (Fig. 2B and C). ELISA experiments showed that rCTB-Bet v 1a retained its IgE binding capacity compared to rBet v 1a (Fig. 2B). Furthermore, the fusion protein inhibited IgE binding to rBet v 1a in a manner comparable to equimolar amounts of rBet v 1a (Fig. 2C).

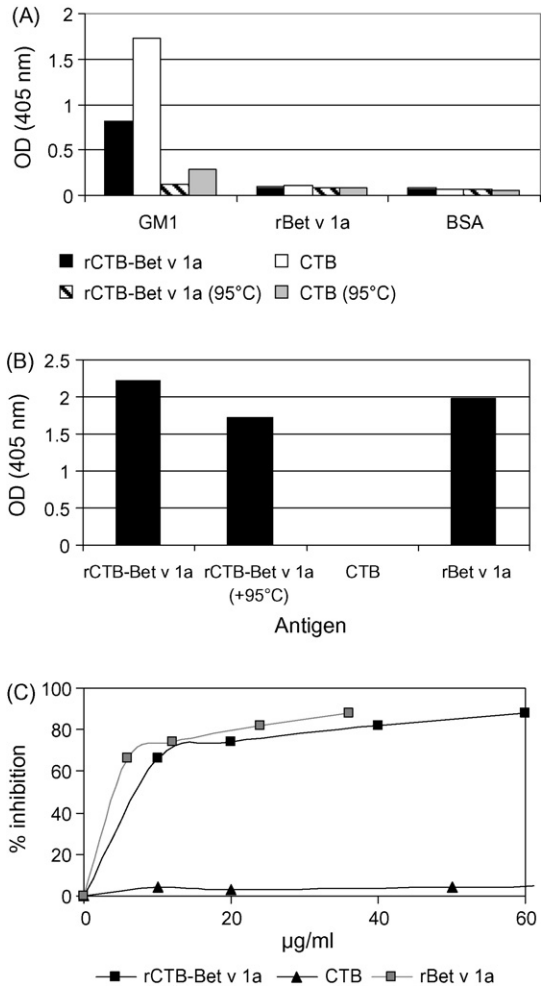

Fig. 2. Biological and immunological activity of rCTB-Bet v 1a. (A) GM1 ganglioside binding ability of unheated and heated rCTB-Bet v 1a and CTB. rBet v 1a and BSA served as negative controls. (B) IgE binding of unheated and heated rCTB-Bet v 1a. (C) Inhibition of IgE binding to rBet v 1a using equivalent molar amount of rBet v 1a and rCTB-Bet v 1a and CTB.

3.3. Antibody levels

To test the immunomodulatory capacity of the rCTB-Bet v 1a fusion molecule, mice were i.n. pretreated either with the fusion molecule, rBet v 1a, or CTB prior to allergic sensitisation. As demonstrated by the basophil release assay Bet v

Table 1

In vitro T-cell proliferation and cytokine production in i.n. pretreated mice

	Sensitised	rCTB-Bet v 1a	rBet v 1a	CTB
Stimulation index[a]	3.84 ± 1.62	3.99 ± 2.06	0.83 ± 0.43**	2.67 ± 2.13
IFN-)'[b]	141.0 ± 118.1	850.8 ± 434.8*	207.0 ± 75.0	484.7 ± 312.3
IL-5[b]	375.0 ± 200.3	370.2 ± 173.6	62.1 ± 46.3**	242.5 ± 92.9
IL-4[b]	146.0 ± 68.0	205.4 ± 132.6	28.0 ± 14.2*	8.4 ± 16.8**
IL-10[b]	1255.7 ± 898.8	1259.5 ± 781.0	132.5 ± 73.6**	905.0 ± 676.3

[a] Spleen cells were cultured with rBet v 1a for 4 days (stimulation index).
[b] Cytokine levels (pg/ml) were measured by ELISA.
* *p*-Values <0.05; using Kolmogorov–Smirnov tests.
** *p*-Values <0.01; using Kolmogorov–Smirnov tests.

