# universität wien

# DISSERTATION

Titel der Dissertation

## „Allergen-specific antibodies"

Verfasserin

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# ACKNOWLEDGEMENTS

# CONTENT

# ZUSAMMENFASSUNG

Typ I Allergie ist eine Überempfindlichkeitsreaktion die ungefähr 25% der Bevölkerung betrifft. Die sofortigen Symptome (z.B. allergische Rhinitis, Konjunktivitis, Asthma oder Anaphylaxie) sind das Resultat der Freisetzung von Entzündungsmediatoren welche durch die Kreuzvernetzung von rezeptorgebundenem IgE auf Effektorzellen (z.B. Mastzellen, basophile Granulozyten) durch das spezifische Allergen induziert wird.

Typ I Allergie wird oft mit Medikamenten behandelt welche die allergischen Symptome lindern können, allerdings nicht auf die zugrundeliegenden Mechanismen abzielen. Die bis jetzt einzige krankheitsmodulierende aber zeitaufwändige Behandlung ist die allergenspezifische Immuntherapie (SIT). Im Verlauf der SIT werden blockierende IgG Antikörper produziert, und deren Induktion wurde als ein Hauptmechanismus für die für die Reduktion der allergischen Entzündung beschrieben. Diese IgG Antikörper verhindern die Mastzell- und Basophilendegranulation, IgE-vermittelte Allergenpräsentation an T Zellen und IgE Produktion durch Kompetition mit IgE um die Allergenbindung.

Ziel der Arbeit war die Analyse allergenspezifischer Antikörper.

Dazu wurden zunächst die krankheitsauslösenden IgE Antikörper sowie die durch SIT induzierten IgG Antikörper charakterisiert.

Manuskript 1 zeigt die Analyse von Antikörperantworten spezifisch für rekombinante Gräserpollenallergene des Lieschgrases in den Seren von zwei Gruppen von Lieschgraspollenallergikern. Die Patienten der ersten Gruppe erhielten eine Behandlung subkutane Injektionstherapie (SCIT) mit an Aluminiumhydroxid adsorbierten Lieschgraspollenextrakten, die Patienten der zweiten Gruppe nur entzündungshemmende Medikamente. Wir zeigen dass nur SCIT aber nicht die Behandlung mit entzündungshemmenden Medikamenten allergenspezifische blockierende IgG Antikörper induziert die in der Lage sind einen Anstieg in der IgE

Produktion zu reduzieren. Weiters berichten wir dass positive Langzeiteffekte mit nur einem Durchgang der SCIT nicht erzielt werden können, wir können damit eine Periode von mehr als zwei Behandlungszyklen als wichtig für den Langzeiteffekt der SIT definieren.

Manuskript 2 analysiert allergenspezifische IgE Antikörper welche aus einem Lieschgraspollenallergiker isoliert worden sind und nun in ein „single chain" Format (ScFv) konvertiert wurden was das Austauschen von schweren und leichten Ketten erleichterte. Im Verlauf der B Zell-Entwicklung wird die Diversität von IgE Antikörpern in einem analogen Prozess zu den weiteren Antikörperisotypen generiert. Dabei ist die zufällige Kombination der schweren und leichten Antikörperketten ein wichtiger diversifizierender Faktor neben V(D)J-Rekombination, junktionaler Diversifikation und dem Hinzufügen von somatischen Mutationen. Wir untersuchten die Bedeutung der schweren und leichten Kette für die Spezifität und Affinität der Antikörper. Es zeigte sich, dass die Spezifität in allergenspezifischen IgE Antikörpern hauptsächlich von der schweren Kette bestimmt wird und dass verschiedene Kombinationen von schweren und leichten Ketten in verschiedenen Affinitäten resultieren können. Dieser letztere Aspekt könnte klinische Bedeutung haben und erklären warum verschiedene Patienten die gegen das gleiche Allergen sensibilisiert sind mit verschiedener Intensität reagieren.

In Manuskript 3 beschreiben wir letztendlich ein Antikörper „single chain" Fragment (ScFv) spezifisch für ein Hauptallergen aus Lieschgras, Phl p 2. Dieses Phl p 2-spezifische ScFv behindert die IgE Bindung von Patienten an das Allergen und könnte damit für therapeutische Zwecke zum Einsatz kommen.

# SUMMARY

Type I allergy represents a hypersensitivity reaction that affects around 25% of the population worldwide. The immediate symptoms (*e.g.* allergic rhinitis, conjunctivitis, asthma or anaphylaxis) are the result of an inflammatory mediator release that is induced by the cross-linking of receptor-bound IgE on effector cells (*e.g.* mast cells, basophils) via the specific allergen.

Type I allergy is often treated with drugs that mitigate allergic symptoms but do not target the underlying mechanisms. The only disease-modifying but time-consuming treatment till now is allergen-specific immunotherapy (SIT). During the course of SIT allergen-specific blocking IgG antibodies are produced. These IgG antibodies inhibit mast cell and basophil degranulation, IgE-facilitated allergen-presentation to T cells and IgE production due to competition with IgE for allergen binding.

The aim of the thesis was to analyze allergen-specific antibodies.

At first, disease-eliciting IgE antibodies as well as SIT-induced IgG antibodies were characterized.

Manuscript 1 describes the analysis of antibody responses specific for recombinant timothy grass pollen allergens in sera from two groups of timothy grass pollen allergic patients. Patients in the first group received one course of subcutaneous injection immunotherapy (SCIT) with timothy grass pollen extracts adsorbed to aluminium hydroxide and patients in the second group received anti-inflammatory treatment. We demonstrate that only SCIT but not anti-inflammatory treatment induced allergen-specific blocking IgG antibodies that were able to reduce boosts of IgE production. Further we report that long-term beneficial effects cannot be obtained with only one course of SCIT and thus define that at least two courses of SCIT are required to obtain long-term beneficial effects.

Manuscript 2 analyzes allergen-specific IgE antibodies that have been isolated from a timothy grass pollen allergic patient and were then converted into single chain

format (ScFv) to be able to generate new heavy and light chain combinations. In the course of B cell development diversity in IgE antibodies is created in an analogous way to the other antibody isotypes. The random combination of antibody heavy and light chains is one important diversity creating factor next to V(D)J-recombination, junctional diversity and the addition of somatic mutations. We investigated the contribution of heavy and light chains for specificity and affinity by shuffling heavy chains with light chains. We report that the specificity in allergen specific IgE is determined mainly by the heavy chain and it was found that different heavy chain-light chain combinations result in different affinities. This latter finding may have clinical implication as it may explain why different patients sensitized to the same allergens show different sensitivity.

Finally, in Manuscript 3 we characterize an antibody single chain fragment that would be suitable for clinical application. This fragment is directed against one of the major timothy grass pollen allergens, Phl p 2, and inhibits patients' IgE binding to the allergen.

# 1. Some milestones in allergy research

| | |
|---|---|
| 1906 | Clemens von Pirquet creates the term allergy [1]. |
| 1911 | Leonard Noon introduces immunotherapy with pollen extracts in hay fever patients [2]. |
| 1921 | Otto Carl W. Prausnitz and Heinz Küstner describe the passive transfer of immediate skin reactivity by injection of serum from allergic patients ("reagins") [3]. |
| 1935 | Robert A. Cooke describes the development of blocking antibodies in the course of immunotherapy [4]. |
| 1940 | Mary Hewitt Loveless characterizes blocking antibodies [5]. |
| 1963 | Robin A. Coombs and Philip George Houthem Gell classify hypersensitivity reactions [6]. |
| 1966 | Kimishige and Teruko Ishizaka characterize reagins as IgE [7]. Hans Bennich and Gunnar Johansson identify myeloma ND as IgE [8, 9]. |
| 1988 | First successful cloning of allergen-specific cDNA [10, 11]. |
| 1991 - 92 | First usage of recombinant allergens for *in vitro* allergy diagnosis [12-14]. |
| 1993 - 97 | Engineering of recombinant hypoallergens for allergy vaccination [15-19]. |
| 1996 | First population studies with recombinant allergens [20]. |
| 2002 | Allergy diagnosis with microarrayed recombinant allergens [21]. |
| 2001 - 04 | First immunotherapy trial with recombinant hypoallergens [22]. |
| 2002 - 05 | First immunotherapy trial with recombinant wild-type allergens [23, 24]. |

## 2. The term "Allergy"

*"What we need is a new generalized term, which prejudices nothing but expresses the change in condition which an animal experiences after contact with any organic poison, be it animate or inanimate… For this general concept of changed reactivity, I propose the term Allergy… The term Immunity must be restricted to those processes in which the introduction of the foreign substance into the organism causes no clinically evident reaction, where, therefore, complete insensitivity exists.[1]"*

(Clemens von Pirquet 1874-1929)

Clemens von Pirquet was an Austrian paediatrician working at the Paediatric Department in Vienna. Together with Bela Schick he observed that some patients who received antiserum developed a series of systemic and local symptoms which they termed "serum sickness". Similar findings have been recorded for diphtheria and tetanus antisera. Obviously, serotherapy not only produced immunity (protection) but also hypersensitivity. Both situations had in common that an external agent induced "changed or altered reactivity". Von Pirquet created the term "Allergy" from the Greek *allos* ("other") and *ergon* ("work") to describe a deviation from the original state. Today the definition of allergy is often used synonymously with the more specific IgE-mediated allergy.

## 3. The Coombs and Gell classification of hypersensitivity reactions

The classification of type I-IV hypersensitivity reactions described by Coombs and Gell [6] in 1963 is in a slightly modified version still in use today since it groups allergic reactions according to fundamented pathomechanisms.
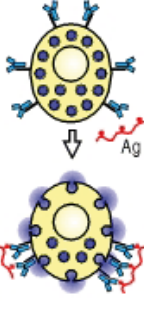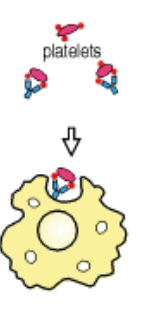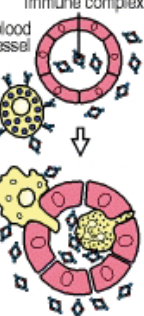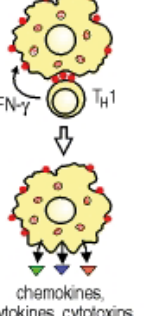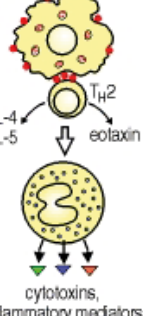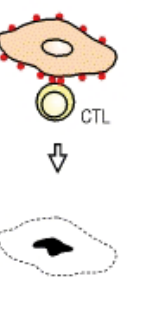
| | Type I | Type II | Type III | Type IV | | |
|---|---|---|---|---|---|---|
| Immune reactant | IgE | IgG | IgG | $T_H1$ cells | $T_H2$ cells | CTL |
| Antigen | Soluble antigen | Cell- or matrix-associated antigen | Soluble antigen | Soluble antigen | Soluble antigen | Cell-associated antigen |
| Effector mechanism | Mast-cell activation | FcR+ cells (phagocytes, NK cells) | FcR+ cells Complement | Macrophage activation | Eosinophil activation | Cytotoxicity |
| | | | | | | |
| Example of hypersensitivity reaction | Allergic rhinitis, asthma, systemic anaphylaxis | Some drug allergies (e.g., penicillin) | Serum sickness, Arthus reaction | Contact dermatitis, tuberculin reaction | Chronic asthma, chronic allergic rhinitis | Contact dermatitis |

Figure 1. Type I-IV hypersensitivity reactions [25]

### 3.1. Type I or immediate hypersensitivity reaction

Type I allergy is a classical IgE-mediated disease affecting around 25% of the population worldwide [26, 27]. There are several explanations for the worldwide epidemic of allergic disease. Increased hygiene conditions lead to decreased stimulation of the immune system in critical periods of development [28]. Air pollution due to urbanization which *e.g.* leads to high levels of vehicle emissions [29] as well as

climate change and global warming [30] also increase the susceptibility to develop allergies.

The ability of allergic individuals compared to non-allergics to produce IgE antibodies (*i.e.*, atopic condition) depends also on genetic predisposition [31]. In general, the personal or familial tendency to produce IgE antibodies has been defined by the term "atopy".

### 3.1.1. Sensitization
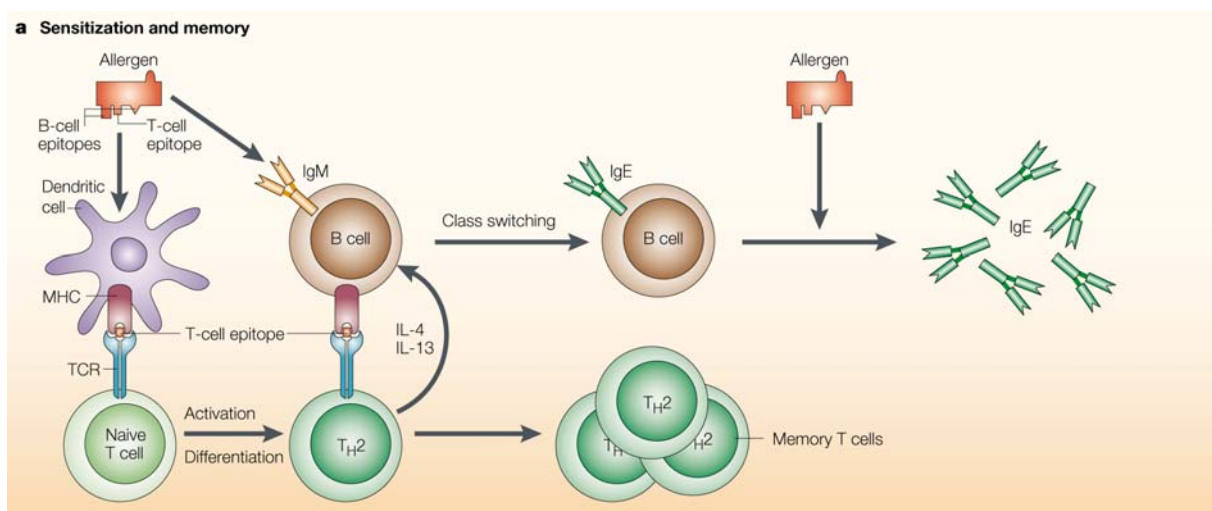


Figure 2. Sensitization [33]

Allergic sensitization happens in early childhood and it is the first immune response which leads to the development of IgE-mediated allergy [32]. It is caused by minute amounts of allergens that in principal do not show harmful properties. However, in allergic individuals the first allergen contact that happens in the case of respiratory allergies on mucosal sites leads to allergen uptake by antigen-presenting cells like dendritic cells. Further, allergen-specific $TH_2$ cells are primed that can help to activate allergen-specific B cells via T cell-B cell interaction. These B cells induce class-switch recombination to IgE under the influence of the $TH_2$ cytokines IL-4 and IL-13 as well as secondary signals coming from T cells. Resulting from this

sensitization process, allergic patients produce allergen-specific IgE antibodies via their plasma cells that have evolved from B cells [33, 34].

### 3.1.2. The immediate phase reaction

These IgE antibodies bind to their high affinity receptors FcεRI that are situated amongst others on mast cells and basophils. A repeated contact with the allergen leads to the cross-linking of allergen-bound IgE antibodies on effector cells. A cascade of following events results in the release of preformed inflammatory mediators like histamine and leukotrienes that elicit within minutes allergic symptoms such as rhinitis, conjunctivitis or asthma [33, 34].
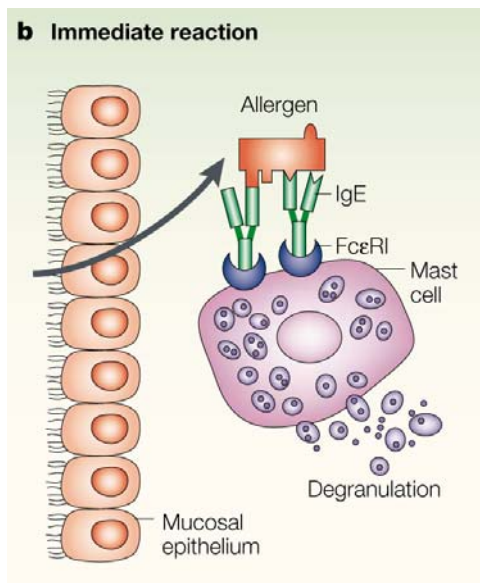
Figure 3. Immediate reaction [33]

### 3.1.3. The late phase reaction

Late phase reactions are described in allergic patients who suffer from chronic manifestations of atopy. They are caused by strong activation of allergen-specific T cells as well as by eosinophil activation. This occurs after hours to days after allergen contact. The whole process is caused by the presentation of allergen to allergen-specific T cells that are activated, proliferate and produce proinflammatory cytokines like IL-4, IL-5 and IL-13. These TH2 cytokines are responsible for the induction of tissue eosinophilia and finally the production of inflammatory mediators by eosinophils. The participation of IgE may have an enhancing effect in this process as

IgE bound to FcεRI or FcεRII on antigen presenting cells is able to ease allergen uptake and therefore facilitates allergen presentation to T cells [35].
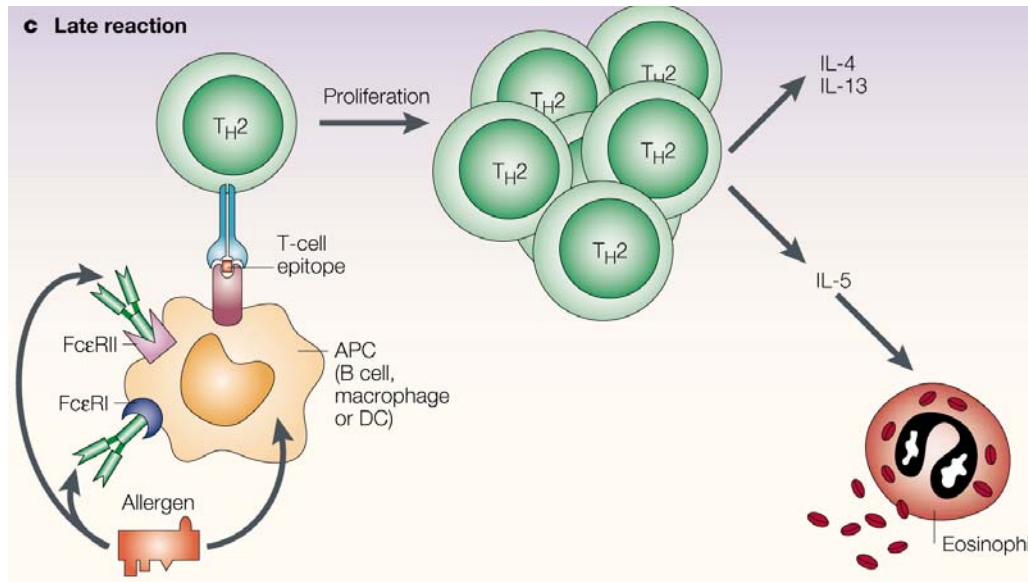


Figure 4. Late phase reaction [33]

3.2. Type II or antibody-mediated hypersensitivity reaction

Type II reactions are mediated by IgG but also IgM antibodies that target cell- or matrix-associated antigens. This opsonisation leads to cellular destruction via Fc-mediated effector mechanisms that include phagocytosis by macrophages or neutrophils, complement activation by the classical pathway or antibody-dependent cellular cytotoxicity mediated by macrophages and NK cells. The most common causes for Type II hypersensitivity reactions are some medications like penicillin, cephalosporins, hydrochlorothiazide which become associated with red blood cells or platelets [36].

## 3.3. Type III or immune complex-mediated hypersensitivity reaction

In this reaction, antibodies (IgG) bind soluble antigens thereby forming immune complexes that become deposited in tissues where they initiate inflammatory reactions. Effector mechanisms are again Fc-mediated and include complement activation as well as the activation of FcR+ cells resulting in tissue edema, cell recruitment and finally tissue destruction. Examples for Type III reactions comprise serum sickness and Arthus reaction [36].

## 3.4. Type IV or delayed-type hypersensitivity reaction

Type IV reactions are in contrast to the other three hypersensitivity reactions mediated by T cells that are exposed to an antigen during sensitization and develop adaptive responses. These reactions can be subdivided into three disease patterns. In the classical reaction Th1 cells are activated. They home to the site of antigen exposure and there they are stimulated to induce a Th1 response. This leads to tissue inflammation mediated primarily by macrophages. Tuberculin skin reaction, contact dermatitis or rheumatoid arthritis are examples for classic reactions.

The second disease pattern is cell-mediated eosinophilic hypersensitivity which was already described before in section 3.1.3.

The third type leads to tissue injury by cytotoxic T lymphocytes that recognize cell-associated antigens, a prominent example would be graft rejections or contact dermatitis [36].

# 4. The mediators of type I allergy

## 4.1. The IgE molecule and its receptors

### 4.1.1. IgE

#### 4.1.1.1. Historical aspects

The experiments by Prausnitz and Küstner (1921) [3] demonstrated for the first time that there is a blood factor that is able to transfer sensitivity to allergens. More than fourty years later (1966) this factor was described by Kimishige and Teruko Ishizaka as a novel class of antibody that acts "as a carrier of reaginic activity" [7]. At this time, Gunnar Johansson and Hans Bennich discovered a myeloma protein (IgND) which did not belong to any of the other immunoglobulin classes, and an antiserum prepared against it had the same specificity as the anti-IgE prepared by Ishizaka [9]. In 1968 it was agreed to call the new class of protein "IgE" [8].

#### 4.1.1.2. Generation of the IgE repertoire

The IgE repertoire is formed similar as the other antibody isotypes by a series of diversity creating factors. Initially, combinatorial diversity is created by heavy chain gene arrangement at chromosome 14. Thereby one family member per V, D and J gene family is linked to each other [37]. Few studies exist regarding the detailed analysis of germ line gene usage in allergen-specific IgE antibodies. They reveal a broad usage of VH genes in IgE molecules [38-42].

Further variability in the IgE repertoire is achieved by junctional diversity that happens via the changing of sequences in junctional regions between V, D and J genes and by the pairing of antibody heavy and light chains.

This random pairing is induced during B cell development via the signalling of the pre-B cell receptor which consists of a functional heavy chain paired with a surrogate light chain. Signalling results in kappa light chain gene rearrangement on chromosome 2 in pre-B cells. The generated kappa light chain binds to the heavy chain. If this combination fails, lambda light chain gene rearrangement on chromosome 22 is induced. Only if the chains are compatible, a functional immunoglobulin is expressed on the cell surface that then undergoes selection processes [37]. Already from this development process in B cells one could speculate that the heavy chain plays a dominant role in a functional antibody molecule. Previous studies in human IgE antibodies indicate the importance of the heavy chain for the determination of binding specificity [40, 41, 43]. On the other hand, allergen-specific IgE molecules have been described where the same light chain combines with different heavy chains and therefore seems to be the specificity determining factor [44, 45].

Manuscript 2 addresses the importance of heavy chain and light chain contribution for the determination of binding specificity and binding affinity in allergen-specific human IgE antibodies.

The final step in creating diversity in antibodies is obtained by the introduction of somatic mutations into germ line genes [37]. in fact, the analysis of allergen-specific IgE Fabs revealed a varying degree of somatic mutations in the VH regions [42], interestingly these regions remain unchanged in patients after several years of allergen exposure [42].

All B cells first express IgM whereas the $V_H$ region is linked to the $C_\mu$ gene. Other $C_H$ genes are arranged downstream on chromosome 14 and can be recombined with the expressed $V_H$ region in class switch recombination [37]. The isotype class switch to IgE, a process that is induced via $TH_2$ cytokines IL-4 and IL-13 as well as secondary

signals coming from T cells. Some authors believe that this happens in the target organs of allergic disease [46, 47].

### 4.1.1.3. Structure of IgE

The basic structure of the IgE molecule is very similar to that of other antibody classes. It consists of two antigen-binding Fab regions and a receptor-binding Fc part that in contrast to IgG has no flexible hinge region but an additional constant domain (Cε2) in each heavy chain. The antigen-binding sites in the V domain are represented by three adjacent hypervariable loops per heavy and light chain that are described as complementary determining regions (CDRs). Their DNA sequences are very susceptible to allergen-triggered somatic hypermutation as described before which leads to affinity maturation and further increases diversity.

### 4.1.2. IgE receptors

Two cell surface receptors have been identified. The high affinity receptor FcεRI is found amongst others on mast cells and basophils. Cross-linking of FcεRI-bound IgE by complex allergens is responsible for the induction of the immediate phase reaction in type I allergy. The binding of FcεRI to IgE happens with an affinity ($K_A$) of $10^{10}$ $M^{-1}$, which is higher then that of the other antibodies for their receptors [48]. FcεRI consists of four or three chains on human cells. The form containing four chains is expressed on effector cells of allergic responses such as mast cells and basophils [49] and comprises an IgE binding α chain (FcεRIα), a β chain signalling subunit (FcεRIβ) and two γ chain signalling subunits (FcεRIγ) that exist as a homodimer. Both β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAM) for signal transduction [50]. While the γ subunits are essential for surface expression and signal transduction, the β chain principally acts as amplifier for both functions. The form

consisting of three chains lacks the β subunit and is found on antigen-presenting cells.

The second receptor is termed FcεRII or CD23. It may appear as soluble fragments (sCD23) which are released from the cell membrane by proteolysis or as membrane-bound CD23 which belongs to the family of trimeric cell-surface receptor molecules. It binds IgE with lower affinity ($K_A \approx 10^8$ M$^{-1}$) than FcεRI and was first identified on B cells but was also found on a variety of inflammatory cells and follicular dendritic cells. It plays a major role in the induction of allergic late phase reaction as binding of loaded IgE to CD23 on antigen-presenting cells may lead to allergen uptake and further to the presentation of allergen-derived peptides to allergen-specific CD4+ T cells [35] that are responsible for late phase inflammation.

## 4.1.3. The binding of IgE to its receptors

The binding of IgE to its receptors was studied in great detail. The binding site for FcεRI was evaluated using recombinant fragments [51] and by solving the crystal structure of the complex between Fcε3-4 and the soluble FcεRI alpha chain (sFcεRIα) [52] which revealed that the high affinity receptor binds to both Cε3 domains. Further, an NMR study with the Cε2-region and sFcεRIα identifies also a cluster of amino acids in the Cε2-region that make contact with the receptor [53]. Spectroscopic studies in solution revealed that IgE – in free condition or bound to its high affinity receptor – has a bent structure [54] in which the Cε2-regions are bent back onto the Cε3-regions [55].
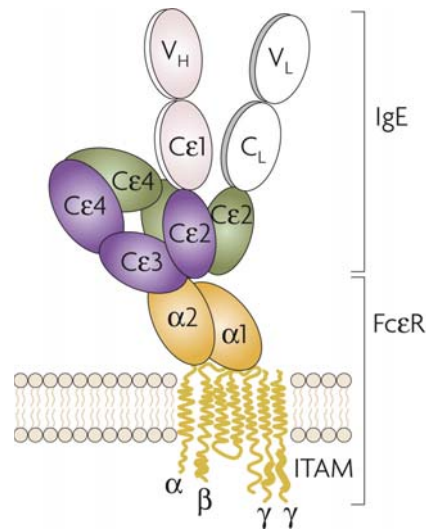
Figure 5. Schematic representation of IgE binding to the FcεRI [56].

Successful binding but also disengagement requires conformational changes in the IgE molecule (in the Cε2 and Cε3 region) and in the high affinity receptor. This may be one of the reasons for the remarkably slow dissociation rate of IgE from its high affinity receptor, which is at least three orders of magnitude slower than that of IgG for its receptor [57].
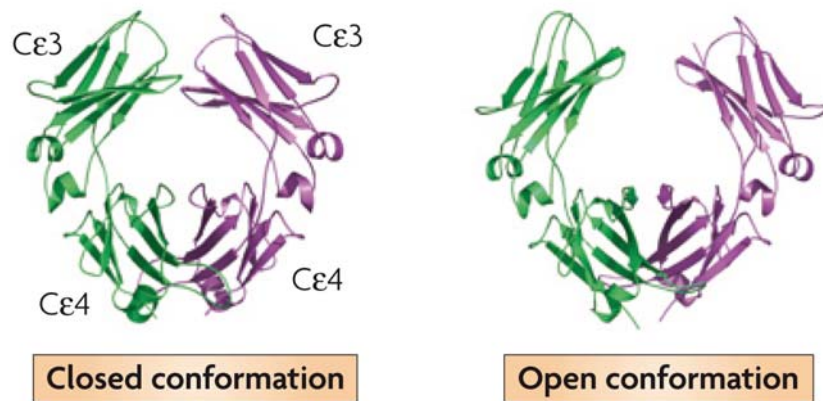


Figure 6. The conformations of the IgE-Fc fragment.

Structure of the uncomplexed Fcε3-4 fragment showing the Fcε3 domains in closed conformation (left) and in open conformation (right) after binding to the FcεRI alpha chain [56].

The complex between CD23 and IgE has not been solved so far with X-ray crystallography or NMR. However, a panel of experiments have been performed that mapped the binding site in the Cε3 region of IgE [58-60].

4.2. Mast cells

Mast cells (MC), like other myeloid cells, derive from the bone marrow. They are found near blood and lymphatic vessels, nerve fibres and of a range of immune cells. In addition to their potential physiological roles (*e.g.* host defence [61, 62], repair mechanisms [63, 64]) they are key players in IgE-mediated allergic disease. Human mast cells can be divided according to the expression of the proteases into $MC_T$ (tryptase) and $MC_{TC}$ (tryptase, chymase).

The best studied activation of MC is induced via the engagement of a multivalent allergen to FcεRI-bound IgE which leads to receptor crosslink. This process ends in the induction of a signalling cascade which results in two waves of mediator release. Initially, insoluble granules and their associated preformed mediators are exocytosed within seconds, a process that is known as degranulation. Some mediators such as histamine become released immediately; others remain in insoluble particles (heparin). Proteases are the most abundant mediators associated with MCs. Beta-tryptase, the major protease released in this activation process, is described to act as a natural mechanism for controlling allergic inflammation as it was shown to cleave FcεRI-bound IgE [65]. Further, the *de novo* production of mediators leads to the rapid synthesis of prostaglandins and leukotrienes and in a second wave to the expression of cytokines and chemokines [66].

## 4.3. Allergens

### 4.3.1. General description

Almost all clinically relevant allergens are soluble proteins or glycoproteins with a molecular mass of 5 – 80kDa. IgE responses are therefore initiated in a T cell dependent manner [67]. A great panel of these protein allergens have been characterized in great detail concerning their three-dimensional structure and also their biological function [33]. An example for the relation of function and allergenic activity delivers the major house-dust mite pollen allergen Der p 1. This allergen acts as a protease and is able to cleave immune cell-surface structures or to damage respiratory epithelial cell layers [68, 69]. However, allergens have also other biological functions which lack the connection to allergenic activities [67, 70].

IgE responses can also be induced to carbohydrate moieties. In these cases T cell help is provided by peptides that are derived from the protein backbone [71]. Although carbohydrates are able to bind IgE they are weak inducers of mast cell or basophil degranulation [72] and may therefore be defined as "IgE reactive antigens" in contrast to "allergens". IgE reactivity to cross-reactive carbohydrates has been described as a problem for allergy diagnosis. In insect venom allergy, the usage of recombinant allergens that lack the cross-reactive carbohydrates has been proposed to overcome these diagnosis problems [73].

Mast cell and basophil degranulation is induced by allergens and requires the presence of at least two epitopes for IgE binding on the allergen. More precisely, the extent of degranulation depends on the number of epitopes and the levels of specific IgE [74]. It has already been demonstrated that many important allergens contain several different IgE epitopes [75, 76], and clustering of these epitopes on the allergens may facilitate receptor crosslink [77].

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Basically, two types of epitopes have been determined. Epitopes that consist of a stretch of amino acids are termed sequential whereas those that include at least two different areas of the molecule which need to be brought together belong to the conformational type. The epitopes on most allergens are created via their three-dimensional architecture and are therefore conformational [67]. Sequential epitopes in allergy are also described, but mainly in food allergens [78-81].

The three-dimensional structures of allergens are very diverse. Whereas some allergens are composed of α-helices (*e.g.* Phl p 5 [82]) or β-sheets (*e.g.* Phl p 2 [83]), others comprise a mix of both structures (*e.g.* Phl p 1, PDB entry 1N10). Allergens can exhibit structural similarities with each other or with human proteins, these accounts for the phenomenon of cross-reactivity or autoimmunity [67].

### 4.3.2. Allergen sources

Allergic patients from different parts of the world are exposed to different allergen sources depending on climate, culture or socioeconomic factors. These allergen sources may vary regarding their allergen composition. Whereas *e.g.* sources from birch pollen [84] contain one major allergen that is recognized by the majority of the patients, other sources contain multiple different important allergens present in varying amounts. The allergenic potential of allergen sources is not only determined by the allergen itself but also by the release from the pollen grain. As shown for grasses and birch, pollen rupture in moisture and form respiratory particles that according to their small size should deposit easily in the human airways [85, 86]. The release of allergen from the pollen also happens very quickly after direct contact with the mucosa [87, 88]. After their release it is possible that allergens are bound to certain materials like diesel exhaust particles which may act as carriers or may have adjuvant activity and thus augment $Th_2$ responses [89, 90]. Further, allergen sources

have been shown to include other substances like phytoprostanes that promote the development of allergic disease [91].

# 5. Allergen-specific immunotherapy

## 5.1. General aspects

Allergen-specific immunotherapy (SIT) is defined as the repeated administration of the sensitizing allergen. It is performed by subcutaneous injection (SCIT), and also by alternative routes such as sublingual application (SLIT). The first description of SIT goes back to 1911 where Leonard Noon performed subcutaneous injection of grass pollen extract in order to prevent allergy to grass pollen [2]. In larger clinical trials performed with John Freeman he reported an improvement in allergic symptoms that could be maintained for one year [92].

SIT is the only causative way of allergy treatment that has a disease-modifying effect and induces active immunity [34]. It improves the quality of life of treated patients by reducing symptoms of allergy and the use of rescue medication. SIT is connected with improved tolerance to allergen challenge and it has been described to be long-lasting. In the case of the subcutaneous administration, clinical effect can be reported after three years of discontinuation or even longer [93-96]. Long-lasting effects have also been described concerning the disease-modifying effect of SCIT like the prevention of asthma [97-99]. All these effects have been achieved by the performance of a treatment protocol that last for three years, however it still needs to be determined whether shorter treatment periods would also be beneficial. Therefore, manuscript 1 addresses this question and analyzes sera from grass pollen allergic patients who received one course of SCIT in comparison to sera from patients who received only anti-inflammatory treatment. Immunological alterations and resulting clinical implications are discussed before and after SCIT and after five years of discontinuation [100].

## 5.2. Sublingual versus subcutaneous immunotherapy

The sublingual administration of allergen (SLIT) has recently been focused as an alternative way to the subcutaneous route because it has the advantage of self-administration. As underlying immunological mechanisms increases of specific IgG, and moreover increases of blocking antibodies in patients' sera are reported [101]. The effect of SLIT on T cell responses is controversial and ranges from reduced proliferation in response to allergen to failure in the detection of changes of T cell parameters [102, 103]. SLIT further reduces the number of infiltrating inflammatory cells like eosinophils and neutrophils [104].

Although SLIT seems to have some clinical benefits [101], the effects were moderate compared to those obtained by subcutaneous administration of allergen preparation.

## 5.3. From allergen extracts to recombinant allergens and allergen derivatives

SIT with natural extracts as introduced by Leonard Noon [2] is still performed today but has several disadvantages. One of the main problems is that the administration of allergen extracts leads to local and systemic side effects [67] (urticaria, asthma shock, anaphylactic shock). Therefore, several attempts have been made to increase their efficacy and reduce these side effects. Allergen extracts were adsorbed to adjuvants in order to immobilize the allergens and increase their immunogenicity [105], extracts were chemically modified in order to reduce their allergenic activities [106, 107] or different administration routes were considered.

Another critical point in the usage of allergen extracts is their varying allergen content [108-111]. Therefore, various forms of standardization have been considered in order to improve the quality of the extracts like determination of protein contents, assessment

of biological activity or antibody-based measurement of allergens. Companies marketing allergen extracts for therapy or diagnosis still use different in-house methods that are displayed by company-specific units, therefore it is impossible to compare products from diverse companies [112].

Nevertheless, the problem of underrepresentation of certain allergens in the preparation still remains [110, 111]. Commercial allergen extracts have also been described to contain allergens from other sources [113], contaminations with endotoxin, [114] or other undefined compounds [91]. Therefore, the preparation of defined, pure proteins as recombinant allergens offers a promising alternative.

Since 1988 [10, 11], a lot of effort has been put into the recombinant expression of allergens from varying sources, and today recombinant allergens for the most important allergen sources have become available [115]. With these tools the reactivity profiles from allergic patients can be determined by component-resolved diagnosis, and the disease-eliciting allergen molecules can be identified [116]. Consequently, patients can be selected more efficiently for immunotherapy. The monitoring of antibody responses in allergic patients has greatly been facilitated by the introduction of multi-allergen test systems based on micro-array technology [21].

Basing on recombinant DNA technology it is possible not only to work with pure allergens showing the same characteristics as their natural counterparts, but also to modify the molecules in order to obtain hypoallergenic variants with reduced IgE reactivity. The first generation of variants was made to preserve allergen-specific T cell epitopes and induce allergen-specific IgG that inhibit IgE recognition [117]. Therefore, strategies have been developed that succeed in the destruction of conformational IgE epitopes. These include fragmentation [18], denaturation of the recombinant wild-type allergen (folding variant) [118] or recombination of allergen fragments to form mosaic molecules [119]. Site-directed mutagenesis may also be used

to reduce IgE reactivity [17] and is has been found that recombinant oligomerization of allergens results in the loss of allergenicity by altered presentation of IgE epitopes although IgE reactivity could be preserved [120].

Today, another strategy arises with the aim to eliminate IgE- or T cell-mediated side-effects. This approach is based on allergen-derived peptides that originate from conformational IgE binding sites of the allergen and lack IgE reactivity and are fused with nonallergen-derived carrier proteins (*e.g.* viral proteins). These vaccines focus on the production of IgG antibodies directed against IgE binding sites without activating allergen-specific T cells as T cell help is provided by the epitopes on the carrier protein [121, 122].
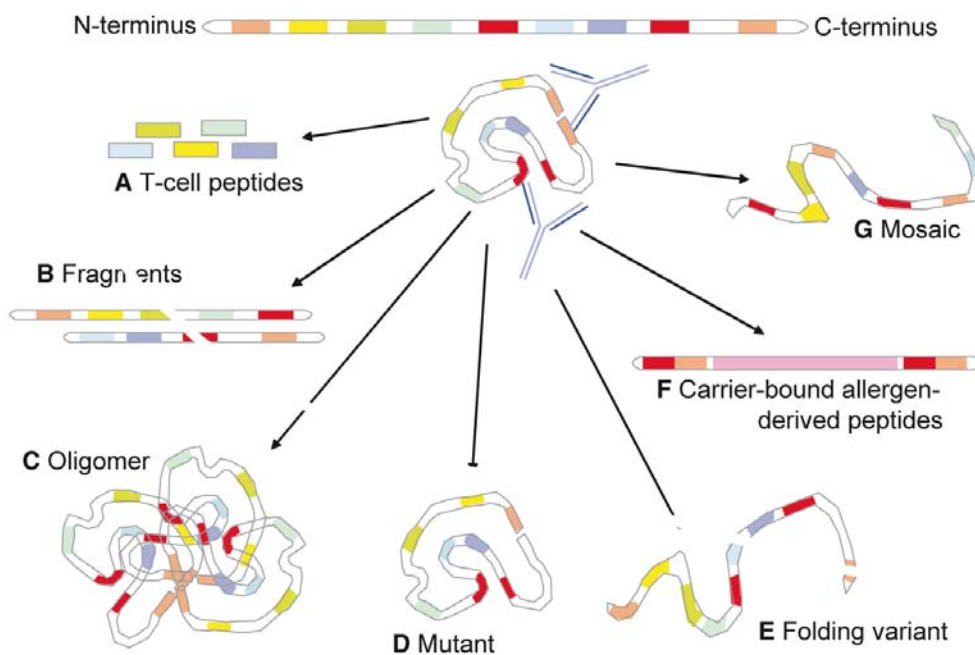


Figure 7. Generation of hypoallergenic variants [117].

The first immunotherapy trials that were performed in allergic patients were interestingly conducted with recombinant hypoallergenic allergen derivatives [22] and then continued with a recombinant hypoallergenic folding variant [118] of the major birch pollen allergen, Bet v 1. Beside these trials, alternative approaches were

started that used recombinant wild-type allergens for administration to allergic patients [23, 24]. All of these trials showed successful outcomes in terms of immunological and clinical parameters.

5.4. Effects of allergen-specific immunotherapy
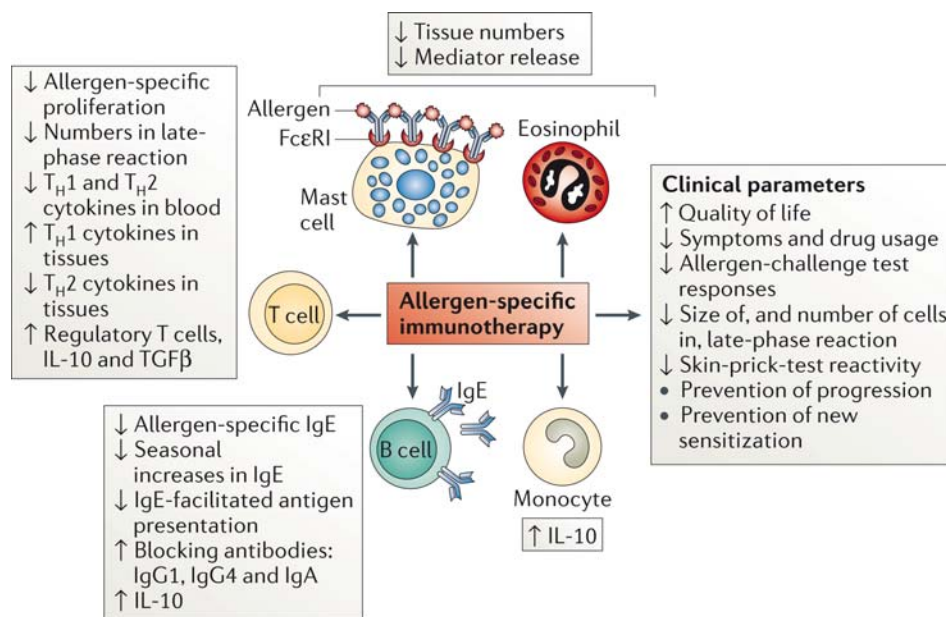


Figure 8. Effects of allergen-specific immunotherapy on clinical and experimental immune parameters [34].

The underlying mechanisms in SIT seem to be diverse and besides induction of allergen-specific blocking IgG may include effects on antigen presenting cells, T cells and B cells [34].