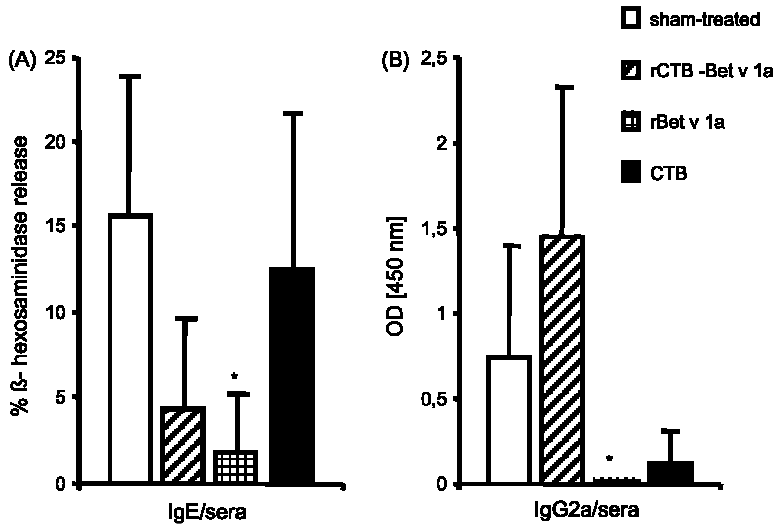

Fig. 3. (A) IgE-dependent allergen-specific basophil degranulation (RBL assay) and (B) allergen-specific IgG2a antibody levels in sera from rCTB-Bet v 1 (hatched bars), rBet v 1 (checkered bars) and CTB (black bars) pretreated mice compared with sham-treated controls (white bars). $^{*}p < 0.05$, $^{**}p < 0.01$, pretreated vs. sham-treated.

1a-specific IgE levels were reduced in the rCTB-Bet v 1a pretreated mice compared to sensitised controls (Fig. 3A), while Bet v 1a-specific IgG2a levels (Fig. 3B) were enhanced. In mice pretreated with rBet v 1a alone both allergen-specific IgE and IgG2a were significantly reduced compared to the sensitised controls. In contrast, pretreatment with CTB alone did not influence allergen-specific IgE antibody levels in serum (Fig. 3A and B).

3.4. Lymphocyte proliferation and cytokine production in the spleen

Proliferation of splenocytes after rBet v 1a stimulation was significantly lower in mice pretreated with rBet v 1a alone compared to sensitised control mice (Table 1). Proliferative responses of mice pretreated with the fusion molecule or CTB alone did not differ from sensitised controls. IL-4, IL-5 and IL-10 levels in spleen cell cultures of rCTB-Bet v 1a pretreated mice remained unchanged in comparison to the control group (Table 1). In contrast, IFN-)' levels were significantly enhanced in fusion molecule pretreated mice. Pretreatment with CTB alone led to enhanced IFN-)' levels and significantly reduced IL-4 levels. IL-5 and IL-10 production in the latter group was decreased in comparison to the sensitised controls. In mice pretreated with Bet v 1a alone Th2 like cytokines (IL-4, IL-5, IL-10) were significantly decreased, whereas IFN-)' production did not markedly differ from those of sensitised controls (Table 1).

3.5. Airway inflammation and local immune response

The number of eosinophils in BAL did not change in mice pretreated with rCTB-Bet v 1a compared to sensitised controls, while IL-5 production was reduced (Fig. 4A and B). Pretreatment with CTB alone led to an increase in the number of eosinophils and IL-5 levels. Eosinophilia in rBet v 1a pretreated mice was completely reduced along with significantly decreased IL-5 levels compared to the sensitised controls (Fig. 4A and B). In mice pretreated with rCTB-Bet v 1a or CTB alone, rBet v 1a-specific IgA antibody levels in BAL were three-fold higher compared to sensitised controls. IgA antibody levels in rBet v 1a pretreated mice were sig-

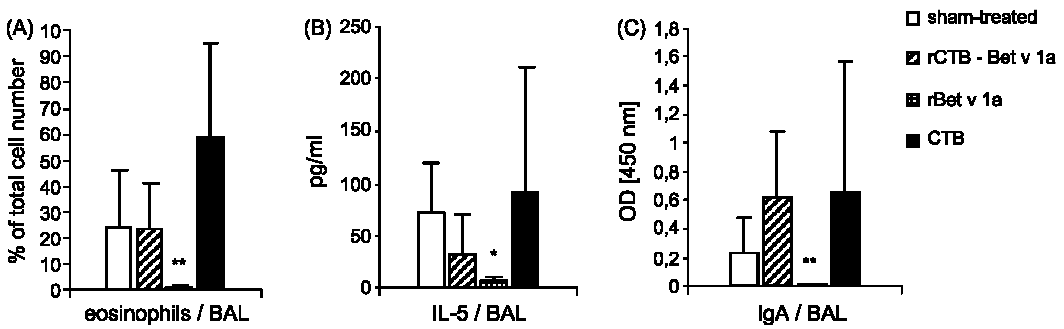

Fig. 4. (A) Number of eosinophils, (B) IL-5 levels and (C) allergen-specific IgA antibody levels in BAL from rCTB-Bet v 1 (hatched bars), rBet v 1 (checkered bars) and CTB (black bars) pretreated mice compared with sham-treated controls (white bars). $^{*}p < 0.05$, $^{**}p < 0.01$, pretreated vs. sham-treated.

Table 2

Th1-, Th2- and regulatory pathway related mRNA expression in spleen and lung of pretreated mice

	Spleen				Lung			
	Sham	rCTB-Bet v 1a	rBet v 1a	CTB	Sham	rCTB-Bet v 1a	rBet v 1a	CTB
TH1								
T-bet	1	0.87	0.03	0.34	1	0.60	0.19	0.72
TH2								
GATA-3	1	1.02	2.41	1.06	1	0.006	0.012	0.068
Regulatory								
IL-10	1	1.13	1.89	1.52	1	0.13	0.42	0.15
TGF-β	1	0.98	3.29	1.41	1	0.45	0.17	0.40
Foxp3	1	0.34	1.49	1.04	1	0.91	0.23	0.68
CTLA4	1	1.16	1.57	0.81	1	0.40	0.07	0.59

Relative expression of the target genes to the housekeeping gene ALAS1was determined by quantitative real time RT-PCR.

nificantly decreased in comparison to sensitised control mice (Fig. 4C).

3.6. Mechanisms of immunomodulation

In splenocytes from mice pretreated with the fusion molecule mRNA expression of T-bet and GATA3 remained unchanged in comparison to sensitised mice. Pretreatment with CTB alone was associated with decreased T-bet mRNA expression while GATA3 mRNA levels remained unchanged compared to sensitised controls. T-bet mRNA expression in splenocytes from mice pretreated with Bet v 1a alone was markedly decreased, while GATA3 expression was upregulated compared to sensitised controls (Table 2). In lung cells, expression of both T-bet and GATA3 mRNA were decreased after pretreatment with the fusion molecule, Bet v 1 alone or CTB alone compared to the sensitised controls (Table 2).

In splenocytes of mice pretreated with the fusion molecule expression of TGF-β, IL-10 and CTLA-4 remained unchanged, only mRNA levels of the transcription factor Foxp3 were decreased compared to sensitised controls (Table 2). Pretreatment with CTB did not induce significant changes of mRNA expression of regulatory cytokines as well as Foxp3 and CTLA-4 (Table 2). Pretreatment with Bet v 1a alone led to upregulation of TGF-β and IL-10 mRNA, whereas Foxp3 and CTLA-4 mRNA levels were unmodified in the spleen compared to sensitised control mice. In lung cells pretreatment with the fusion molecule, CTB or rBet v 1a alone led to a decrease of mRNA expression of the regulatory molecules IL-10, TGF-β, Foxp3 and CTLA-4 compared to the sensitised controls (Table 2).