## 5.5. Blocking antibodies

It was already demonstrated in 1935 by experiments from Robert A. Cooke that SIT-treated patients develop allergen-specific factors that prevent immediate allergic skin inflammation [4]. These factors were then reported to belong to the IgG class and to bind to the very same allergen thereby preventing the binding of the sensitizing antibody [5, 123] and thus were termed „blocking antibodies".

However, not every IgG is a blocking one and therefore the induction of IgG in the course of an immunotherapy trial is no sufficient parameter to evaluate the trial outcome. Former studies tried to correlate serum IgG concentrations with a positive clinical outcome and came to different conclusions [124, 125].

In general, the SIT-induced IgG response is described as very heterogeneous concerning epitope specificity. To show a blocking effect, IgG has to be directed directly to the very same epitope as IgE thereby competing with IgE for allergen binding or IgG has to bind in close vicinity of the IgE epitope to hamper IgE binding via steric hindrance. IgG directed against other epitopes may fail to inhibit IgE binding and it has even been reported that IgG enhances binding of IgE to the allergen maybe due to the induction of conformational changes in the allergen [126, 127].

## 5.6. Mechanisms of allergen-specific blocking antibodies
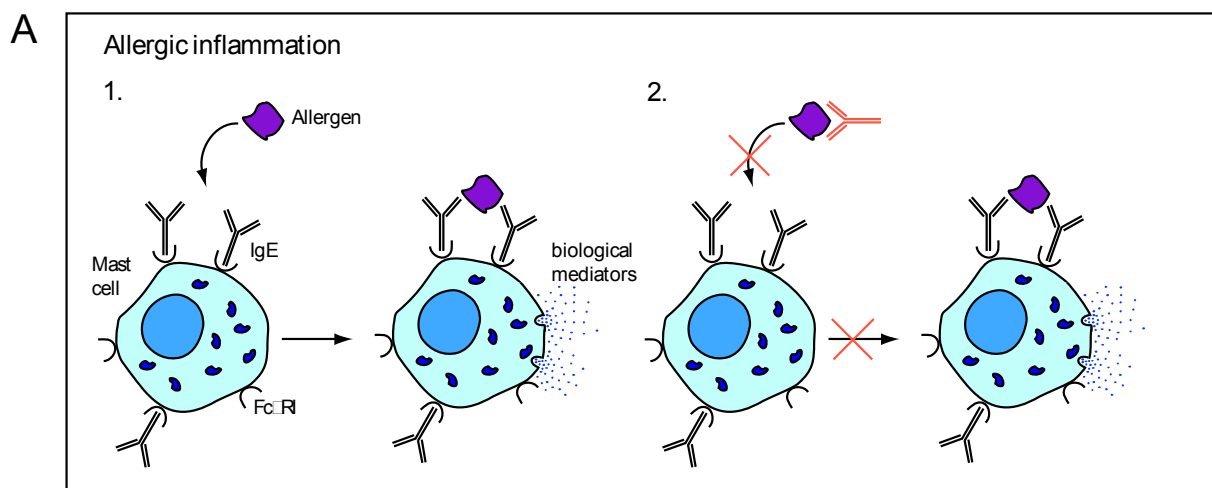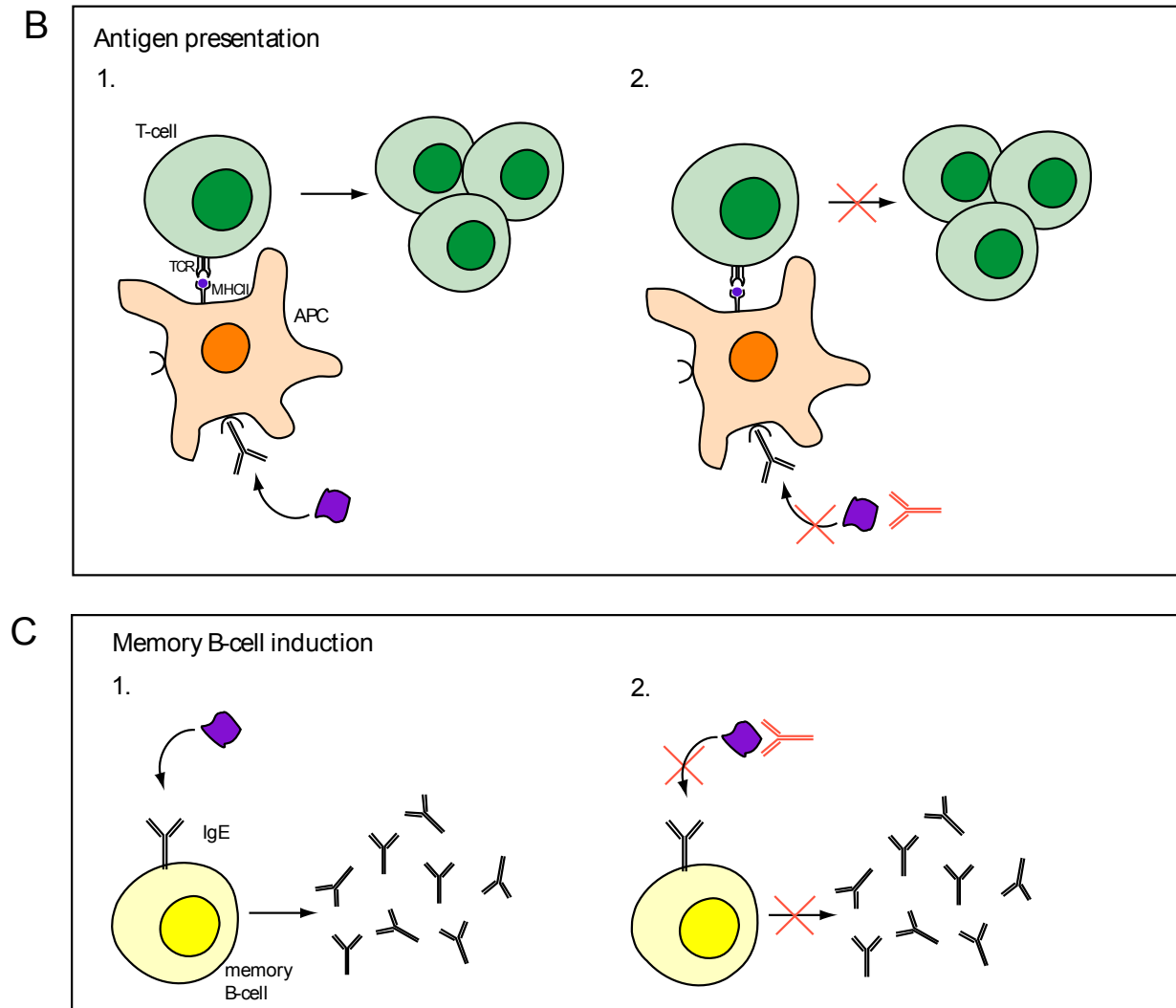
Figure 9. Three different modes of action for allergen-specific blocking IgG antibodies [128].

Allergen-specific blocking IgG antibodies have been shown to operate in different ways. They inhibit allergen-induced mast cell and basophil activation which result in reduced immediate phase reactions (Figure 9A) [128]. There are several clinical trials which show that SIT-induced IgG were able to suppress immediate allergic inflammation [22-24]. It was further shown that SIT induces IgG mainly of the subclasses $IgG_1$ and $IgG_4$ that were directed against new epitopes [129, 130] and that were able to block IgE binding and further cross-react with structurally related allergens [22, 130].

Moreover, blocking IgG antibodies inhibit IgE-facilitated allergen presentation of antigen presenting cells (APC) to T cells [131, 132] (Figure 9B). This results in the decreased activation of T cells that play a crucial role on the one hand in the

induction and maintenance of allergic disease and on the other hand in late phase responses.

Finally, blocking IgG can suppress the boost of memory IgE production induced by allergen contact (Figure 9C) [22, 133, 134].

## 5.7. Allergen-specific blocking antibodies for allergy treatment

As blocking IgG show a central role in the treatment of allergic inflammation they may be used for passive immunization [128]. Today, techniques and materials for the isolation and construction of allergen-specific therapeutic IgG are available. Hybridoma technology [135], combinatorial cloning and phage display [136-138] in combination with the now existing clinically relevant major purified allergens that are used for isolation [139, 140] have enabled the generation of allergen-specific recombinant antibodies. Therapeutic IgG would be especially suitable for the treatment of seasonal allergies like grass or tree pollen allergies due to their long serum half life of approximately 21 days. An example for a possible allergen-specific therapeutic IgG is the already described anti-Phl p 2 $IgG_1$. This antibody has been isolated from a combinatorial IgE Fab library and was converted into a whole $IgG_1$. It is directed against one of the major timothy grass pollen allergens, Phl p 2 [43, 141]. In manuscript 3 we suggest a variant of this antibody for topical application. Therefore, an easy to produce single chain version of anti-Phl p 2 $IgG_1$ with low immunogenicity was constructed and characterized. It inhibited the binding of patients' IgE to the allergen and could therefore be a suitable tool for clinical application.

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Type I allergy represents a hypersensitivity reaction that affects around 25% of the population worldwide. The immediate symptoms are the result of an inflammatory mediator release induced by the cross-linking of receptor-bound IgE on effector cells by the specific allergen.

The only disease-modifying but time-consuming treatment till now is allergen-specific immunotherapy (SIT). During the course of SIT allergen-specific blocking IgG antibodies are produced which act by inhibiting mast cell and basophil degranulation, IgE-facilitated allergen-presentation to T cells and IgE production due to competing with IgE for allergen binding.

The aim of the thesis was the analysis of allergen-specific antibodies.

At first, manuscript 1 describes allergen-specific IgE- as well as SIT-induced IgG antibodies. In an observational study we show the dissection of antibody responses specific for recombinant timothy grass pollen allergens in sera from two groups of timothy grass pollen allergic patients. Patients in the first group received one course of subcutaneous injection immunotherapy (SCIT) with timothy grass pollen extracts adsorbed to aluminium hydroxide whereas patients in the second group received anti-inflammatory treatment. We demonstrate that allergen-specific blocking IgG antibodies that were able to reduce boosts of IgE production were only induced during SCIT but not during anti-inflammatory treatment. Further we report that long-term beneficial effects cannot be obtained with only one course of SCIT.

Next, manuscript 2 analyzes allergen-specific IgE antibodies that have been isolated from a timothy grass pollen allergic patient by combinatorial cloning and were then converted into single chain format (ScFv) to be able to generate new heavy and light chain combinations. We investigated the contribution of heavy and light chains for specificity and affinity by shuffling heavy chains with light chains. We show that the

specificity in allergen specific IgE is determined mainly by the heavy chain and that different heavy chain-light chain combinations result in different affinities.

Finally, in Manuscript 3 we characterize an antibody single chain fragment that is directed against one of the major timothy grass pollen allergens, Phl p 2, and would be suitable for clinical application.

**Manuscript 1**

Gadermaier E, Staikuniene J, Scheiblhofer S, Thalhamer J, Kundi M, Westritschnig K, Swoboda I, Flicker S, Valenta R. **Recombinant allergen-based monitoring of antibody responses during injection grass pollen immunotherapy and after 5 years of discontinuation**. *Allergy.* 2011;66:1174-1182.

Manuscript 1 describes an observational study where sera from two groups of grass pollen allergic patients were analyzed. The first group received one course of subcutaneous injection immunotherapy (SCIT) with timothy grass pollen extracts adsorbed to aluminium hydroxide while the second group obtained anti-inflammatory treatment. Sera were analyzed before and directly after treatment as well as after five years of discontinuation for $IgG_{1-4}$, IgA, IgM, IgE and kappa and lambda light chain responses to a panel of recombinant timothy grass pollen allergens. We report an induction of $IgG_1 > IgG_4 > IgG_2 > IgA$ specific for the major timothy grass pollen allergens, an inhibition of allergen-induced basophil degranulation only with sera that contain therapy-induced IgG, and further a reduction of IgE levels as well as a reduction of symptom and medication scores in the SCIT group after one course of immunotherapy but not in the group of patients who received anti-inflammatory treatment. After five years of treatment, antibody levels had returned to baseline

levels and there was no difference anymore in the SCIT and anti-inflammatory group regarding symptoms and medication.

**Contribution to Manuscript 1**:

ELISA measurements of $IgG_{1-4}$, IgA and IgM antibodies and of kappa and lambda light chains specific for a panel of recombinant allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 7, Phl p 12, Phl p 13), quantitative RAST-based non-denaturing dot blot assay for the determination of IgE reactivity to the same panel of allergens, analyzes of the data, writing of the manuscript.

**Manuscript 2**

Gadermaier E, Flicker S, Lupinek C, Steinberger P, Valenta R. **Determination of allergen-specificity by heavy chains in grass pollen allergen-specific IgE antibodies.** *J Allergy Clin Immunol.* In revision.

Manuscript 2 describes the conversion of human IgE Fabs which have been isolated from a timothy grass pollen allergic patient via combinatorial cloning and which are specific for the three non-cross reactive major timothy grass pollen allergens Phl p 1, Phl p 2 and Phl p 5 into single chain format (ScFv). Additionally, the heavy chains specific for one allergen were recombined with light chains specific for another allergen resulting in shuffled ScFvs. Possible ancestor genes for the heavy and light chain variable genes were determined and the recombinant ScFv representing the original combinations of heavy and light chains as isolated from the combinatorial library as well as the shuffled ScFvs were analyzed for binding specificities and affinities. We report that promiscuous pairing between heavy and light chains is possible and that binding specificity to the allergen the heavy chain is directed to can

be maintained. Therefore, the heavy chain plays a dominant role in allergen-specific human IgE antibodies in the determination of binding specificity. Further, different heavy and light chain combinations result in different binding affinities to the corresponding allergen which may explain different clinical reactivities in patients sensitized to the same allergens.

**Contribution to Manuscript 2**:

Determination and mapping of possible ancestor genes for Phl p 1, Phl p 2 and Phl p 5-specific human IgE Fabs, multiple sequence alignments of light chain protein sequences, conversion of Fabs into ScFvs and shuffling of light chains, expression of all recombinant ScFvs, determination of binding specificities in ELISA, affinity measurements and evaluations using BIAcore technology, analysis of the data, writing of the manuscript.

**Manuscript 3**

<u>Gadermaier E</u>, Flicker S, Blatt K, Valent P, Valenta R. **A single chain fragment specific for group 2 grass pollen allergens with therapeutic potential.** *Submitted*

Manuscript 3 describes the expression and characterization of an allergen-specific ScFv fragment. We show that this ScFv binds in a very stable way to the major timothy grass pollen allergen Phl p 2 and blocks the binding of patients' IgE to Phl p 2. Further, the ScFv inhibits allergen-induced basophil activation. This recombinant ScFv fragment may be used for therapeutic purposes.

**Contribution to Manuscript 3**:

Cloning and expression of the Phl p 2-specific ScFv, determination of association and dissociation rate constants and evaluation using BIAcore technology, analysis of the potential to inhibit the binding of allergic patients' IgE in ELISA, evaluation of the data, writing the manuscript.

**Manuscript 4**

Flicker S, <u>Gadermaier E</u>, Madritsch C, Valenta R. **Passive Immunization with Allergen-Specific Antibodies**. *Curr Top Microbiol Immunol*. 2011;141-159.

Manuscript 4 reviews the current knowledge of allergen-specific IgG antibodies that are induced in the course of allergen-specific immunotherapy and play a major part in the reduction of allergic inflammation. The article summarizes mechanisms of action as well as engineering strategies of allergen-specific antibodies and antibody fragments and discusses applications for treatment and prophylaxis of allergy.

**Contribution to Manuscript 4:**

Creation of figures and tables, proofreading of the manuscript.

**Manuscript 5**

<u>Gadermaier E</u>. **In Shape – The Art of Mapping Conformational Epitopes**. *Int Arch Allergy Immunol*. 2012;157:321-322.

Manuscript 5 is an Editorial reviewing the current possibilities to map conformational epitopes. These play an important role in type I allergy as the huge majority of allergens contain conformational epitopes that are recognized by IgE. It is of great

importance to better understand the interface between IgE and the corresponding allergen in order to gain more knowledge about the elicited immune responses and further to design effective active and passive treatment strategies. The manuscript discusses these issues on the basis of a recently published article by Tiwari *et al.* (*Int Arch Allergy Immunol.* 2012;157:323-330) that reports the mapping of a conformational epitope on the major cockroach allergen Bla g 2.


**Contribution to Manuscript 5:**

Review of the article by Tiwari *et al.* (Int Arch Allergy Immunol. 2012;157:323-30), writing of the editorial.

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# Recombinant allergen-based monitoring of antibody responses during injection grass pollen immunotherapy and after 5 years of discontinuation

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ORIGINAL ARTICLE                    EXPERIMENTAL ALLERGY AND IMMUNOLOGY

# Recombinant allergen–based monitoring of antibody responses during injection grass pollen immunotherapy and after 5 years of discontinuation

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## Abstract

**Background:** Subcutaneous injection immunotherapy (SCIT) is considered as antigen-specific and disease-modifying treatment with long-lasting effect.

**Methods:** We used a panel of recombinant grass pollen allergens for analyzing allergen-specific IgE, $IgG_1$-$IgG_4$, IgM, IgA, and light-chain (kappa, lambda) responses in grass pollen–allergic patients who had received one course of injection immunotherapy (SCIT) with an aluminum hydroxide-adsorbed grass pollen extract or only anti-inflammatory treatment. Serum samples were analyzed before and after 5 months of treatment as well as after 5 years.

**Results:** After 5 months of SCIT but not of anti-inflammatory treatment, $IgG_1 > IgG_4 > IgG_2 > IgA$ antibody responses using both kappa and lambda light chains specific for major grass pollen allergens (Phl p 1, Phl p 5, Phl p 6, Phl p 2) increased significantly, whereas specific IgM or $IgG_3$ levels were unaltered. Allergen-dependent basophil degranulation was only inhibited with SCIT sera containing therapy-induced allergen-specific IgG antibodies. Likewise, decreases in Phl p 1- and Phl p 5-specific IgE levels and significant ($P < 0.001$) reduction in symptom and medication scores were found only in the SCIT group but not in the group of patients receiving anti-inflammatory treatment. After 5 years, allergen-specific IgG antibody levels in the SCIT group had returned to baseline levels and there was no significant difference regarding symptoms between the SCIT and non-SCIT groups.

**Conclusion:** The results from our observational study demonstrate that only SCIT but not anti-inflammatory treatment induces allergen-specific IgG and reduces boosts of allergen-specific IgE production but that one SCIT course was not sufficient to achieve long-term immunological and clinical effects.

Subcutaneous injection immunotherapy (SCIT) is considered as antigen-specific, disease-modifying treatment for IgE-mediated allergies (1). It is accompanied by alterations in the humoral and cellular allergen-specific immune response which are thought to reduce allergen-specific hypersensitivity leading to tolerance. Subcutaneous injection immunotherapy has

been reported to have long-lasting clinical effects which can be detected for 5 years and even longer after discontinuation of treatment. In this context, it has been reported that patients having received venom immunotherapy for at least 3 years tolerated stings even after 5 years without treatment (2, 3). Similar findings have been made for grass pollen injection

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immunotherapy. It could be demonstrated that SCIT for 3 to 4 years induced prolonged clinical remission for at least 3 years after discontinuation of injections (4). In an independent study, Eng et al. described a significant clinical benefit 6 years after discontinuation of 3 years of preseasonal grass pollen injection immunotherapy (5).

Long-lasting effects have been also described for the disease-modifying potential of SCIT, in particular for the prevention of asthma in patients suffering from allergic rhinoconjunctivitis in the PAT study (6). A preventive effect for the development of asthma was also observed in follow-ups of the PAT study conducted after 5 and 10 years (7, 8). Although several studies have demonstrated long-lasting clinical effects of SCIT, several important questions remain open. For example, it has not been investigated whether long-lasting effects can be achieved when SCIT is performed for shorter periods than 3 years and whether such effects may even be observed after one course of treatment. Furthermore, only few studies demonstrating long-lasting effects have analyzed immunological alterations that might be associated or responsible for the long-lasting clinical effects. The studies by Golden and Lerch have shown for venom SCIT that long-term protection was associated with decreases in allergen-specific IgE levels and skin sensitivity, whereas allergen-specific IgG levels had declined after treatment (2, 3). Similar results were obtained by Durham et al., who found trends toward decreases in immediate-type allergen-specific conjunctival sensitivity, a reduction in late-phase skin responses, and reduced activation of Th2 cells in biopsies taken from allergen-challenged skin (4).

We performed an observational study in a group of patients who had received only one course of preseasonal grass pollen injection immunotherapy and in a group of patients who had received only anti-inflammatory pharmacotherapy. Symptoms and medication were recorded before treatment, shortly after treatment, and 5 years after the single preseasonal SCIT course. Furthermore, we performed a detailed analysis of allergen-specific antibody responses using a panel of recombinant allergens at the same time points.

## Materials and methods

### Recombinant allergens

Recombinant allergens, i.e., rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, rPhl p 7 (timothy grass pollen), and rBet v 1 (birch pollen), were purchased from Biomay (Vienna, Austria), and rPhl p 12 and rPhl p 13 (timothy grass pollen) were purified as described (9, 10).

### Patients, blood samples, treatment, and monitoring of clinical effects

Measurements of allergen-specific antibody responses were taken in grass pollen–allergic patients ($n = 19$) who had been recruited in summer 1999.