4. Discussion

This work describes the construction and characterisation of a genetically engineered inhalative allergen-CTB conjugate, CTB-Bet v 1, as a candidate vaccine against allergic disease. So far, a protective effect of an allergen conjugated to a mucosal adjuvant has only been shown for a commonly experimentally used food allergen, ovalbumin was chemically coupled to CTB or LTB [11–13,17,25]. In contrast to these results, our earlier findings showed that mucosal administration of Bet v 1 chemically coupled to CTB resulted in the induction of a predominant IgE response upon subsequent sensitisation with birch pollen [13]. This may indicate a varying degree of sensitivity to tolerisation depending upon the nature of the specific antigen studied as previously suggested [13]. Furthermore, the process of chemically conjugating an antigen to CTB and the multiple steps of exposure to cross-linking agents may potentially denature and inactivate the antigen. Differences concerning the nature of the antigen–CTB coupling might influence the outcome, i.e. active immunity or tolerance [16]. It was speculated that, even though the ganglioside binding ability, which is thought to be essential for uptake by immune cells, of either chemical or genetically coupled constructs is preserved, the detailed molecular mechanism of uptake and intracellular processing by antigen presenting cells could be different [16,26,27]. Along these lines, it was recently demonstrated that the antigen presentation process to naive T cells, particularly of macrophages, was ameliorated when influenza virus hemagglutinin H1 subunit was genetically and not chemically coupled to CTB [16].

The clear advantage of a genetically engineered fusion protein over a chemical conjugate is its homogeneity. The molecular composition and position of the antigen do not vary and the amount of antigen is increased to five molecules per one molecule of CTB pentamer. Conversely, chemical conjugates yield complexes of ill-defined and somewhat variable molecular structures with a maximum of two antigen molecules per one molecule CTB pentamer [17,28].

In the present study, we generated a construct by the fusion of the Bet v 1a cDNA and the CTB encoding DNA via a hinge region coding for a flexible linker tetrapeptide at the C-terminus of CTB (Fig. 1A). The hinge was introduced to reduce steric hindrances between the CTB and Bet v 1 moieties facilitating CTB subunit assembly [29]. Since the N-terminal region of CTB is crucial for B-subunit assembly into pentamers and stabilization as well as for GM1-ganglioside binding, Bet v 1 was fused to the C-terminal site of CTB [30]. The obtained fusion protein (rCTB-Bet v 1a) was

active and correctly folded as shown by binding to GM1 and the ability to inhibit IgE-binding to rBet v 1a (Fig. 2).

When applying the fusion molecules via the mucosal/intranasal route prior to allergic sensitisation with Bet v 1, we demonstrated that the rCTB-Bet v 1 fusion protein led to the reduction of Bet v 1-specific IgE as indicated by a decreased IgE mediated basophile degranulation (Fig. 3A). Surprisingly, *in vitro* IL-4 production was reduced in spleen cell cultures of CTB-pretreated, but not of CTB-Bet v 1-pretreated mice. In this respect we have already previously described that IgE production and Th2 cytokine production do not always concur after co-administration of the mucosal adjuvant LTB with an allergen [20]. However, as it has been suggested that under certain conditions IgE can be produced independently of IL-4 [31], the IgE mediated basophile release, as an equivalent to allergic manifestations *in vivo*, might be the measurement of higher clinical relevance than *in vitro* production of the particular cytokine.

The present data are in contrast with our previous study describing that Bet v 1 chemically coupled to CTB enhances rBet v 1-specific IgE response [13]. We speculate that this opposite effect of the fusion protein to the chemical conjugate on IgE production is related to chemical modifications induced to the Bet v 1 molecules by the coupling procedure. Alternatively, or additionally, the applied antigen dose might have differed between the chemical coupled Bet v 1 and the Bet v 1 fusion molecules. Production of recombinant fusion molecules secures a constant linkage of one molecule Bet v 1 to one molecule CTB, a situation that cannot be guaranteed by chemical coupling processes. It is known that the dose of antigen applied via the mucosa is one of the key factors for successful tolerance induction. Therefore, recombinant production of a CTB-allergen fusion molecule seems advantageous for improved treatment efficacy.

We also observed a significant increase in Bet v 1-specific IgG2 in mice intranasally pretreated with rCTB-Bet v 1. This effect was due to the fusion of CTB and Bet v 1, since intranasal application of rBet v 1 or CTB alone reduced IgG2a antibody levels (Fig. 3B). In addition, the *in vitro* response of birch pollen-challenged spleen cells from the rCTB-Bet v 1 pretreated mice were strongly IFN-)' dominated (Table 1). Altogether, reduced allergen-specific IgE versus enhanced IFN-)' and IgG2 production corroborate the capacity of the fusion molecule to force the immune response towards a Th1-like response.

In BAL, IL-5 levels were reduced after intranasal pretreatment with either the fusion molecule or rBet v 1. A significant reduction of eosinophils in BAL, was achieved after intranasal pretreatment with rBet v 1, but not with the fusion molecule (Fig. 4A and B). However, intranasal pretreatment with rCTB-Bet v 1 increased the production of Bet v 1-specific IgA in BAL (Fig. 4C) demonstrating the potential of the fusion molecule not only to induce a systemic but also a mucosal immune response. This could be of importance, since it has been shown that secretory IgA could act as blocking antibody at the site of allergen exposure and inhibit IgE mediated histamine release [32,33]. These results are in agreement with our previous study and indicate that the immunomodulating effect was due to the CTB component of the fusion molecule, since intranasal application of CTB alone also enhanced antigen-specific IgA production, whereas unconjugated Bet v 1 suppressed antigen-specific IgA production [5].

Among the many signals that influence the development of Th cells, the transcription factors T-bet and GATA-3 have been suggested to be important regulators of Th1/Th2 cells [34]. As pretreatment with the fusion molecule induced a clear shift towards Th1 responses at the humoral and cellular levels, a corresponding enhancement of the T-bet expression in splenocytes or lungs cells was expected. However, the data obtained by real time PCR did not indicate a significant change in the expression of T-bet or GATA-3 in splenocytes from CTB-fusion molecules treated mice and sensitised controls. The obvious disproportion between T-bet expression and IFN-)' levels might be a matter of kinetics, as it has been demonstrated that upregulation of T-bet is only seen within the first days of Th1 stimulation in rat spleen cells, but not under long term Th1 polarizing conditions with high IFN-)' production [35]. The latter situation might more closely reflect the time point after treatment in our model. It has further been shown that the GATA-3 gene, which directly controls IL-5 gene expression, is more sensitive to Th-differentiation than to expression of T-bet [34]. Along these lines, GATA-3 expression in lung cell populations of mice, which showed reduced IL-5 production within the airways after treatment with the fusion molecule as well as with Bet v 1 alone, also displayed markedly reduced expression of this transcription factor. Moreover, it was demonstrated that the relative expression of T-bet and GATA-3 ratio, rather than the expression of either transcription factor alone, was more representative for the Th1/Th2 cytokine balance [35]. Accordingly, we found that particularly in lung cell populations the pretreatment with the fusion molecule was associated with a markedly higher T-bet/GATA-3 ratio compared to Bet v 1 or CTB pretreated and sensitised control mice.

A recent study by Sun et al. demonstrated that intragastric application of CTB chemically coupled to ovalbumin led to an increase in Foxp3+ and Foxp3− T regulatory cells along with enhanced TGF-β levels in Peyer's patches and mesenteric lymph nodes, but to a lesser degree in spleen [25]. In our hands, tolerance induction with the CTB fusion molecule was not associated with an increased expression of Foxp3, CTLA4 or the suppressive cytokines IL-10 and TGF-β in lymphocyte population of spleens or lungs. Similarly, as demonstrated by Sun et al., preliminary data indicate that regulatory pathways/cytokines can be spotted within the inductive site and draining lymph nodes, such as NALT and submandibular lymph nodes, rather than in peripheral lymph organs and effector organs (Hufnagl et al., unpublished data). In contrast to the fusion molecule, the present data also