The diagnosis of grass pollen allergy in all 19 patients was based on case history, specific IgE measurements, and positive skin prick test results. Twelve patients suffered for more than 2 years from allergic rhinoconjunctivitis (one of them from concomitant asthma) and had no contraindications for subcutaneous immunotherapy (SCIT). These patients agreed to receive immunotherapy by subcutaneous injections of a mix of timothy and orchard grass pollen extracts adsorbed to aluminum hydroxide gel (Allpyral; Bayer Corporation, Spokane, WA, USA) in one course of preseasonal treatment (SCIT group) as recommended by the manufacturer (Fig. 1). On average, patients received 13.8 injections (between 11 and 17) and a mean cumulative dose of 5512.9 PNU was administered. Seven patients with allergic rhinoconjunctivitis, two of them with concomitant asthma, received only treatment with anti-inflammatory drugs (non-SCIT group) during the pollinosis season.

Blood samples were taken before treatment in January/February 2000 (time point T0), after one course of SCIT in May/June 2000 (T1), and 5 years later in July/August/September 2005 (T2) (Fig. 1).

The clinical effects of immunotherapy and of anti-inflammatory treatment were evaluated on the basis of symptom scores and the need for rescue medication. Symptoms were determined according to a scale ranging from 0 to 3 points (0 – no, 1 – mild, 2 – moderate, 3 – severe symptoms) for the following symptoms: nasal obstruction, itching, sneezing, rhinorrhea, and eye symptoms (as well as cough and dyspnea). After SCIT, patients kept a diary for 2 weeks during the pollen season (June 2000) where they reported their daily symptoms and medication. For these patients, the mean daily symptom score was calculated as follows: the sum of the daily symptom scores per patient was calculated for the period of the 2-week monitoring and divided by 14.

The scores for patients from the non-SCIT group at T1 were determined during consultations. Symptom scores at T0 as well as at T2 were collected for both groups during consultations.

Patients from both groups were allowed to take the antihistamine loratadine, the nasal glucocorticoid beclomethasone, and the $\beta_2$-adrenergic receptor agonist salbutamol as rescue medications. The number of days with rescue medica-
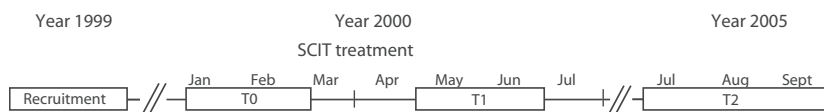


**Figure 1** Patients were recruited in summer 1999 to establish a baseline symptom score. At time point T0 (January/February 2000), patients from the subcutaneous injection immunotherapy group received vaccination treatment. Vaccination was stopped after 5 months in the grass pollen season 2000, and patients were monitored immediately thereafter (T1) and after 5 years (T2).

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tion during the 2-week assessment period in summer 2000 (T1) was noted by each patient and used to calculate the total days and mean days medication/patient.

### ELISA measurements of $IgG_{1-4}$, IgA, and IgM

Sera were analyzed for $IgG_{1-4}$, IgA, and IgM responses against recombinant allergens from timothy grass pollen as described (11). Each allergen (5 μg/ml in 0.1 M bicarbonate buffer, pH 9.6) was coated to ELISA plates (Nunc, Roskilde, Denmark) and was incubated with sera diluted as follows: $IgG_{1-4}$: 1 : 50, IgA and IgM: 1 : 100, or with buffer alone. IgA and IgM detections were also performed at lower serum dilutions (1 : 10, 1 : 25, 1 : 50). Bound antibodies were detected with mouse monoclonal anti-human $IgG_1$ (clone JDC-1), $IgG_2$ (clone G18-21), $IgG_4$ (clone JDC-14), $IgA_{1/2}$ (clone G18-1), and IgM (clone JDC-15) (PharMingen, San Diego, CA, USA) or mouse monoclonal anti-human $IgG_3$ (clone HP-6050) (Sigma-Aldrich, St Louis, MO, USA) (12–15). All determinations were performed in duplicates, and results are displayed as mean values with a mean coefficient of variation of 6.5%. Serum from a patient with defined antibody levels was added to each ELISA plate for calibration. Cutoffs were determined using the buffer control, which yielded mean OD values of 0.025 for IgG, 0.023 for IgA, and 0.022 for IgM determinations.

### Allergen-specific quantitative IgE measurements

Serum IgE reactivities against recombinant allergens and, for control purposes, against human serum albumin were determined in a quantitative RAST-based, nondenaturing dot blot assay under conditions of allergen excess (16). Allergens were immobilized to nitrocellulose by dot blotting (0.2 μg/dot). Bound IgE antibodies were detected with $^{125}$I-labeled anti-human IgE (Phadia AB, Uppsala, Sweden) diluted 1 : 15 in blocking buffer and were visualized by autoradiography and quantified (counts per minute: cpm) using a gamma counter (1277 Gammamaster; LKB, Wallac, Gaithersburg, MD, USA) (16).

The percentages of increase or reduction in the median IgE binding were determined according to the following formula: % increase/reduction = 100 × median $cpm_{T1}$/median $cpm_{T0}$ − 100, where $cpm_{T1}$ represent the cpm after incubation of the dotted allergen with post-treatment sera and $cpm_{T0}$ represent the cpm after incubation with pretreatment sera.

### ELISA measurement of allergen-specific light-chain binding

Patients' sera were analyzed for light chain-specific binding to purified recombinant allergens. ELISA plates (Nunc) were coated with allergens (5 μg/ml) and were incubated with 1 : 50 diluted sera (or buffer).

Serum from a patient with defined antibody levels was added to each ELISA plate to allow a comparison of results from different plates. Bound antibodies were detected with mouse monoclonal anti-human kappa or anti-human lambda light-chain antibodies (Sigma) and were traced with a

horseradish peroxidase-labeled sheep anti-mouse antiserum (Amersham Biosciences) diluted 1 : 2000. Results are means of duplicate determinations with a mean coefficient of variation of 1.85%. Cutoff of the ELISA was determined using the buffer control (mean OD value of 0.029).

### Basophil degranulation experiments

T0, T1, and T2 sera from two SCIT group patients and from two non-SCIT patients where sufficient serum was available were heat-inactivated for 4 h at 56°C in order to remove IgE reactivity (17). Then, sera were diluted 1 : 10 and pre-incubated overnight with 0.01 μg/ml rPhl p 5 in Tyrode's buffer (18). In parallel, RBL cells expressing human FcεRI (19) ($1 \times 10^5$/well) were loaded with serum IgE from non-heat-inactivated T0 serum samples at a dilution of 1 : 10 overnight. Cells were washed three times with Tyrode's buffer and exposed to the pre-incubated T0, T1, and T2 serum samples containing allergen immune complexes for 1 h. Supernatants were analyzed for β-hexosaminidase activity as described (18). Results (single determinations owing to serum shortness) are reported as percentages of total β-hexosaminidase released after addition of 10% Triton X-100.

### Statistical analysis

Because of the skewed distribution of OD and cpm values, data were log-transformed before analysis, resulting in distributions of residuals not deviating significantly from a normal distribution in the Lilliefors corrected Kolmogorov–Smirnov test. These data were subjected to ANOVA with repeated measurements. Homogeneity of variances was tested by Levene's tests. Comparisons between time points were made by linear contrasts against the baseline. No correction for multiple testing was applied. *P*-values below 0.05 were considered significant.

### Results

#### Subcutaneous injection immunotherapy patients develop $IgG_1$, $IgG_2$, and $IgG_4$ responses primarily against Phl p 1, Phl p 2, Phl p 5, and Phl p 6 but no relevant allergen-specific IgA or IgM antibody levels

As shown in Fig. 2, vaccinated patients significantly increased their $IgG_1$ responses against each of the analyzed allergens except against Phl p 12 after one course of SCIT. The increases in $IgG_1$ and $IgG_4$ against Phl p 1, Phl p 2, Phl p 5, and Phl p 6 were strong (Fig. 2A,C), whereas the increases in allergen-specific $IgG_2$ were more moderate but nevertheless highly significant ($P < 0.001$) (Fig. 2B). No relevant changes in allergen-specific IgA (serum dilution 1 : 100; Fig. 2D) and IgM (serum dilutions: 1 : 10, 1 : 25, 1 : 50) responses were observed for the vaccinated group. Only a slight increase in allergen-specific IgA was noted at a serum dilution of 1 : 10.

Five years after vaccination, the IgG antibody responses were comparable to those found in the pretreatment serum samples (Fig. 2A–D). Patients from the group receiving only
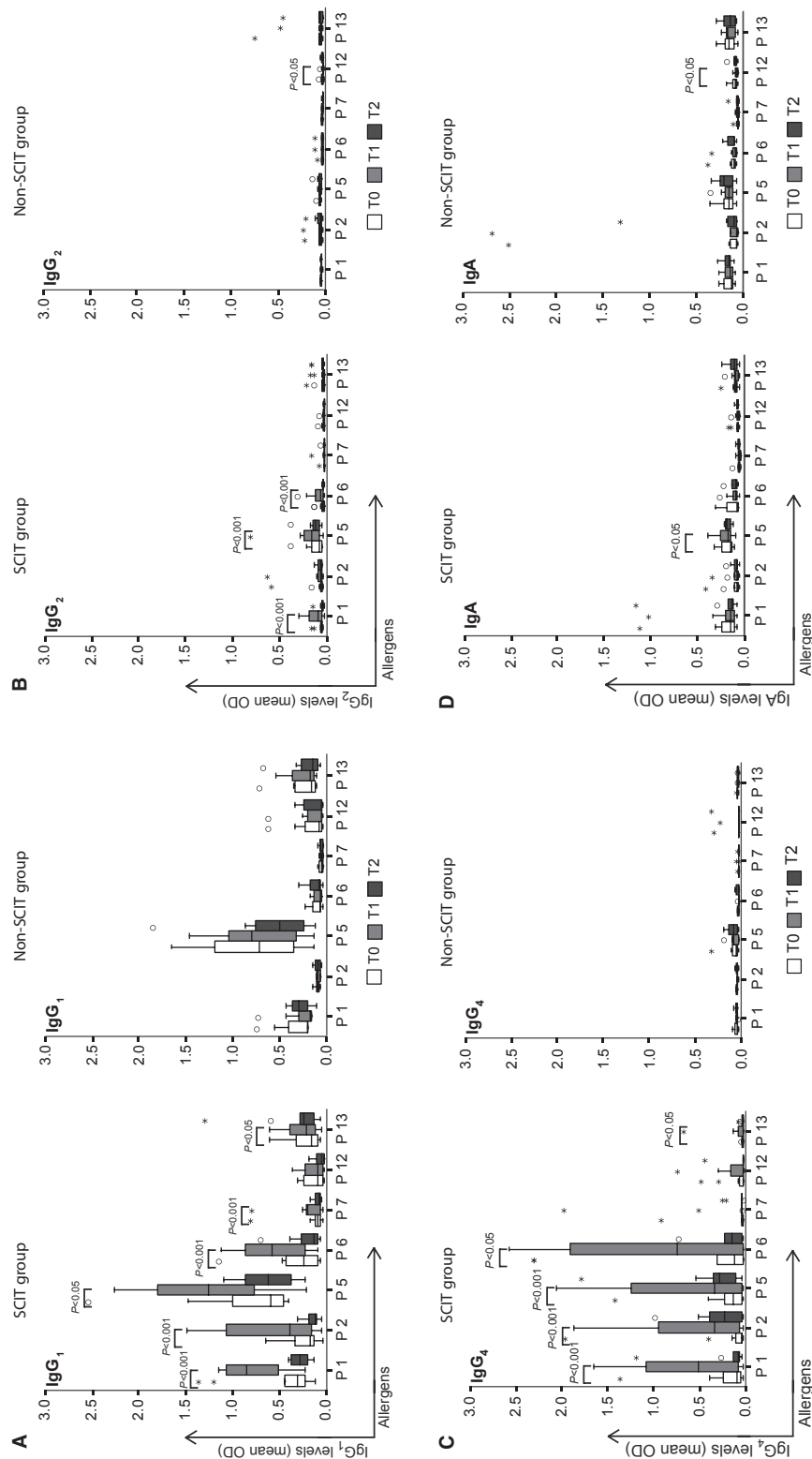
**Figure 2** Box plot representation of $IgG_1$ (A), $IgG_2$ (B), $IgG_4$ (C), and IgA (D) reactivities (OD values: y-axes) to recombinant timothy grass pollen allergens (rPhl p 1: P1, rPhl p 2: P2, rPhl p 5: P5, rPhl p 6: P6, rPhl p 7: P7, rPhl p 12: P12, rPhl p 13: P13) are shown for the subcutaneous injection immunotherapy (SCIT) group and non-SCIT group patients. The courses of antibody reactions are shown for three time points: T0: serum obtained at the start of the trial, T1: serum obtained approximately 5 months after T0, T2: serum 5 years after T0. Boxes include 50% of the values, and nonoutliers are located between the bars. Circles and asterisks indicate outliers and extremes, respectively. Lines within the boxes denote median values. P-values of <0.05 or <0.001 respectively are displayed.

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anti-inflammatory treatment showed no relevant increases in allergen-specific IgG, IgA, or IgM responses compared with the vaccinated group (Fig. 2A–D).

### Subcutaneous injection immunotherapy blunts the boost of allergen-specific IgE responses

Figure 3 shows the development of allergen-specific IgE levels for the major timothy grass pollen allergens Phl p 1 and Phl p 5 and for the unrelated birch pollen allergen Bet v 1. By comparing the median IgE levels in serum samples at T0 and T1, we found a reduction in the median Phl p 1-specific IgE levels in the range of 16.45% for the vaccinated group, whereas the median Phl p 1-specific IgE levels increased slightly in the group of patients receiving only anti-inflammatory treatment. This effect was much stronger for the highly immunogenic grass pollen allergen Phl p 5 (20). A median decrease in Phl p 5-specific IgE levels in the range of 30% was observed for the vaccinated group, whereas strong boost of Phl p 5-specific IgE of 136% was noted for the patients with only anti-inflammatory treatment. Concerning Bet v 1, the comparison of allergen-specific IgE levels at T0 and T1 showed a similar increase in median IgE levels in the vaccinated group and in patients with anti-inflammatory treatment only (i.e., SCIT group: +122%; non-SCIT group: +139%). Phl p 5-specific IgE levels in T2 were higher in both groups compared with those in T0, whereas Phl p 1-specific IgE levels in T2 were comparable with those in T0 in both groups as observed for the unrelated birch pollen allergen Bet v 1 (Fig. 3).

### Therapy-induced antibodies utilize both kappa and lambda light chains

In order to investigate the contribution of kappa and lambda chains in the vaccine-induced IgG responses, we determined allergen-specific kappa and lambda reactivities in the sera. Figure 4 shows that only vaccinated patients exhibit a significant induction of kappa and lambda responses against the major allergens Phl p 1 and Phl p 5 from T0 to T1. This response decreased after 5 years. For the minor allergens, Phl p 7 and Phl p 12, no significant development of light chain responses could be detected. The time courses/kinetics and levels of allergen-specific $IgG_1$ and $IgG_4$ responses shown in Fig. 2A,C are similar to those of the light-chain responses but not to those of the other antibody classes and subclasses, suggesting that the detected light chains belong to the corresponding $IgG_1$ and $IgG_4$ heavy chains.

### Reduction in allergen-induced basophil degranulation in SCIT-treated patients

We found that serum samples obtained after SCIT which had increased allergen-specific IgG inhibited basophil degranulation strongly (Fig. 5). At T1, serum from patient 4 caused an 89% inhibition of basophil degranulation and serum from patient 5 a 42% inhibition compared with that at T0. Serum from patient 4 showed reduced blocking activity after 5 years, which was associated with lower allergen-specific IgG levels at T2 in this serum ($IgG_1$: T0: 0.547, T1: 1.673, T2: 0.226; $IgG_4$: T0: 0.244, T1: 1.646, T2: 0.107; Fig. 5). Serum from patient 5 obtained at T2 showed even stronger inhibition of basophil degranulation than serum obtained at T1. This effect was associated with maintained allergen-specific IgG levels in this serum ($IgG_1$: T0: 0.463, T1: 1.981, T2: 1.096; $IgG_4$: T0: 0.116, T1: 0.414, T2: 0.353; Fig. 5).

Sera obtained from patients who had received only anti-inflammatory medication did not show any relevant inhibition of allergen-induced basophil degranulation at T1 or T2 compared with that at T0 (Fig. 5, right).
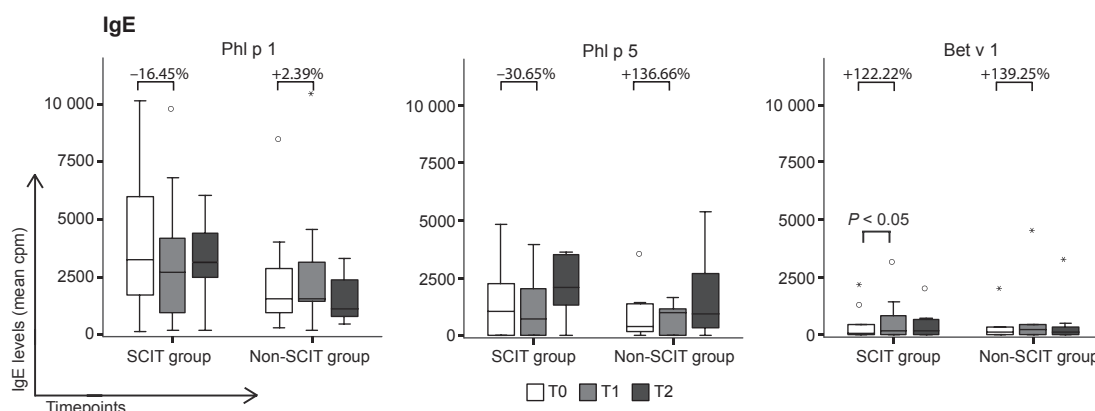


**Figure 3** Box plot representation of IgE levels (cpm: *y*-axes) to rPhl p 1, rPhl p 5, and rBet v 1 is shown for the subcutaneous injection immunotherapy (SCIT) group and non-SCIT group patients before the trial (T0), 5 months after T0 (T1), and 5 years later (T2). Alterations of the median allergen-specific IgE levels between T0 and T1 are expressed as percentage increases (+) or decreases (−). Boxes include 50% of the values, and nonoutliers are located between the bars. Circles and asterisks indicate outliers and extremes, respectively. Lines within the boxes denote median values. *P*-values of <0.05 are displayed.

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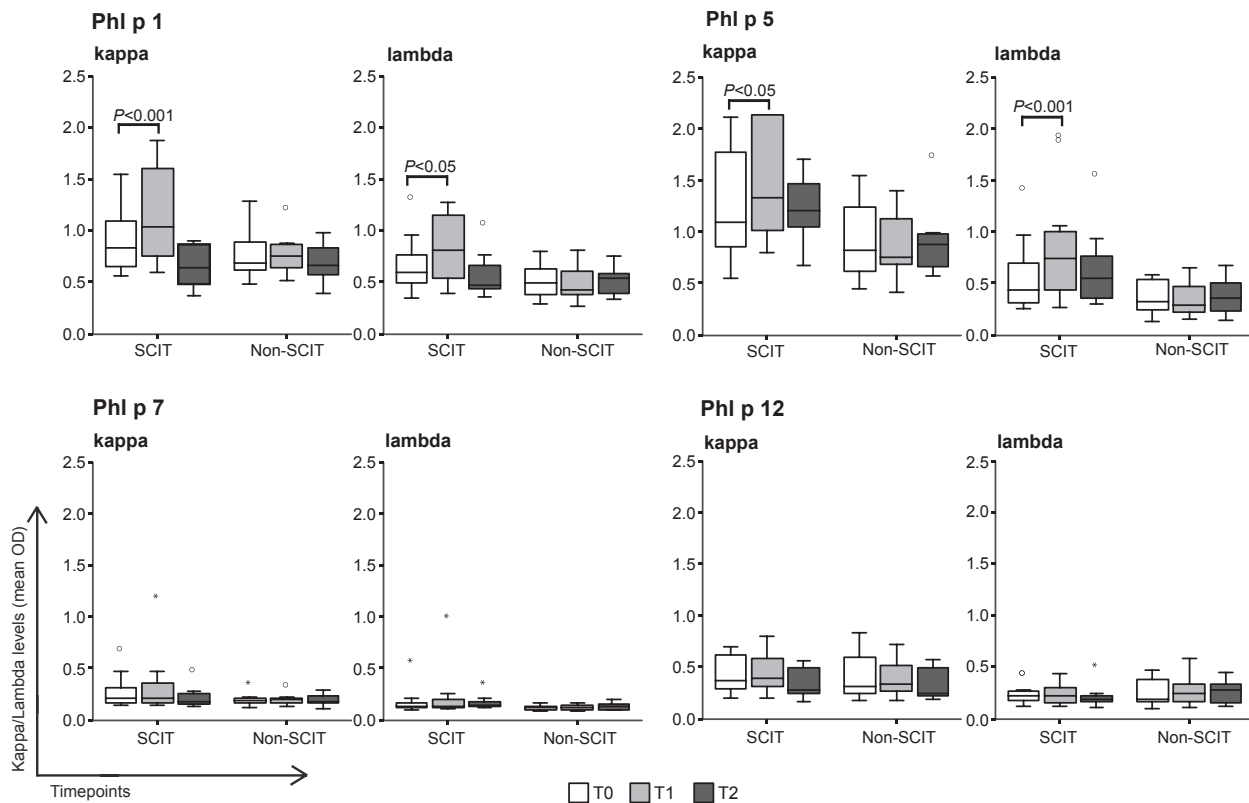


**Figure 4** Box plot representation of kappa and lambda light chain levels (OD values: *y*-axes) to recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12) is shown for the subcutaneous injection immunotherapy (SCIT) group and non-SCIT group patients. Reactions are displayed before the trial (T0), 5 months after T0 (T1), and 5 years later (T2). Boxes include 50% of the values, and nonoutliers are located between the bars. Circles and asterisks indicate outliers and extremes, respectively. Lines within the boxes denote median values. *P*-values of <0.05 or <0.001 respectively are displayed.
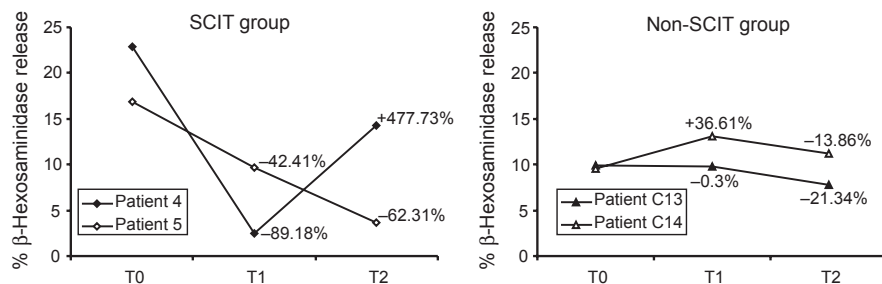


**Figure 5** Influence of allergen-specific IgG in patients sera on rPhl p 5-induced basophil degranulation. RBL cells were loaded with pretreatment serum IgE from two treated (left: 4, 5) and two non-treated (right: C13, C14) patients. Cells that had been loaded with IgE from a patient were then exposed to IgE-depleted sera from the very same patient obtained before the trial (T0), 5 months after T0 (T1), or 5 years thereafter (T2). ß-Hexosaminidase releases are displayed as percentages of total releases. Alterations of the releases are indicated as percentage increase or decrease compared with those of the earlier time point.