illustrated that tolerance induction with Bet v 1 alone is associated with enhanced IL-10, TGF-[3 and Foxp3 expression in spleen cells, thereby confirming previous findings [3]. It thus seems reasonable to assume that receptor-associated uptake of antigen results in different mechanisms of oral tolerance induction for CTB conjugated Bet v 1 or unconjugated Bet v 1. Whereas CTB has a high affinity for GM1 ganglioside receptors present on all mammalian cells that are directly responsible for the trafficking of the antigen to the endosome [36], the tolerogenic properties of Bet v 1 could be mediated by an enhanced antigen uptake due to its ability to bind and permeabilise cell membranes, as recently described by Mogensen et al. [37]. Based on this assumption, a genetically construction of allergen–CTB vaccine against allergic reactions could be of advantage in the cases where the induction of mucosal tolerance by the antigen itself is less or not at all effective.

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IV. CONCLUDING DISCUSSION

With more than 25% of affected people, type I allergy belongs to the most common disorders in industrialized countries. Allergen-specific immunotherapy is an effective treatment strategy in many cases of allergic disease (28). Nevertheless, low compliance of patients due to the long duration of the treatment requiring frequent allergen injections, and the low efficacy in the constantly increasing number of multi-sensitized patients are main reasons to modify the treatment modalities and develop novel strategies.
Within this thesis 3 different approaches based on mucosal tolerance induction were developed as new strategies for primary and secondary prevention of allergic diseases.

The “basic steps” to develop an immunotherapeutica for allergen-specific immunotherapy is to identify and characterize the relevant allergens.
Many atopic individuals with allergic asthma suffer from indoor allergy, a very complex type of allergy which includes allergic reactions to several allergens from house dust mites, animal dander, cockroach, or moths. Binder et al. proved that 52 of 102 indoor allergic patients had developed IgE antibodies against larval extract from the Indianmeal moth *Plodia interpunctella* (57). Indianmeal moths represent a very common food pest in our households, and have been described as relevant elicitors of indoor allergy, as well as inhalation-associated seafood allergy. The same group identified the moth allergen Plo i 1 as cross-reactive panallergen for multi-sensitized patients (57).
As part of this thesis a novel Indianmeal moth allergen, the thioredoxin Plo i 2 was identified, produced as a recombinant protein and immunologically characterized together with the Indianmeal moth allergen Plo i 1 (Publication I). *In vivo* immunization studies in mice demonstrated that the thioredoxin Plo i 2 as well as the arginine kinase Plo i 1 act as classical type I allergens, which induced Th2-biased immune responses with significantly enhanced IgE and IgG1 levels at the humoral site and increased IL-5 and IL-4 levels at cellular site. IgE Immunoblot studies with sera from indoor- and seafood allergic patients confirmed the allergenic potential of moth allergens in multi-sensitized patients, notably with 95% frequency of IgE binding among seafood allergic patients (Publication I).
Recombinant forms of allergens are promising tools for allergen-specific immunotherapy, especially for animal allergens, where immunotherapeutic preparations are extracts from natural tissues with variable composition (184). Recombinant products facilitate allergen preparations of

consistent pharmaceutical quality, deprived of any non-allergenic proteins and non-protein compounds as present in allergen extracts of natural source materials (184).
The production of recombinant proteins like Plo i 2 is the basis for the development of alternative, advanced tools for allergen-specific immunotherapy, such as hypoallergenic variants with reduced IgE reactivity (116, 117, 119), or fusion proteins with adjuvants (Publication III) or multi-allergen constructs for multiple immunotherapy (Publication II).