## Development of symptoms following SCIT treatment and after 5 years

In the SCIT group, median symptom scores declined significantly ($P < 0.001$) from 8.5 points to 2.1 points after 5 months of SCIT (Fig. 6). Five years later, this effect showed

only moderate significance ($P < 0.05$) and the reported median scores went back to 6.5 points. The median score at time point T0 in the non-SCIT group was 9 and thus was very similar to the 8.5 points in the SCIT group before treatment. Patients receiving only anti-inflammatory treatment showed almost no clinical improvement with a symptom score of 8

**Figure 6** Box plot representation of the development of symptoms in the subcutaneous injection immunotherapy (SCIT) and non-SCIT group. Symptom scores (y-axis) are shown for each group before treatment in summer 1999, at time point T1 in summer 2000, and 5 years later in summer 2005 (T2). Boxes include 50% of the values, and nonoutliers are located between the bars. Circles and asterisks indicate outliers and extremes, respectively. Lines within the boxes denote median values. Significant differences (P-values of <0.05 or <0.001) are displayed.
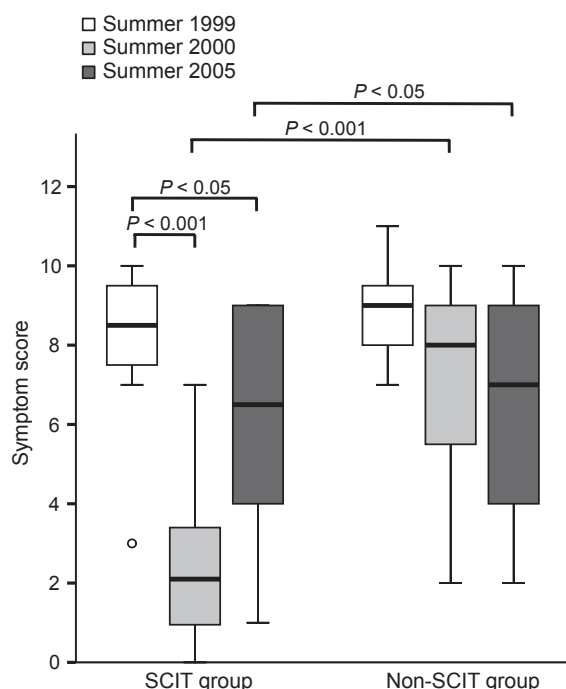
points in summer 2000 and of 7 points in summer 2005, 5 years after treatment. Five years after discontinuation of the study, patients from the SCIT group showed a lower symptom score than the patients from the non-SCIT group, which was of moderate significance ($P < 0.05$). The number of patients with asthma did not increase in the two groups at T2.

Table 1 shows the number of days during which patients had consumed rescue medication and the mean number of days/patient with rescue medication at time point T1. Patients in the non-SCIT group took more often loratadine

(75 days; mean 10.7 days/patient) than patients from the SCIT group (58 days; mean 4.8 days/patient). This was partly outweighed by a higher consumption of nasal beclomethasone in the SCIT group (38 days; mean 3.2 days/patient) compared with the non-SCIT group (0 days). When the total medication including salbutamol was calculated, we found that the SCIT group had even consumed less often rescue medication (101 days; mean 8.4 days/patient) than the non-SCIT group (77 days; mean 11 days/patient). These observations indicate that clinical improvement was not caused by higher consumption of rescue medication.

## Discussion

Here, we performed an observational pilot study in which we used a panel of recombinant timothy grass pollen allergens to study allergen-specific antibody levels in grass pollen–allergic patients who had received one course of injection immunotherapy and in patients who had received anti-inflammatory treatment. We found a strong induction of specific $IgG_1 > IgG_4 > IgG_2$ but no relevant induction of IgM or $IgG_3$ levels specific for allergens included in the vaccine (11, 21–26). As described by other authors (27), a slight increase in allergen-specific IgA was noted, but given the fact that it was obvious at a very low serum dilution of 1 : 10, the clinical relevance of this increase is uncertain. IgG antibodies were not induced against all grass pollen allergens, and the levels of allergen-specific IgG varied between the individual allergens. Increases in IgG responses against allergens (e.g., Phl p 7, Phl p 12, Phl p 13) which may be absent from allergen extracts or exhibit poor immunogenicity were low or not detectable (28). The testing for the usage of kappa and lambda light chains provided a hitherto unknown result, i.e., that SCIT induces allergen-specific reactivity of both kappa and lambda light chains. We think that the increase in light-chain reactivity is primarily attributable to the occurrence of lambda and kappa light chains in the allergen-specific IgG fraction because there was a parallel increase in similar magnitudes in IgG and light chain binding. Furthermore, free light chains reportedly have anaphylactic but not protective activity as shown in our basophil experiment (29). This finding indicates that SCIT induces a *de novo* immune response via a balanced activation of kappa and lambda bearing B cells.

In the majority of SCIT-treated patients, allergen-specific IgG responses declined to pretreatment levels 5 years after

**Table 1** Days with rescue medication (loratadine, salbutamol, beclomethasone) are reported for subcutaneous injection immunotherapy (SCIT) group ($n = 12$) and non-SCIT group patients ($n = 7$). For each single drug as well as for total medication, the total days of usage are shown and further the mean days per patient are indicated

|  |  | Days with loratadine | | Days with salbutamol | | Days with beclomethasone | | Total | |
|---|---|---|---|---|---|---|---|---|---|
|  |  | Total days | Mean days/patient | Total days | Mean days/patient | Total days | Mean days/patient | Total days | Mean days/patient |
| SCIT group | $n = 12$ | 58 | 4.8 | 5 | 0.42 | 38 | 3.2 | 101 | 8.4 |
| Non-SCIT group | $n = 7$ | 75 | 10.7 | 2 | 0.3 | 0 | 0 | 77 | 11 |

treatment. In a study performed with hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, it was also found that therapy-induced IgG decreased already 1 year after treatment and that at this time point patients had regained sensitivity when subjected to nasal allergen provocation (24, 30). Using a recently established basophil degranulation assay that allows determining the effects of blocking antibodies on basophil sensitivity for serum samples obtained before and after treatment, we show that the reduction in basophil degranulation depends on the presence of allergen-specific IgG antibodies, because no relevant alterations of allergen-specific antibody levels were noted for other immunoglobulin classes. Pauli et al. recently showed in a double-blind placebo-controlled SCIT trial performed with purified recombinant Bet v 1, natural Bet v 1, and birch pollen extract that clinical improvement and reduction in skin/mast cell sensitivity are associated with the induction of allergen-specific IgG (26).

Three studies demonstrated that patients who have developed allergen-specific IgG during SCIT exhibit reduced boosts of allergen-specific IgE (23, 24, 31) or a decrease in allergen-specific IgE when treatment was performed for several years (2, 3). One possible explanation for the decrease in allergen-specific IgE is that patients develop allergen-specific IgG which prevent boosts of allergen-induced IgE production (32–34).

If elevated allergen-specific IgG can be maintained for a long period by SCIT, allergen-specific IgE levels may decline as under lack of allergen exposure. This may lead to reduced loading of mast cells with IgE antibodies and reduced clinical sensitivity. The latter mechanisms may play a role for the long-lasting effects of SCIT, which have been reported to be associated with reductions of allergen-specific IgE levels and skin sensitivity (2, 3). Also, the decrease in Th2 cell activation observed for grass pollen immunotherapy (4) may be associated with the effects of blocking antibodies that block IgE-facilitated allergen presentation to T cells (35–39).

Although it is known that SCIT and SLIT can induce rises in allergen-specific IgE, we found a trend toward a reduction in allergen-specific IgE levels after one course of SCIT but not in the patients receiving anti-inflammatory treatment. This effect may be explained by the suppression of boosts of IgE production owing to grass pollen exposure by SCIT-induced IgG as reported earlier (23, 24, 31). However, the reduction in IgE levels was only detected shortly after treatment but not after 5 years, which may explain why only a moderate long-term clinical effect was achieved in this study.

In summary, our results may indicate that more than one course of preseasonal SCIT is necessary to maintain high levels of allergen-specific IgG for a period long enough to achieve a sustainable reduction in allergen-specific IgE levels leading to long-lasting and relevant clinical effects.

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# Determination of allergen-specificity by heavy chains in grass pollen allergen-specific IgE antibodies

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# Determination of allergen-specificity by heavy chains in grass pollen allergen-specific IgE antibodies

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## Abstract

Background: Affinity and clonality of allergen-specific IgE antibodies are important determinants for the magnitude of IgE-mediated allergic inflammation.

Objective: To analyze the contribution of heavy and light chains of human allergen-specific IgE antibodies for allergen-specificity and to test if promiscuous pairing of heavy and light chains with different allergen specificity allows binding and may affect affinity.

Methods: Ten IgE Fabs specific for three non-cross-reactive major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5) obtained by combinatorial cloning from a grass pollen allergic patient were used to construct stable recombinant ScFvs representing the original Fabs as well as shuffled ScFvs in which heavy chains were recombined with light chains from IgE Fabs with specificity for other allergens using the pCANTAB 5 E expression system. Possible ancestor genes for the heavy chain and light chain variable region-encoding genes were determined by sequence comparison with the IMGT data base and their chromosomal locations were determined. Recombinant ScFvs were tested for allergen-specificity and affinity by ELISA and surface plasmon resonance experiments, respectively.

Results: The shuffling experiments demonstrate that promiscuous pairing of heavy and light chains is possible and maintains allergen-specificity which is mainly determined by the heavy chains. ScFvs consisting of different heavy and light chains exhibited different affinities for the corresponding allergen.

Conclusion: Our results indicate that allergen-specificity of allergen-specific IgE is mainly determined by the heavy chains. Different heavy and light chain pairings in allergen-specific IgE antibodies affect affinity and thus may influence clinical reactivity to allergens.

Key message: Allergen-specificity in IgE-derived grass pollen allergen-specific ScFvs were dictated by the heavy chains. Different pairings of heavy and light chains resulted in differences in affinity of allergen recognition and thus may affect clinical sensitivity.

Capsule summary: Heavy chains seem to determine allergen-specificity in allergen-specific IgE antibodies. Promiscuous pairing of heavy and light chains may contribute to polyclonality as well as to diversity, modulate affinity and thus clinical sensitivity.

Key words: Allergy, allergen, IgE antibodies, specificity, affinity.

Abbreviations used:

VH: heavy chain variable region

VL: light chain variable region

ScFv: single-chain variable fragment

aa: amino acid

## Introduction

Immunoglobulin E (IgE) is the key immunoglobulin mediating allergic inflammation [1]. When allergens cross-link mast cell- or basophil-bound IgE, the release of inflammatory mediators, cytokines and proteases is induced [2]. Furthermore, IgE facilitates the presentation of allergens to T cells and thus may contribute to T cell-mediated chronic inflammation [3].

Both the levels of IgE antibodies in allergic patients as well as the amounts of allergen to which allergic patients are usually exposed are extremely low [1]. Nevertheless, small amounts of IgE-allergen immune complexes are able to induce strong inflammatory reactions which may be explained by the high affinity of IgE to FcεRI on mast cells and basophils and also by high affinity recognition of allergens by IgE [1, 4, 5]. In fact, the three dimensional structures of two IgE-allergen complexes have recently been solved by X-ray crystallography and revealed high affinity binding of allergen-specific human IgE to conformational epitopes on the corresponding allergens [4, 5].

Several studies suggest that the mode of interaction between IgE and allergens has profound effects on clinical symptoms in allergic patients. It has been shown that the magnitude of basophil degranulation increases with the number of IgE epitopes on a given allergen and thus with the clonality of the IgE response as well as with the levels of allergen-specific IgE and with the affinities of IgE antibodies for the allergen [6, 7]. Despite the importance of the IgE allergen interaction in allergy, only limited information regarding the structure of allergen-specific human IgE antibodies and their mode of interaction with the allergens is available.

The analysis of the primary structure of allergen-specific IgE from allergic patients has mainly become possible through combinatorial cloning because so far no technology is available to establish a representative repertoire of IgE producing cell

lines from allergic patients [4, 5, 7-16]. The analysis of allergic patients' allergen-specific IgE Fabs or ScFvs shows that these molecules bind with high specificity and affinity to the corresponding allergens, show a varying degree of somatic mutations and there seems to be quite a broad usage of the individual VH regions [4, 5, 7-17]. Analyzing allergen-specific IgE Fabs it has been noted that certain IgE heavy chains can recombine with different light chains and retain the specificity for the allergen [9, 10, 16]. On the other hand human IgE-derived allergen-specific Fabs or ScFvs have been described in which different heavy chains combined with the same light chain [7, 8]. In this study we analyzed the contribution of heavy chains and light chains for determining the specificity of allergen-recognition by recombining IgE heavy chains from grass pollen allergen IgE Fabs with different light chains. For this purpose we engineered stable ScFvs representing the original IgE Fabs isolated from allergic patients and shuffled versions in which light chains with specificities for other allergens were inserted. The recombinant ScFvs were then tested for allergen-specificity and affinity by ELISA and surface plasmon resonance measurements.

## Material and Methods

**Recombinant allergens**

Recombinant grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6 and rPhl p 7 (timothy grass) and recombinant major birch pollen allergen, rBet v 1, were purchased from Biomay (Vienna, Austria), rPhl p 12 and rPhl p 13 (timothy grass) were expressed and purified as described [18, 19].

**Characterization of grass pollen allergen-specific IgE Fabs**

IgE Fabs specific for major timothy grass pollen allergens Phl p 1, Phl p 2 and Phl p 5 were isolated from a combinatorial pComb 3H-phagemid library constructed from PBMCs of a grass pollen allergic patient [16]. In total, four IgE Fab clones specific for Phl p 5 (clones 5, 14, 28, 31), three specific for Phl p 2 (clones 94, 60, 100) and three specific for Phl p 1 (clones 25, 43, 10) were used as starting material for the engineering of defined ScFvs [11, 14, 16].

DNA sequences coding for the heavy and light chain variable regions were aligned with human germ line gene sequences (allele*01) from the ImMunoGeneTics (IMGT) database using V-Quest software [20]. For each of the analyzed heavy and light chain sequences the corresponding germ line V, D and J genes with the highest sequence identities and those with sequence identities with no less than 5% to the latter (highlighted in bold) (Table E1) were mapped on chromosome locus 14q32.33 (heavy chains) [21] or on chromosome locus 2p11.2 (light chains) [22] (Fig 1). The light chain DNA sequences were translated (ExPASy, Swiss Institute of Bioinformatics, Geneva, Switzerland) and the deduced amino acid sequences were aligned (Fig 2) in order to compare the individual chains. Sequence identities were calculated using the multiple sequence alignment program clustal W [23] (Table E2).

**Conversion of allergen-specific IgE Fabs into ScFvs and light chain-shuffled ScFvs**

Using the pComb 3H phagemid DNA from clones 5 (Phl p 5), clone 94 (Phl p 2) and clone 25 (Phl p 1) as template the heavy chain cDNAs were amplified by PCR using 20pmol of an equimolar mix of IgHV- and IgHJ-family specific ScFv primers [24]. In parallel, cDNAs coding for 10 light chains of the original IgE Fabs (Phl p 5: clones 5, 14, 28, 31; Phl p 2: clones 94, 60, 100; Phl p 1: clones 25, 43, 10) were PCR amplified using IgKV and IgKJ-family specific primers [24]. PCR products were purified on a 1% agarose gel.

Fifty ng of DNA coding for VH and VL fragments were assembled to obtain ScFv cDNAs. The combination of heavy and light chain fragment-encoding cDNAs of the original Fab clones (clones 5, 94, 25) resulted in corresponding ScFv cDNAs giving rise to ScFvs specific for Phl p 5 (5VH/5VL), Phl p 2 (94VH/94VL) and Phl p 1 (25VH/25VL). In parallel, cDNAs coding for the VH fragments were recombined with light chains from IgE Fabs with other specificities to obtain shuffled ScFvs which were designated according to the clones from where the heavy chain and light chain cDNAs were obtained (heavy chain/light chain: 5VH/94VL, 5VH/60VL, 5VH/100VL, 5VH/25VL, 5VH/43VL, 5VH/10VL, 94VH/5VL, 94VH/31VL, 94VH/14VL, 94VH/28VL, 94VH/25VL, 94VH/43VL, 94VH/10VL, 25VH/5VL, 25VH/31VL, 25VH/14VL, 25VH/28VL, 25VH/94VL, 25VH/60VL, 25VH/100VL).

The PCR assembly was performed by introducing a linker DNA coding for a 15 amino acids linker $(Gly_4Ser)_3$ [24]. The production of the human linker DNA is described in the online repository material. Assembly PCR was run for seven cycles (Mouse ScFv module/Amersham Biosciences, Little Chalfont, UK) and the assembled sequence was further amplified by adding an equimolar mix of 20 pmol

IGHV and IGKJ outer primers containing restriction sites for *Sfi*I (IGHV) and *Not*I (IGKJ) [24]. Products were purified on a 1% agarose gel and eluted as described.

Purified ScFv cDNA was digested with *Sfi*I and *Not*I (New England Biolabs, Ipswich, MA, USA), purified on a 1% agarose gel and further ligated into the phagemid vector pCANTAB 5 E (Amersham Biosciences). Recombinant phagemid DNA was transformed into the *E. coli* strain HB2151 by electroporation for the production of soluble ScFv fragments (Expression module/Recombinant phage antibody system, Amersham Biosciences). *E. coli* cells were grown on SOBAG plates (Expression module, Amersham Biosciences) at 30°C over night.

ScFv expressing clones were identified by sequencing (Eurofins MWG Operon, Ebersberg, Germany) using the ScFv-specific primers pCANTAB 5 E_fwd (5' CCA TGA TTA CGC CAA GCT TTG GAG CC 3') and pCANTAB 5 E_rev (5' GTA AAT GAA TTT TCT GTA TGA GG 3') and by testing supernatants for reactivity to the allergens. For this purpose, transformed bacteria were induced with 1 mM IPTG to express soluble ScFv and tested for allergen specificity by ELISA.

ScFvs were further tested for reactivity with an antibody directed to the C-terminal peptide tag (E tag) by immunoblotting (Schleicher & Schuell, Dassel, Germany) [25]. Membrane-bound ScFvs were traced with a monoclonal mouse anti-E tag antibody (Amersham Biosciences) followed by a horseradish peroxidase-labelled sheep anti-mouse antiserum (Amersham Biosciences). Blots were developed with the ECL Plus Western Blotting Detection System (GE Healthcare) followed by exposure to Kodak XOMAT films with intensifying screens (Kodak, Heidelberg, Germany).


**Determination of binding specificities by ELISA**

Bacterial lysates containing soluble ScFvs were analyzed for specific binding to recombinant timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 7,

Phl p 12, Phl p 13), to the major birch pollen allergen Bet v 1 and to HSA as controls. Five µg/ml of the proteins in PBS were coated to ELISA plates (Nunc, Roskilde, Denmark) and incubated with the ScFv-containing lysates diluted 1:2 in blocking buffer (PBS/3% w/v BSA) or, for control purposes, with blocking buffer alone. For calibration, lysates from the 5VH/5VL clone were added to each plate. Bound ScFv fragments were detected with the monoclonal mouse anti-E tag antibody (Amersham Biosciences) and traced with a horseradish peroxidase-labelled sheep anti-mouse antiserum (Amersham Biosciences). The colour reaction was read at 405 nm [26]. All samples were analyzed in duplicates, the means were calculated and after subtraction of the baseline signal (i.e., signal obtained with the unrelated allergen Bet v 1) were displayed as mean OD values.

**Determination of kinetics and binding affinities of ScFvs**

All measurements were performed on a BIACORE 2000 instrument at 25°C. In order to activate the surface of a CM5 sensor chip flow cell (BIACore AB), a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was injected at a flow rate of 5 µl/min for 7 minutes. To serve as capturing antibody, 60 µg/ml of the monoclonal anti-E tag antibody (Amersham Biosystems) diluted in 10 mM sodium acetate (pH 5) were injected at a flow rate of 10 µl/min until the maximal immobilization level of approximately 10 000 resonance units (RU) was reached. The flow cell was then deactivated by injection of 1 M ethanolamine-HCl (pH 8.5) (5 µl/min) for 7 minutes. The reference cell was loaded with an isotype control antibody in an analogous way. Lysates containing ScFvs diluted in HBS-EP (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005 % v/v surfactant P20, pH 7.4) were injected at 5 µl/min for different periods of time followed

by a stabilization phase of 30 minutes in order to capture 100 RU of ScFv for a calculated $R_{max}$ of 100 RU.

For the determination of kinetics and binding affinities, multi cycle kinetic was performed. For this purpose, the tested allergen was diluted in HBS-EP in 2-fold increasing concentrations (0.125 – 128 nM) and injected in random order at 30 µl/min for 5 minutes. Dissociation was determined by injection of HBS-EP buffer at 30 µl/min for 30 minutes. For control purposes, two runs were performed with the same allergen dilutions. In addition, two cycles were run with HBS-EP alone in order to subtract background signals. Regeneration of the sensor chip was performed by injection of 10 mM glycine-HCl, pH 2.5 at 30 µl/min for 30 seconds. BIAEvaluation 3.2 (BIACore AB) software was used to calculate kinetics and affinity constants, fittings were performed with a 1:1 (Langmuir) binding model.

For ScFvs showing weak binding in the ELISA (i.e., 25VH/14VL, 25VH/28VL) Biacore experiments were performed in order to verify binding reactions. After capturing of 100 RU of ScFv, 256 nM of the respective allergens were injected at a flow rate of 30 µl/min for 5 minutes. For double referencing, running buffer (HBS-EP) was injected under the same conditions.

## Results

**Sequence analysis of human IgE Fabs specific for three non-cross-reactive grass pollen allergens.**

VH and kappa VL cDNAs from the ten IgE Fabs specific for three non-cross-reactive allergens obtained from the combinatorial library were compared with human germ line genes from the IMGT database.

Sequence analysis and comparison with the germ line genes revealed that the four Phl p 5-specific IgE Fabs used the very same heavy chain (5VH) which had recombined with four different light chains (5VL, 14VL, 28VL, 31VL) (Table E1A). The heavy chains of the three Phl p 2-specific IgE Fabs (94VH, 60VH, 100VH) seemed to utilize the same IGHV genes (Table E1A). Interestingly, two (94VH, 60VH) seemed to have recombined with similar light chains (94VL, 60VL) which were also used by Phl p 5-specific IgE Fabs (14, 28, 31) and one (100VH) used a light chain identical to that of one of the four Phl p 5-specific IgE Fab (5VL) (Tables E1A, B, E2). All three Phl p 1-specific IgE Fabs were derived from the same IGHV gene and also contained light chains based on IGKV genes which appeared similar to those used by three of the Phl p 5-specific IgE Fabs (i.e., 14, 28, 31) (Tables E1A, B). However, the alignment of the light chains in Figure 2 showed that their similarity was mainly due to the usage of the same V genes (Table E1B), whereas most of them showed high diversity in the amino acid composition within CDR1 and CDR3 (Fig 2).

Possible ancestor genes for Phl p 5-, Phl p 2- and Phl p 1-specific IgE VHs are distributed along the heavy chain locus on chromosome 14 (14q32.33) (Fig 1A), and arise from different VH genes belonging to the IGHV3 and IGHV4 family (Fig 1A, Table E1A). In Table E1 the closest related IGHV germ line gene and those germ line genes with a difference in sequence of less than 5% are coloured. Within this range, 4 and 2 of the 23 functional IGHV3 genes may be considered as possible ancestors

for Phl p 5-specific (IGHV3-30-3, IGHV3-30, IGHV3-33, IGHV3-NL1) and Phl p 1-specific (IGHV3-9, IGHV3-20) variable regions, respectively. Considering the same range of sequence identity at least 5 of the 11 functional genes may be considered as possible progenitors (IGHV4-31, IGHV4-30-4, IGHV4-30-2, IGHV4-61, IGHV4-59) for the Phl p 2-specific VH genes.

The sequence comparison for the CDR3 regions with the possible corresponding IGHD and IGHJ genes was not possible due to their junctional diversity. Two of the Phl p 1 IgE Fabs (i.e., 43, 10) used similar IGHD genes whereas the third and the Phl p 2-specific IgE Fabs seemed to use different IGHD genes. The IGHJ genes in the IgE Fabs were similar (mainly IGHJ4*01) whereas the light chains used different IGKJ genes and thus may result from different somatic recombinations (Table E1A, B).

Interestingly, the light chains of the IgE Fabs specific for different unrelated allergens (i.e., Phl p 5, Phl p 2, Phl p 1) seemed to evolve from one germ line gene pool (Table E1B) located on the kappa light chain locus on chromosome 2 (2p11.2) (Fig 1B). The possible germ line genes of the light chains all belonged to the IGKV1 family (IGKV1-12, IGKV1D-12, IGKV1-39, IGKV1D-39, IGKV1D-16, IGKV1-17, IGKV1-9) (Fig 1B, Table E1B) involving 7 of 20 functional genes.

**Conversion of allergen-specific IgE Fab fragments into ScFv fragments with identical binding specificity and shuffling of heavy and light chains**

Expression of recombinant Fab fragments using the pComb 3H system may yield Fab preparations which consist of difficult to define mixtures of correctly formed Fabs and also incorrectly formed homo-dimers consisting of only heavy or light chains. In order to obtain high yields of defined and stable combinations of heavy and light chain fragments for binding experiments [27], recombinant IgE Fabs were converted

into ScFvs. Based on the sequences of the original IgE Fab fragments, stable ScFv fragments were generated in a first step (Fig 3). ScFvs containing the original combinations of heavy and light chains were termed "original" whereas those in which heavy chains were newly combined with light chains taken from Fabs with other specificities were designated "light chain-shuffled".

Table 1 gives an overview of the three original ScFvs (5VH/5VL, 94VH/94VL, 25VH/25VL) and 18 light chain-shuffled ScFvs. The recombinant ScFvs contain an E tag at their C-terminus and therefore could be detected in *E. coli* lysates by immunoblotting as exemplified for certain clones in Fig 4. Light chain-shuffled ScFvs were obtained for all combinations except for 94VH/28VL which carried a frame shift mutation and for 25VH/31VL which contained a substitution mutation and thus resulted in a premature stop of translation (Fig 4; data not shown).


**Light chain shuffling in ScFvs indicates that allergen-specificity is mainly determined by the heavy chain**

The majority of the shuffled clones (12 out of 18) reacted with the allergen that was recognized by the original ScFv when tested by ELISA (Table 1).

The Phl p 5-specific 5VH was successfully recombined with each of the six light chains and each of the light chain-shuffled ScFvs bound to Phl p 5 but not to other timothy grass pollen allergens (i.e., Phl p 1, Phl p 2, Phl p 6, Phl p 7, Phl p 12 and Phl p 13) (Fig 5A, Table 1).

Likewise, the Phl p 2-specific 94VH and Phl p 1-specific 25VH recombined with the light chains and many of the light-chain-shuffled ScFvs bound to the VH-specific allergen but not to the other tested grass pollen allergens (Fig 5B, 5C, Table 1).

**Surface plasmon resonance measurements confirm that light chain-shuffled ScFvs preserve the specificity of the original ScFvs and exhibit affinities in the nanomolar range**

Kinetics (i.e., association and dissociation rate constants) and binding affinities for the interaction between original and light chain-shuffled ScFvs and their respective allergens were measured using surface plasmon resonance. For this purpose, ScFvs were immobilized to the Biacore chip using the anti-E tag antibody for capturing and 2-fold increasing concentrations of allergen were injected into the flow cell allowing interaction with the captured ScFv. Six ScFvs (two original and 4 light-chain-shuffled ScFvs) with positive ELISA results could be analyzed in Biacore experiments because they could be successfully captured with the anti-E tag antibody (Table 2).

Among the ScFvs carrying the Phl p 5-specific 5VH the original ScFv 5VH/5VL showed the highest affinity to Phl p 5 ($K_D$ = 3.74 x $10^{-11}$ M) (Fig 6A, Table 2). The light chain-shuffled ScFv 5VH/25VL (Fig 6B) still bound with high affinity to Phl p 5 ($K_D$ = 2.6 x $10^{-9}$ M) but the affinity was approximately 100-fold reduced compared to the original ScFv (Table 2). Likewise, the other two light chain-shuffled ScFvs (5VH/100VL, 5VH/43VL) showed lower affinities to Phl p 5 than the original ScFv (Table 2).

The original Phl p 1-specific ScFv 25VH/25VL showed an affinity which was comparable to that of the Phl p 5-specific shuffled ScFv (5VH/100VL) (Table 2). The shuffled Phl p 2-specific ScFv 94VH/5VL showed a lower affinity which was comparable to two of the Phl p 5-specific shuffled ScFvs (5VH/25VL, 5VH/43VL).

In this context we noted that certain amino acids which were involved in the binding of the light chain of the Phl p 2-specific IgE Fab to Phl p 2 [4] were different in the shuffled version but binding was retained (Fig 2, printed in red). From the two contacts in the CDR1 of the light chain, one was conserved in all three ScFvs which

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retained binding specificity (94VH/94VL, 94VH/5VL, 94VH/25VL). In CDR2, only one residue made contact which was conserved in each of the three binding ScFvs. Several contacts were observed for CDR3, but none of these amino acids was conserved in all three binding ScFvs. Despite the shuffling, each of the tested ScFvs showed binding affinities in the nanomolar range (Table 2).

## Discussion

In this study the contribution of heavy and light chains to the binding specificity and affinity of ScFvs derived from human IgE antibodies specific to allergens was investigated. Using heavy and light chains from human IgE Fabs [11, 14, 16] with specificity for three different immunologically unrelated major grass pollen allergens, Phl p 1 , Phl p 2 and Phl p 5 [28-30] as a starting material we constructed and characterized stable recombinant ScFvs. Thus a defined experimental model system was obtained for testing the effects of variations in heavy and light chain pairing on allergen-specificity and affinity.

When the various ScFvs were tested for reactivity to seven different grass pollen allergens we found that the original ScFvs bound with high specificity only to the allergens which were recognized by the corresponding original IgE Fabs. Interestingly, several of the shuffled ScFvs in which the original light chain was replaced by a light chain derived from an IgE Fab with specificity for another unrelated allergen retained their specificity to the original allergen in a heavy chain-dependent manner. These results indicate that the heavy chain is important for determining the specificity in human allergen-specific IgE antibodies. According to the sequence comparison of the cDNAs coding for the IgE VHs with the germ line genes it seemed that the analyzed heavy chains are derived from several distinct ancestor genes. Therefore, allergen specificity may originate from several different genes which indicates a certain redundancy in allergen recognition by IgE antibodies and may contribute to diversity.

Shuffled ScFvs containing different heavy and light chains showed different affinities to the corresponding allergens as measured by surface plasmon resonance experiments. This finding suggests that besides junctional diversity and varying affinity maturation by somatic mutations [31], pairing of different heavy and light chains

may contribute to the modulation of affinity in the allergen recognition by allergen-specific IgE antibodies. We have not done epitope mapping of the binding sites of the original and shuffled ScFvs but it is possible that the shuffled versions may recognize different epitopes which may increase clonality. Variations of affinity and clonality through promiscuous heavy and light chain pairing may be of clinical importance because it has been shown that both, affinity and clonality of IgE recognition of allergens determines the intensity of effector cell degranulation [6, 7] and therefore could have an effect on the magnitude of immediate allergic inflammation. Promiscuous heavy and light chain pairing may thus represent a novel mechanism responsible for varying clinical sensitivities in allergic patients.

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## Table 1

Characterization of ScFvs regarding expression and binding specificities

| combined with | Phl p 5-specific 5VH | | | | | | | Phl p 2-specific 94VH | | | | | | | Phl p 1-specific 25VH | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Phl p 5-specific | Phl p 2-specific | | | Phl p 1-specific | | | Phl p 5-specific | | | Phl p 2-specific | Phl p 1-specific | | | Phl p 5-specific | | | Phl p 2-specific | | | Phl p 1-specific |
| | 5VL | 60VL | 94VL | 100VL | 10VL | 25VL | 43VL | 5VL | 31VL | 14VL | 94VL | 10VL | 25VL | 43VL | 5VL | 14VL | 28VL | 60VL | 94VL | 100VL | 25VL |
| E tag detection | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | +/- | + | + | + | + | + |
| ELISA | + | + | + | + | + | + | + | + | - | - | + | - | + | - | - | +/- | +/- | + | + | - | + |
| Biacore | + | + | + | + | + | + | + | + | n.d. | n.d. | + | n.d. | + | n.d. | n.d. | - | - | + | + | n.d. | + |
| binding to | Phl p 5 | | | | | | | Phl p 2 | | | | | | | Phl p 1 | | | | | | |

n.d. not done;

# Table 2

Summary of Biacore results for ScFvs with allergen-specifity

| combined with | Phl p 5-specific 5VH | | | | | | | Phl p 2-specific 94VH | | | Phl p 1-specfic 25VH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Phl p 5-specific | Phl p 2-specific | | | Phl p 1-specific | | | Phl p 5-specific | Phl p 2-specific | Phl p 1-specific | Phl p 2-specific | | Phl p 1-specific |
| | 5VL | 60VL | 94VL | 100VL | 10VL | 25VL | 43VL | 5VL | 94VL | 25VL | 60VL | 94VL | 25VL |
| ka (1/Ms) | $2.23 \times 10^5$ | n.a. | n.a. | $1.84 \times 10^5$ | n.a. | $9.91 \times 10^4$ | $1.31 \times 10^5$ | $9.94 \times 10^4$ | n.a. | n.a. | n.a. | n.a. | $2.23 \times 10^5$ |
| kd (1/s) | $8.34 \times 10^{-6}$ | n.a. | n.a. | $6.46 \times 10^{-5}$ | n.a. | $2.57 \times 10^{-4}$ | $5.23 \times 10^{-4}$ | $6.66 \times 10^{-4}$ | n.a. | n.a. | n.a. | n.a. | $1.23 \times 10^{-4}$ |
| KD (M) | $3.74 \times 10^{-11}$ | n.a. | n.a. | $3.5 \times 10^{-10}$ | n.a. | $2.6 \times 10^{-9}$ | $4.01 \times 10^{-9}$ | $6.71 \times 10^{-9}$ | n.a. | n.a. | n.a. | n.a. | $5.5 \times 10^{-10}$ |

n.a. not analyzable due to unstable capturing

Figure legends

**FIG. 1.** Localization of the possible functional ancestor genes of the allergen-specific ScFvs in the human genome. *A*, Representation of the human IGH locus (14q32.33) and *B*, of the human IGK locus (2p11.2). Functional genes are boxed and possible germ line ancestors for the isolated allergen-specific ScFvs are indicated on top. Symbols (Phl p 5-specific: *; Phl p 1-specific: #; Phl p 2-specific: +) represent the germ line genes with high sequence identity to the allergen-specific variable regions (see Tables E1A and B). The closest germ line genes are indicated with big symbols. Gene IGHV3-NL1 on 14q32.33 could not be located with the IMGT/GENE-DB software.

**FIG. 2.** Amino acid sequence alignment of VL regions used in the allergen-specific IgE Fabs with the Phl p 1-specific region 25VL. Identical amino acids are indicated by dots and complimentarity determining regions (CDR) are highlighted in grey. Amino acids involved in the binding of the light chain of the Phl p 2-specific Fab with Phl p 2 are represented in red.

**FIG. 3.** Schematic illustration of the light chain shuffling approach. Allergen-specific IgE Fabs (A) were converted into the ScFv format (B). Subsequently the heavy chains were recombined with the light chains from the original Fabs (C).

**FIG. 4.** Detection of complete ScFvs in nitrocellulose-blotted bacterial lysates with an anti-E tag antibody. The analysis of a representative number of bacterial clones expressing original and shuffled ScFvs (top) is shown. Molecular weight markers (kDa) are indicated on the left margin.

**FIG. 5.** Reactivities of ScFvs to a panel of recombinant timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 7, Phl p 12 and Phl p 13) by ELISA. OD values (y-axes) corresponding to the binding intensities are shown for the allergens (x-axes) and the ScFvs containing heavy chains from IgE Fabs specific for Phl p 5 (*A*: 5VH), Phl p 2 (*B*: 94VH) and Phl p 1 (*C*: 25VH) (right margins).

**FIG. 6.** Sensor chip-based studies of the interaction between Phl p 5 and Phl p 5 specific ScFvs. ScFvs (*A*: 5VH/5VL; *B*: 5VH/25VL) were captured on the chip and Phl p 5 was injected in 2-fold increasing concentrations from 0.5nM to 16nM (*A*) or 2nM to 64nM (*B*). Recorded (coloured lines) and calculated curves (black lines) which represent fittings of the data to a 1:1 binding model were superimposed. Signal intensities (RU) are displayed (y-axes) versus time (s) (x-axes). Association ($k_a$) and dissociation ($k_d$) rate constants as well as dissociation constants ($K_D$) are indicated.

**Table 1.** Summary of ScFvs regarding expression and binding characteristics. ScFvs grouped by their heavy chains and combined light chains (top) were classified according to expression (E tag detection) and binding to the allergens (bottom) by ELISA and Biacore (positive: +; weak: +/-; negative: -). Original clones were highlighted in red.

**Table 2.** Summary of Biacore results for ScFvs with allergen-specificity as determined by ELISA. Association ($k_a$) and dissociation ($k_d$) rate constants as well as dissociation constants ($K_D$) calculated by fitting the analyzed data with a 1:1 binding model are displayed for those ScFvs which could be analyzed by Biacore.

14q32.33

Huma IGH locus on chromosome 14 with functional genes
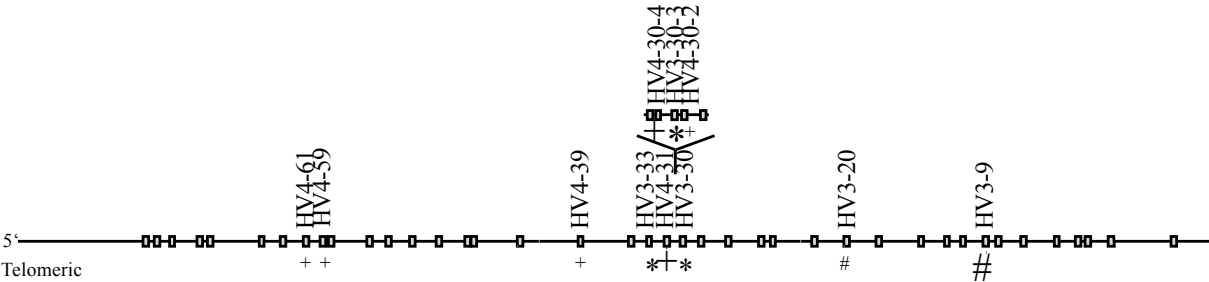


∗ HV3-NL1: precise location on 14q32.33 unknown

∗ Phl p 5-specific VH
+ Phl p 2-specific VH
# Phl p 1-specific VH

FIG. 1A

2p11.2
Huma IGK locus on chromosome 2 with functional genes



* Phl p 5-specific VH
+ Phl p 2-specific VH
# Phl p 1-specific VH

FIG. 1B

FIG. 2

A

VH Wildtype IgE Fab

B

ScFv

C

Light chain shuffling

FIG. 3

FIG. 4

A



B



C



FIG. 5

A

5VH/5VL

$k_a = 2.23 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$
$k_d = 8.34 \times 10^{-6} \text{ s}^{-1}$
$K_D = 3.74 \times 10^{-11} \text{ M}$

B

5VH/25VL

$k_a = 9.91 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$
$k_d = 2.57 \times 10^{-4} \text{ s}^{-1}$
$K_D = 2.6 \times 10^{-9} \text{ M}$

FIG. 6

## Online Repository Material

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Methods

**Construction of linker DNA for the assembly of VH and VL fragments**

For the construction of linker DNA, 24 separate PCR reactions (Platinum PCR Supermix, Invitrogen, Life Technologies, Carlsbad, CA, USA) were performed where all possible combinations of the four HJ- and six VL-specific primers [24] were used to amplify a DNA sequence coding for a 15 amino acids linker $(Gly_4Ser)_3$. 24 linker products were purified on a 2% agarose gel, eluted as described, and ligated into the pre-cut pSTBlue-1 AccepTor Vector (Novagen) that allows direct ligation of a PCR product with 3'-dA overhangs produced by Taq-Polymerase that anneal with 3'-dU on the vector. Sequences were confirmed by double-strand sequencing (Microsynth, Balgach, Switzerland) performed with the standard T7 and SP6 primers and were further used as templates for another PCR step followed by purification as described. Concentrations of the 24 separate linkers were determined via Nanodrop (Peqlab, Erlangen, Germany) and an equimolar mix was produced.

Figure legends

**Table E1.** Comparison of VH (Table E1A) and VL (Table E1B) sequences from the original IgE Fabs with germ line genes (allele *01) from the IMGT database. The closest related germ line genes and those with sequence identities with no less than 5% of the latter are displayed in bold together with their accession numbers and nucleotide sequence identities. Likewise, variable (IGHV), diverse (IGHD) and joining (IGHJ) genes of heavy chain sequences were identified in Table E1A as well as variable (IGKV) and joining (IGKJ) genes of light chain sequences in E1B.

**Table E2**. Amino acid sequence identities (%) and mean % sequence identities between the analyzed VL regions to each other are displayed.