The prevalence of multiple sensitizations is constantly increasing recent studies confirm that more than 70% of birch pollen allergic individuals show allergic symptoms to birch pollen-related food allergens (185). Unfortunately, the common allergen-specific immunotherapy has low effectiveness in reducing symptoms of food allergy in these patients.
To improve the simultaneous induction of tolerance to multiple allergens, a multi-allergen chimer (Publication II) was constructed.
Previous studies of our lab demonstrated that co-application of a mixture of recombinant allergens does not elicit the same potent effect of poly-tolerance induction to multiple allergens compared to a multi-allergen construct (122, 127). Therefore we genetically engineered a multi-allergen chimer composed of the whole major birch pollen allergen Bet v 1 and the immunodominant T cell epitopes of Dau c 1 (major carrot allergen) and Api g 1 (major celery allergen), for treatment of the highly prevalent birch pollen-related food allergy (Publication II). Mucosal application of the pollen-food-chimer in a murine model of the oral allergy syndrome, showed an effective suppression of allergic immune responses to all three allergens, modulated by a shift towards Th1. The role of regulatory T cells as immunomodulators of tolerance was demonstrated with locally enhanced expression of IL-10 TGF-β and Foxp3. Such multi-allergen vaccines represent a promising tool to prevent multi-sensitization in multiple allergic patients.

In order to enhance the tolerogenicity of mucosally applied allergens, recombinant protein can be combined with mucosal adjuvants. These mucosal antigen delivery systems such as the nontoxic B subunit of cholera toxin (CTB) or the heat labile toxin of *E. coli (LTB)*, transport small amounts of allergens directly to APCs by binding to the asialo-GM-1 receptor, thereby modulating the allergen-specific immune responses towards a tolerogenic state (176). Smits et al. demonstrated the immunomodulatory potential of CTB to suppress allergy through the induction of IgA-secreting B cells (176). In several studies we and

others confirmed the strong benefit of the mucosal adjuvants CTB, compared with administration of the antigen alone (170, 186), already in clinical trials.
According to these promising data we produced a fusion protein composed of the potent mucosal adjuvant CTB genetically linked to the major birch pollen allergen Bet v 1 (Publication III). Intranasal application of the CTB-Bet v 1 fusion protein in a murine model of birch pollen allergy confirmed the immunomodulatory effectiveness of CTB to shift Th2 immune responses towards Th1, accompanied by local induction of potentially protective IgA antibodies.

Taken together, we demonstrated that novel allergen constructs can be successfully administered via the mucosa to prevent allergic sensitizations, and allergic responses at local and systemic sites. These data give further evidence that prophylactic approaches via mucosa might be a promising non-invasive strategy to prevent allergy development in genetic predisposed risk individuals.

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VI. CURRICULUM VITAE

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Education

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	2003 – 2004	diploma work at the Department of Cell Biology , University of Salzburg
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	2004	Graduation to Mag. rer. nat.
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Memberships

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Publications

Schabussova, I., K. Hufnagl, M. L. Tang, **E. Hoflehner**, A. Wagner, G. Loupal, S. Nutten, A. Zuercher, A. Mercenier, and U. Wiedermann. 2012. Perinatal Maternal Administration of Lactobacillus paracasei NCC 2461 Prevents Allergic Inflammation in a Mouse Model of Birch Pollen Allergy. *PLoS One* 7:e40271.

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Congress & Meetings

06/2006 2nd PhD Symposium, Vienna, Austria
Oral presentation

06/2006 XXV. EAACI Congress 2006, Vienna, Austria

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11/2006 40. Jahrestagung der ÖGTP, Linz, Austria
Poster presentation

12/2006 40. Jahrestagung der ÖGAI „Clemens von Pirquet Symposium“, Vienna, Austria
Poster presentation

02/2007 5th EAACI-GA2LEN Davos Meeting 2007, Davos, Switzerland
Oral presentation

06/2007 XXVI.EAACI Congress 2007, Göteborg, Sweden
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02/2008 WIRM II Davos 2008, Davos, Switzerland
Poster presentation

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Oral presentation

12/2008 42. Jahrestagung der ÖGAI, Wien, Austria
Poster presentation

06/2009 5th PhD Symposium, Vienna, Austria
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Oral presentation

11/2009 43.Jahrestagung der ÖGAI, “Karl Landsteiner Symposium“, Salzburg, Austria
Poster presentation

06/2010 XXIX.EAACI Congress 2010, London, Great Britain

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12/2010 44.Jahrestagung der ÖGAI, Wien, Austria

Poster presentation

12/2010 8th SFB Work-in-progress Meeting, Ottenstein, Austria

Oral presentation

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Poster presentation

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Oral presentation