Table E1A

Comparison of isolated heavy chains with germline genes

| Specificity | Isolated VH | IGHV gene*allele_01 | Nucleotide sequence Identity | Accession No | IGHD gene*allele_01 | Nucleotide sequence Identity | Accession No | IGHJ gene*allele_01 | Nucleotide sequence Identity | Accession No |
|---|---|---|---|---|---|---|---|---|---|---|
| Phl p 5-specific VH | 5VH | IGHV3-30-3*01 | 87.50% | X92283 | IGHD1-1*01 | 88.24% | X97051 | IGHJ3*01 | 94.00% | J00256 |
| | | IGHV3-30*01 | 86.81% | M83134 | IGHD1-14*01 | 82.35% | X13972 | IGHJ4*01 | 75.00% | J00256 |
| | | IGHV3-33*01 | 86.11% | AB019439 | IGHD1-20*01 | 82.35% | X97051 | IGHJ1*01 | 69.23% | J00256 |
| | | IGHV3-NL1*01 | 83.33% | HM855939 | IGHD1-7*01 | 82.35% | X13972 | IGHJ5*01 | 68.63% | J00256 |
| | | IGHV3-7*01 | 81.25% | M99649 | IGHD7-27*01 | 72.73% | J00256 | IGHJ2*01 | 67.92% | J00256 |
| Phl p 2-specific VH | 94VH | IGHV4-31*01 | 92.47% | L10098 | IGHD5-18*01 | 80.00% | X97051 | IGHJ4*01 | 85.11% | J00256 |
| | | IGHV4-30-4*01 | 91.76% | Z14238 | IGHD5-5*01 | 80.00% | X13972 | IGHJ5*01 | 82.00% | J00256 |
| | | IGHV4-30-2*01 | 89.96% | L10089 | IGHD5-24*01 | 70.00% | X97051 | IGHJ3*01 | 75.51% | J00256 |
| | | IGHV4-61*01 | 87.81% | M29811 | IGHD2-15*01 | 60.00% | J00234 | IGHJ1*01 | 74.51% | J00256 |
| | | IGHV4-39*01 | 87.10% | AB019439 | IGHD3-16*01 | 60.00% | X93614 | IGHJ6*01 | 72.41% | J00256 |
| | 60VH | IGHV4-30-4*01 | 91.40% | Z14238 | IGHD1-26*01 | 63.64% | X97051 | IGHJ4*01 | 85.11% | J00256 |
| | | IGHV4-31*01 | 91.40% | L10098 | IGHD5-24*01 | 63.64% | X97051 | IGHJ5*01 | 78.00% | J00256 |
| | | IGHV4-30-2*01 | 89.25% | L10089 | IGHD6-25*01 | 62.50% | X97051 | IGHJ1*01 | 76.47% | J00256 |
| | | IGHV4-61*01 | 87.46% | M29811 | IGHD1-7*01 | 54.55% | X13972 | IGHJ3*01 | 75.51% | J00256 |
| | | IGHV4-39*01 | 87.10% | AB019439 | IGHD5-12*01 | 53.85% | X13972 | IGHJ6*01 | 63.93% | J00256 |
| | 100VH | IGHV4-30-4*01 | 94.27% | Z14238 | IGHD1-26*01 | 60.00% | X97051 | IGHJ4*01 | 87.23% | J00256 |
| | | IGHV4-31*01 | 94.27% | L10098 | IGHD5-24*01 | 60.00% | X97051 | IGHJ5*01 | 80.00% | J00256 |
| | | IGHV4-30-2*01 | 92.11% | L10089 | IGHD6-13*01 | 60.00% | X13972 | IGHJ1*01 | 76.47% | J00256 |
| | | IGHV4-59*01 | 90.48% | AB019438 | IGHD5-5*01 | 60.00% | X13972 | IGHJ3*01 | 73.47% | J00256 |
| | | IGHV4-61*01 | 90.32% | M29811 | IGHD7-27*01 | 55.56% | J00256 | IGHJ6*01 | 68.97% | J00256 |
| Phl p 1-specific VH | 25VH | IGHV3-9*01 | 90.58% | M99651 | IGHD5-5*01 | 54.55% | X13972 | IGHJ4*01 | 91.49% | J00256 |
| | | IGHV3-20*01 | 85.87% | M99657 | IGHD6-13*01 | 53.85% | X13972 | IGHJ5*01 | 80.00% | J00256 |
| | | IGHV3-43*01 | 85.14% | M99672 | IGHD6-6*01 | 53.33% | X13972 | IGHJ1*01 | 76.47% | J00256 |
| | | IGHV3-23*01 | 82.97% | M99660 | IGHD2-15*01 | 52.94% | J00234 | IGHJ3*01 | 69.39% | J00256 |
| | | IGHV3-48*01 | 82.97% | M99675 | IGHD4-23*01 | 52.94% | X97051 | IGHJ2*01 | 69.23% | J00256 |
| | 43VH | IGHV3-9*01 | 93.84% | M99651 | IGHD1-26*01 | 69.23% | X97051 | IGHJ4*01 | 82.98% | J00256 |
| | | IGHV3-20*01 | 88.04% | M99657 | IGHD2-15*01 | 68.75% | J00234 | IGHJ1*01 | 74.51% | J00256 |
| | | IGHV3-43*01 | 88.04% | M99672 | IGHD2-2*01 | 68.75% | J00232 | IGHJ5*01 | 72.00% | J00256 |
| | | IGHV3-h*01 | 83.15% | AJ879484 | IGHD6-25*01 | 61.54% | X97051 | IGHJ3*01 | 71.43% | J00256 |
| | | IGHV3-48*01 | 82.61% | M99675 | IGHD3-10*01 | 58.82% | X13972 | IGHJ2*01 | 69.23% | J00256 |
| | 10VH | IGHV3-9*01 | 92.75% | M99651 | IGHD1-26*01 | 69.23% | X97051 | IGHJ4*01 | 85.11% | J00256 |
| | | IGHV3-20*01 | 86.59% | M99657 | IGHD2-15*01 | 68.75% | J00234 | IGHJ5*01 | 78.00% | J00256 |
| | | IGHV3-43*01 | 85.51% | M99672 | IGHD1-7*01 | 61.54% | X13972 | IGHJ3*01 | 75.51% | J00256 |
| | | IGHV3-h*01 | 83.15% | AJ879484 | IGHD6-25*01 | 61.54% | X97051 | IGHJ1*01 | 74.51% | J00256 |
| | | IGHV3-48*01 | 82.61% | M99675 | IGHD6-6*01 | 56.25% | X13972 | IGHJ2*01 | 69.23% | J00256 |

Table E1B

Comparison of isolated light chains with germline genes

| | Isolated VL | IGKV gene*allele_01 | Nucleotide sequence Identity | Accession No | IGKJ gene*allele_01 | Nucleotide sequence Identity | Accession No |
|---|---|---|---|---|---|---|---|
| **Phl p 5-specific VL** | **5VL** | IGKV1-12*01 | 96.34% | V01577 | IGKJ2*01 | 94.12% | J00242 |
| | | IGKV1D-12*01 | 95.60% | X17263 | IGKJ1*01 | 85.29% | J00242 |
| | | IGKV1D-16*01 | 91.94% | K01323 | IGKJ3*01 | 79.41% | J00242 |
| | | IGKV1-16*01 | 90.84% | J00248 | IGKJ4*01 | 79.41% | J00242 |
| | | IGKV1-9*01 | 90.48% | Z00013 | IGKJ5*01 | 64.71% | J00242 |
| | **14VL** | IGKV1-39*01 | 95.70% | X59315 | IGKJ1*01 | 96.97% | J00242 |
| | | IGKV1D-39*01 | 95.70% | X59312 | IGKJ4*01 | 78.79% | J00242 |
| | | IGKV1-17*01 | 89.25% | X72808 | IGKJ2*01 | 75.76% | J00242 |
| | | IGKV1-6*01 | 89.25% | M64858 | IGKJ3*01 | 75.76% | J00242 |
| | | IGKV1-NL1*01 | 89.25% | Y14865 | IGKJ5*01 | 66.67% | J00242 |
| | **28VL** | IGKV1-39*01 | 93.04% | X59315 | IGKJ1*01 | 90.62% | J00242 |
| | | IGKV1D-39*01 | 93.04% | X59312 | IGKJ3*01 | 78.12% | J00242 |
| | | IGKV1-9*01 | 86.81% | Z00013 | IGKJ4*01 | 75.00% | J00242 |
| | | IGKV1-6*01 | 86.45% | M64858 | IGKJ2*01 | 71.88% | J00242 |
| | | IGKV1D-13*01 | 86.45% | X17262 | IGKJ5*01 | 68.75% | J00242 |
| | **31VL** | IGKV1-39*01 | 97.49% | X59315 | IGKJ2*01 | 94.12% | J00242 |
| | | IGKV1D-39*01 | 97.49% | X59312 | IGKJ1*01 | 73.53% | J00242 |
| | | IGKV1-12*01 | 91.40% | V01577 | IGKJ3*01 | 73.53% | J00242 |
| | | IGKV1-9*01 | 90.68% | Z00013 | IGKJ4*01 | 73.53% | J00242 |
| | | IGKV1D-12*01 | 90.68% | X17263 | IGKJ5*01 | 73.53% | J00242 |
| **Phl p 2-specific VL** | **94VL** | IGKV1-39*01 | 93.77% | X59315 | IGKJ2*01 | 94.87% | J00242 |
| | | IGKV1D-39*01 | 93.77% | X59312 | IGKJ1*01 | 84.21% | J00242 |
| | | IGKV1-12*01 | 88.64% | V01577 | IGKJ4*01 | 84.21% | J00242 |
| | | IGKV1-9*01 | 88.64% | Z00013 | IGKJ3*01 | 81.58% | J00242 |
| | | IGKV1-17*01 | 88.28% | X72808 | IGKJ5*01 | 71.05% | J00242 |
| | **60VL** | IGKV1-39*01 | 92.67% | X59315 | IGKJ3*01 | 86.84% | J00242 |
| | | IGKV1D-39*01 | 92.67% | X59312 | IGKJ2*01 | 82.05% | J00242 |
| | | IGKV1-12*01 | 86.81% | V01577 | IGKJ1*01 | 76.32% | J00242 |
| | | IGKV1-37*01 | 86.81% | X59316 | IGKJ4*01 | 73.68% | J00242 |
| | | IGKV1D-37*01 | 86.81% | X71893 | IGKJ5*01 | 73.68% | J00242 |
| | **100VL** | IGKV1-12*01 | 95.97% | V01577 | IGKJ2*01 | 94.74% | J00242 |
| | | IGKV1D-12*01 | 95.24% | X17263 | IGKJ1*01 | 86.84% | J00242 |
| | | IGKV1D-16*01 | 91.58% | K01323 | IGKJ3*01 | 81.58% | J00242 |
| | | IGKV1-16*01 | 90.48% | J00248 | IGKJ4*01 | 81.58% | J00242 |
| | | IGKV1-9*01 | 90.11% | Z00013 | IGKJ5*01 | 68.42% | J00242 |
| **Phl p 1-specific VL** | **25VL** | IGKV1-39*01 | 95.24% | X59315 | IGKJ5*01 | 97.37% | J00242 |
| | | IGKV1D-39*01 | 95.24% | X59312 | IGKJ2*01 | 71.05% | J00242 |
| | | IGKV1-12*01 | 90.11% | V01577 | IGKJ4*01 | 71.05% | J00242 |
| | | IGKV1-17*01 | 89.38% | X72808 | IGKJ1*01 | 68.42% | J00242 |
| | | IGKV1D-12*01 | 89.38% | X17263 | IGKJ3*01 | 68.42% | J00242 |
| | **43VL** | IGKV1-39*01 | 90.32% | X59315 | IGKJ4*01 | 89.47% | J00242 |
| | | IGKV1D-39*01 | 90.32% | X59312 | IGKJ2*01 | 76.32% | J00242 |
| | | IGKV1-17*01 | 85.66% | X72808 | IGKJ3*01 | 76.32% | J00242 |
| | | IGKV1-9*01 | 85.66% | Z00013 | IGKJ1*01 | 73.68% | J00242 |
| | | IGKV1-12*01 | 84.95% | V01577 | IGKJ5*01 | 65.79% | J00242 |
| | **10VL** | IGKV1-39*01 | 91.76% | X59315 | IGKJ4*01 | 97.37% | J00242 |
| | | IGKV1D-39*01 | 91.76% | X59312 | IGKJ1*01 | 81.58% | J00242 |
| | | IGKV1-12*01 | 86.38% | V01577 | IGKJ3*01 | 81.58% | J00242 |
| | | IGKV1-27*01 | 86.02% | X63398 | IGKJ2*01 | 78.95% | J00242 |
| | | IGKV1-9*01 | 86.02% | Z00013 | IGKJ5*01 | 71.05% | J00242 |

Table E2

Amino acid sequence identities (%) and mean % sequence identities between the analyzed VL regions to each other

| | | Phl p 1-specific | | | | Phl p 2-specific | | | | Phl p 5-specific | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 25VL | 43VL | 10VL | mean | 94VL | 60VL | 100VL | mean | 5AVL | 28VL | 31VL | 14VL | mean |
| Phl p 1-specific | 25VL | 100% | | | | | | | | | | | | |
| | 43VL | 82% | 100% | | 82.7% | | | | | | | | | |
| | 10VL | 83% | 83% | 100% | | | | | | | | | | |
| Phl p 2-specific | 94VL | 83% | 75% | 78% | | 100% | | | | | | | | |
| | 60VL | 82% | 79% | 79% | 78.8% | 82% | 100% | | 81.0% | | | | | |
| | 100VL | 83% | 74% | 76% | | 81% | 80% | 100% | | | | | | |
| Phl p 5-specific | 5AVL | 83% | 74% | 76% | | 81% | 80% | 100% | | 100% | | | | |
| | 28VL | 88% | 81% | 84% | 82.2% | 83% | 84% | 82% | 85.2% | 82% | 100% | | | 86.0% |
| | 31VL | 90% | 82% | 82% | | 86% | 85% | 87% | | 87% | 89% | 100% | | |
| | 14VL | 87% | 79% | 80% | | 88% | 83% | 83% | | 83% | 86% | 89% | 100% | |

# A single chain fragment specific for group 2 grass pollen allergens with therapeutic potential

Elisabeth Gadermaier, Sabine Flicker, Katharina Blatt, Peter Valent, Rudolf Valenta

**Submitted.**

# A single chain fragment specific for group 2 grass pollen allergens with therapeutic potential

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**Capsule summary**: We characterized a recombinant single chain fragment specific for major group 2 grass pollen allergens which inhibits IgE binding to the allergen and allergen-induced basophil activation and hence has therapeutic potential.

**Key words**: Allergy, grass pollen, recombinant single chain fragment, therapy.

*To the Editor:*

Grass pollen is one of the most frequent and potent allergen sources worldwide affecting almost 50% of allergic patients [1]. Allergen-specific immunotherapy (SIT) is a clinically effective, disease-modifying treatment for grass pollen allergy and has long lasting effects [2]. However, it has been demonstrated that current allergen-extract-based SIT induces protective IgG responses mainly against group 1 and group 5 allergens but not sufficiently against group 2 allergens which represent major allergens for more than 60% of grass pollen allergic patients [3, 4]. Moreover, it has been demonstrated in clinical studies that the group 2 allergen from timothy grass pollen, Phl p 2, is besides Phl p 5 the most potent allergen in eliciting immediate type skin and respiratory symptoms [5, 6].

Using combinatorial cloning technology we have identified a Phl p 2-specific IgE Fab which after conversion into a full human $IgG_1$ antibody inhibited allergic patients IgE binding to Phl p 2 and Phl p 2-induced basophil activation and therefore may be useful for treating patients allergic to group 2 grass pollen allergens by passive immunization [7]. However, in the case of grass pollen allergy the major manifestations of the disease are allergic rhinitis, conjunctivitis and asthma for which topical forms of treatment as needed would be advantageous. For such topical application large amounts of antibodies which are easy and inexpensive to produce and which should have low immunogenicity would be needed. Unfortunately complete antibodies may elicit neutralizing antibodies even when they are fully human and their production is very expensive because cell culture systems are needed [8]. In order to decrease immunogenicity it has therefore been suggested to use antibody fragments (*i.e.*, Fabs) for therapeutic purposes [8].

Here we report the expression, purification and characterization of a ScFv fragment, designated Phl p 2-ScFv that is specific for the major group 2 allergen from timothy

grass, Phl p 2. Phl p 2-ScFv could be expressed in the *E. coli* strain HB2151 as soluble ScFv and was purified to homogeneity as a 28 kDa molecule (Fig 1A) by affinity chromatography [9]. The Phl p 2-ScFv specifically recognized group 2 allergens (*i.e.*, Phl p 2) but not other unrelated timothy grass pollen allergens (*e.g.* Phl p 1, Phl p 5, Phl p 6, Phl p 7, Phl p 12, Phl p 13) or the major birch pollen allergen Bet v 1 (Fig E1). Importantly, surface plasmon resonance studies revealed a similar association and dissociation rate constant of the ScFv fragment to Phl p 2 as was found for the complete Phl p 2-specific IgG [7] (Association rate constants $K_a$ for Phl p 2-ScFv: 8.41 * $10^5$ $M^{-1}s^{-1}$; Phl p 2 IgG: 2.96 * $10^5$ $M^{-1}s^{-1}$; dissociation rate constants $K_d$ for Phl p 2-ScFv: 3.26 * $10^{-5}$ $s^{-1}$; Phl p 2 IgG: 3.38 * $10^{-5}$ $s^{-1}$) and demonstrated stable binding to Phl p 2 with a half-life of >19 hours (Fig 1B).

Using a competitive IgE ELISA [10] it is demonstrated that the Phl p 2-ScFv inhibited significantly ($p<0.05$) the binding of grass pollen allergic patients (n=12) IgE to Phl p 2 yielding a maximal inhibition of up to 71% in certain patients (29.4% mean inhibition) (Fig 2A). Perhaps more important, we found that the Phl p 2-ScFv also inhibited Phl p 2-induced basophil activation (Fig 2B). Due to the fact that the Phl p 2-ScFv lacks IgG constant regions, competition of the ScFv with IgE antibodies for their binding sites on the allergen must be the mechanism underlying the inhibition of basophil activation and not co-crosslinking of IgE and IgG receptors [11].

The Phl p 2-ScFv may now be manufactured under conditions of good manufacture practice for clinical trials investigating whether topical application to the eyes and respiratory tract can inhibit group 2 allergen-induced allergic symptoms. Should the clinical trials show efficacy it may be considered as an ad on local therapy for grass pollen allergic patients who are sensitized to group 2 allergens.

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Figure legends


**FIG 1. A,** Coomassie blue-stained SDS-PAGE. Lane M: Protein molecular mass marker (M) (kDa) and lane 1, purified Phl p 2-ScFv. **B,** Sensor chip-based studies of the interaction between Phl p 2 and the Phl p 2-ScFv. Two-fold increasing concentrations (0.125 nM – 4 nM) of the ScFv were injected into the flow cell containing immobilized Phl p 2. Recorded (coloured) and calculated curves (black) which represent a fitting of the response data to a 1:1 interaction model were superimposed. Signal intensities (RU) are shown (y-axis) versus the time (s) (x-axis). Association ($K_a$) and dissociation rate constants ($K_d$) are indicated.


**FIG 2. A,** Patients' IgE binding (OD values: y-axis) to plate-bound Phl p 2 after pre-incubation with bacterial lysates containing the Phl p 2-ScFv or an unrelated ScFv is shown, the mean inhibition is indicated. The p-value of <0.05 is displayed. **B,** A blood sample from a Phl p 2-allergic patient was exposed to increasing doses of Phl p 2 (x-axis) that had been pre-incubated with the Phl p 2-ScFv or an unrelated ScFv. CD203c expression on basophils was determined by FACS analysis and is displayed as stimulation index on the y-axis.
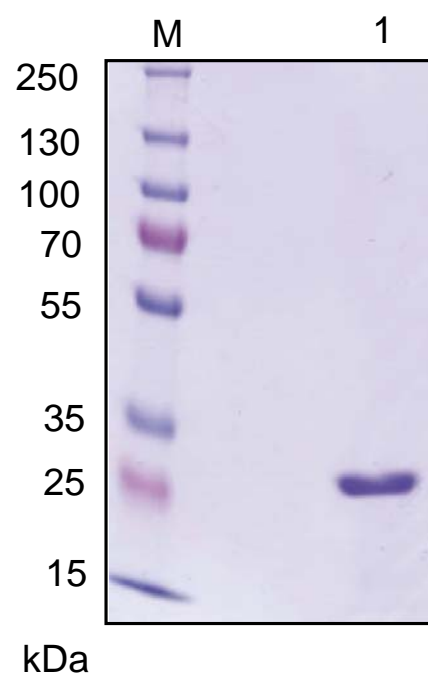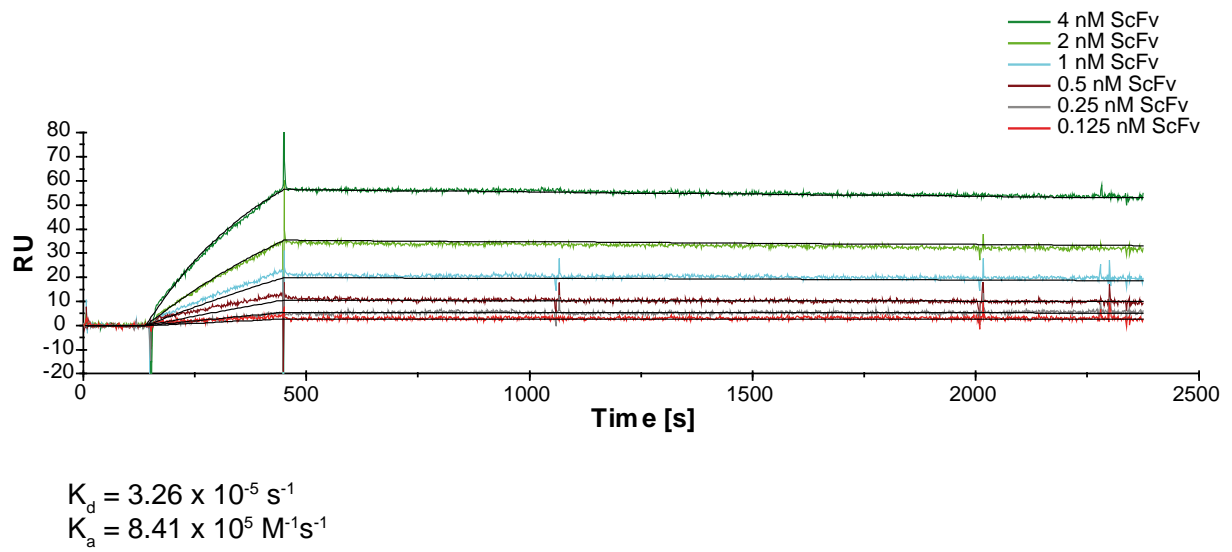
FIG. 1A

$K_d = 3.26 \times 10^{-5}\ s^{-1}$
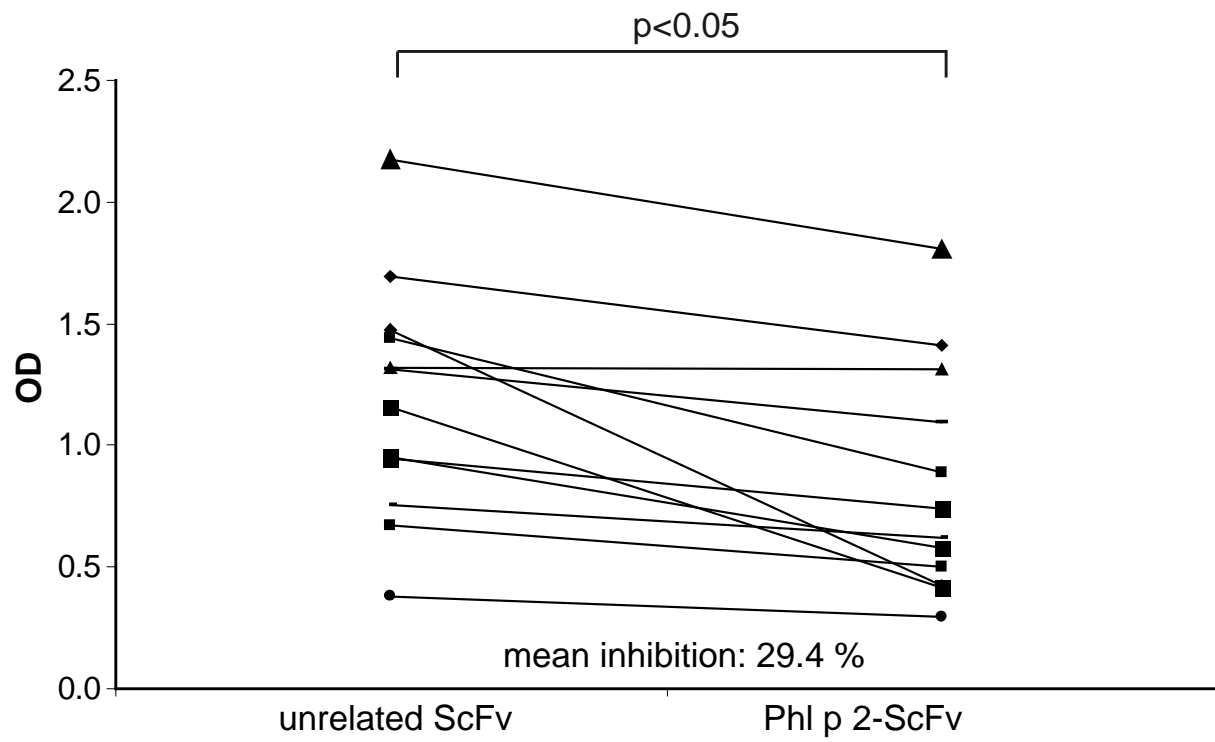$K_a = 8.41 \times 10^5\ M^{-1}s^{-1}$
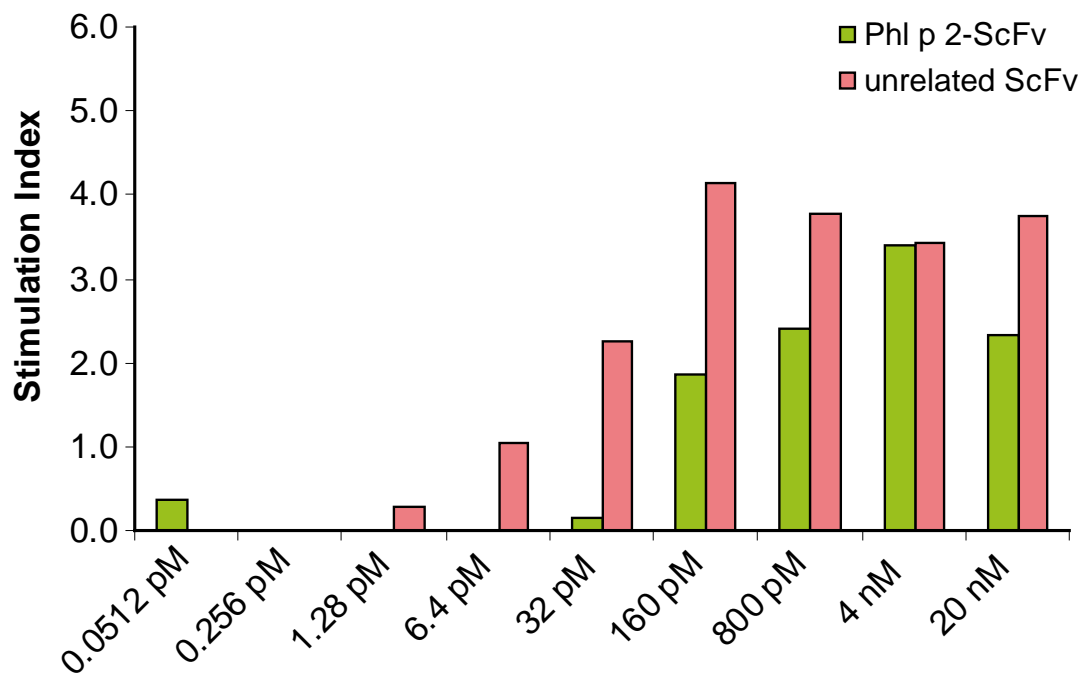
FIG. 1B

FIG. 2A

FIG. 2B

## Online Repository Material

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Figure legends

**FIG E1.** Determination of binding specificities of the Phl p 2-ScFv (OD-values: y-axis) to seven timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 7, Phl p 12, Phl p 13) and to the unrelated birch pollen allergen Bet v 1 as control.
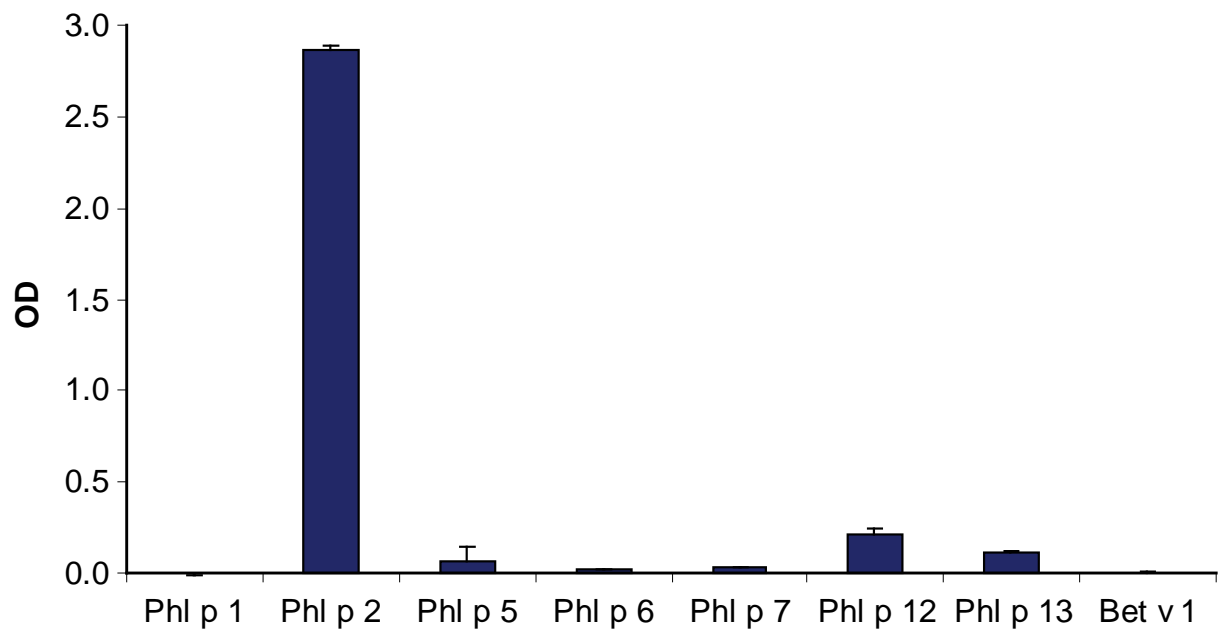
FIG. E1

# Passive Immunization with Allergen-Specific Antibodies

Sabine Flicker, <u>Elisabeth Gadermaier</u>, Christoph Madritsch, Rudolf Valenta

# Passive Immunization with Allergen-Specific Antibodies

**Sabine Flicker, Elisabeth Gadermaier, Christoph Madritsch and Rudolf Valenta**

**Abstract** The induction of allergen-specific IgG antibodies has been identified as a major mechanism responsible for the reduction of allergic inflammation in allergic patients treated by allergen-specific immunotherapy. Several studies suggest that allergen-specific IgG antibodies induced by vaccination with allergens block mast cell and basophil degranulation, IgE-facilitated allergen presentation to T cells and IgE production. The availability of recombinant allergens and technologies for the production of recombinant human antibodies allows engineering of allergen-specific antibodies which can be used for passive immunization (i.e., therapy) and eventually for the prevention of allergy (i.e., prophylaxis). This chapter summarizes data supporting the possible use of allergen-specific antibodies for treatment and prophylaxis. Finally, concrete approaches for the treatment and prevention of allergy based on blocking antibodies are envisioned.

**Abbreviations**

| | |
|---|---|
| SIT | Specific immunotherapy |
| APC | Antigen presenting cell |
| FcεRI | High-affinity IgE receptor |
| scFv | Single-chain antibody fragment |
| Fab | Antibody fragment |
| FcγR | IgG Fc receptor |
| FcRn | Neonatal Fc receptor |
| BAL | Broncho alveolar lavage |
| AHR | Airway hyper-responsiveness |

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OVA     Ovalbumin
i.v.    Intravenous
i.p.    Intraperitoneal
s.c.    Subcutaneous
SPR     Surface plasmon resonance
ADCC    Antibody dependent cell-mediated cytotoxicity

## Contents

## 1  Background

The usefulness of IgG antibodies for passive immunization was demonstrated for the first time nearly 120 years ago. Passive immunization was applied to treat different infectious diseases, e.g., rabies, diphtheria and tetanus (von Behring and Kitasato 1991) (Sawyer 2000). Using the technology of passive serum transfer developed by Prausnitz and Kuestner (1921) for investigating mechanisms of allergic inflammation, Cooke et al. discovered that allergen-specific IgG antibodies that were induced by specific immunotherapy (SIT) were able to inhibit immediate inflammatory responses. In fact, they could demonstrate that allergen-specific IgG antibodies which block allergen-induced immediate allergic inflammation were transferable by simple serum injections from a treated to an untreated patient (Cooke 1935).

Even before it became technically possible to produce large amounts of defined allergen-specific IgG antibodies, a few studies provided evidence for the feasibility of using allergen-specific IgG antibodies for passive immunization. It was demonstrated for immunotherapy with ragweed extract that higher allergen doses were

tolerated by patients who received combined passive and active immunotherapy (Bernstein et al. 1979). In order to prevent side effects, bee venom-allergic patients were pre-treated with beekeeper gammaglobulin to accept higher doses of venom (Muller et al. 1986; Bousquet et al. 1987).

The analysis of mechanisms underlying SIT with natural allergen extracts has been facilitated by the availability of recombinant allergens and defined epitopes. Moreover, several immunotherapy studies have been conducted with purified recombinant allergens, allergen-derived peptides and genetically modified allergens allowing a better understanding of the importance of blocking antibodies for successful treatment (Valenta et al. 2010; Larche et al. 2006).

## 2  Beneficial Role of Allergen-Specific Blocking IgG Antibodies

Allergen-specific IgG antibodies may suppress allergic immune responses and allergic inflammation through at least three different modes of action which are summarized in Fig. 1. First, they can inhibit immediate allergic inflammation by competing with mast cell-bound IgE antibodies for allergen binding and thus inhibit effector cell degranulation. A beneficial effect (i.e., reduction of degranulation) may be also achieved by co-cross-linking of mast cell-bound IgE and IgG by allergens (Fig. 1a). Second, allergen-specific IgG antibodies can compete with IgE bound to antigen presenting cells (APC) and thus prevent IgE-facilitated allergen presentation to T cells and thus T cell activation (Fig. 1b). Finally, allergen-specific IgG may capture allergens before they can induce IgE production (Fig. 1c) (Flicker and Valenta 2003; Wachholz and Durham 2004; Larche et al. 2006).

Cross-linking of allergens by FcεRI-bound IgE on mast cells or basophils is a key event in allergic inflammation. During the last decades it has been repeatedly shown by several clinical studies that SIT-induced allergen-specific IgG antibodies suppress allergen-induced mast cell degranulation and thus immediate allergic inflammation (Cooke 1935; Loveless 1940; Niederberger et al. 2004; Jutel et al. 2005; Klimek et al. 2005; Pauli et al. 2008; Purohit et al. 2008). IgG antibodies induced by SIT with a particular allergen source or allergen may also cross-protect against cross-reactive allergens. In this context it has been found that immunotherapy with birch pollen extract also protects against allergy to hazel and alder pollen (Petersen et al. 1988). More recently, it has been demonstrated that IgG induced by SIT with recombinant hypoallergenic derivatives of the major birch pollen allergen Bet v 1 inhibited basophil degranulation by Bet v 1-related food allergens (Niederberger et al. 2007a). Interestingly, SIT-induced IgG antibodies do not need to recognize exactly the same epitopes as those which are recognized by IgE. In fact, it has been shown that also IgG induced against new epitopes can block IgE binding as long as these antibodies occupy parts of the IgE epitopes or cause steric inhibition of IgE binding (Pree et al. 2007; Gadermaier et al. 2010a). It should be also stated at this point that the ability of a "blocking antibody" to

**(a)** Immediate allergic inflammation

1.

Allergen

Mast cell

IgE

FcεRI

biological mediators

2.

+ blocking IgG

**(b)** Antigen presentation to T cells

1.

T cell

TCR

MHCII

APC

2.

+ blocking IgG

**(c)** IgE production

1.

IgE

memory B cell

2.
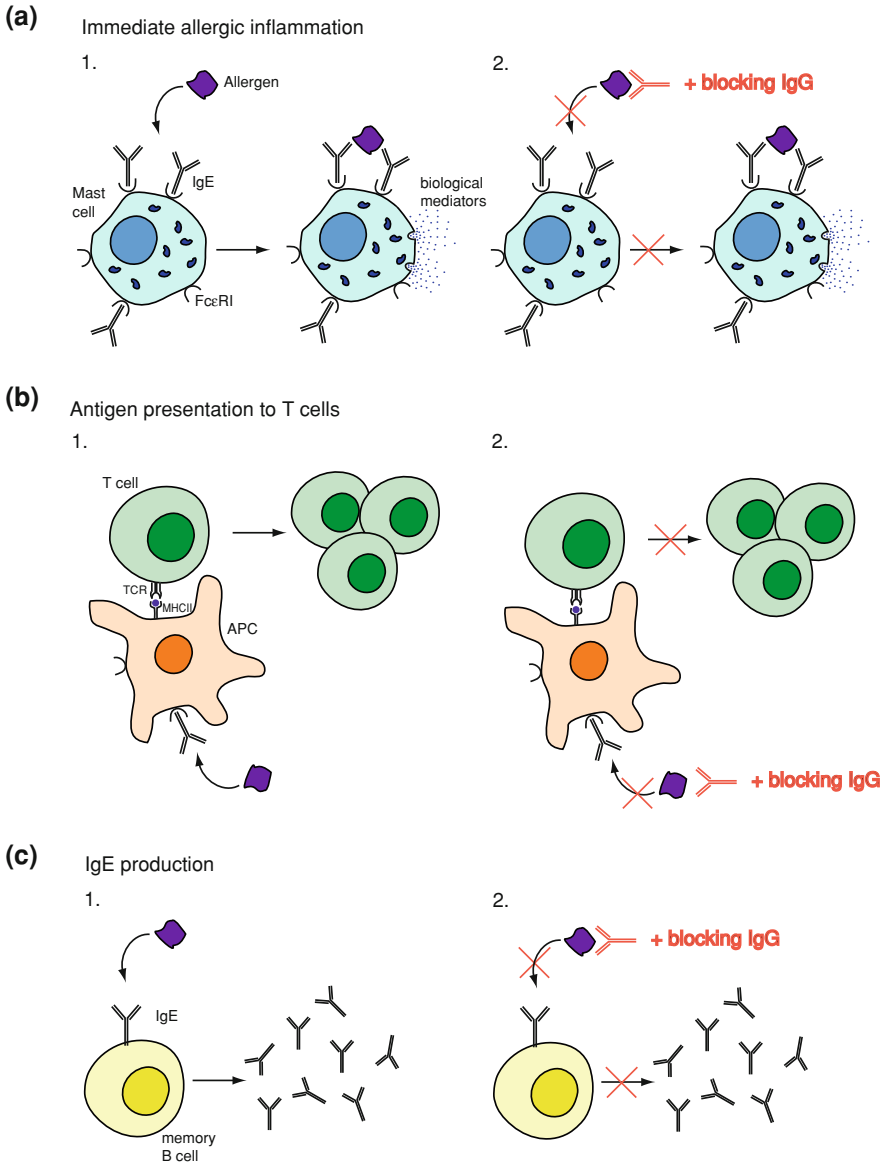
+ blocking IgG

**Fig. 1** *Summary of the beneficial roles of allergen-specific blocking IgG antibodies.* **a** Allergen-specific IgG captures allergen before it can cross-link mast cell-bound IgE and thus suppresses immediate allergic inflammation. **b** Allergen-specific IgG prevents allergens from being presented by IgE-facilitated antigen presentation to T cells and thus reduces T cell activation. **c** Allergen-specific IgG binds allergen and thus prevents allergens to activate IgE-producing cells

**Table 1** Essential features for protective antibodies

| Feature | References |
| --- | --- |
| Epitope specificity and titer | Flicker and Valenta (2003), Flicker et al. (2009) |
| Cross-reactivity | Valenta et al. (1996), Gieras et al. (2011) |
| Affinity | Jakobsen et al. (2004, 2005), Padavattan et al. (2009), Gieras et al. (2011) |
| Half-life and clearance | Akilesh et al. (2007), Jeffris (2009) |
| Binding to Fc receptors | Malbec and Daeron (2007) |
| Safety | Presta (2006), Hansel et al. (2010) |

compete with IgE for allergen recognition does not at all depend on its isotype or subclass but only depends on its epitope specificity, affinity and titer. The latter statement is supported by defined mechanistic experiments performed with purified antibodies and allergens and cultured effector cells (Table 1). For example, defined human monoclonal allergen-specific IgG antibodies gained by hybridoma techniques or combinatorial library technology have been shown to inhibit IgE from binding to allergens and to suppress allergen-induced basophil degranulation (Visco et al. 1996; Flicker et al. 2002; Jylha et al. 2009; Padavattan et al. 2009). However, there is also evidence for another protective effect of allergen-specific IgG. It has been reported that concomitant binding of IgG and IgE antibodies to the same allergen may down-regulate effector cell responses through co-cross-linking of IgE and IgG receptors (Malbec and Daeron 2007; Mertsching et al. 2008). These studies have been performed mainly in murine models whereas for human immunotherapy studies no evidence for down-regulation of effector cell activation by co-cross-linking has been found so far (Ejrnaes et al. 2006).

Moreover, allergen-specific IgG antibodies are responsible for the reduction of IgE-facilitated antigen presentation by preventing the binding of allergen to APC-bound IgE antibodies resulting in decreased T cell activation. T cell activation is crucial for the development and perpetuation of the allergic immune response, and is also directly linked to the late-phase response as well as chronic inflammation of the airways (Larche et al. 2006).

In 1999, it was demonstrated that IgE-mediated allergen presentation to birch allergen-specific T cells was inhibited by IgG from sera of birch SIT patients, but not by IgG from patients who had received grass pollen-specific SIT, suggesting that an allergen-specific factor in their sera inhibited the IgE-mediated allergen presentation (van Neerven et al. 1999). The inhibitory effect was shown to be present in the IgG fraction of the birch SIT sera. This study and a follow-up study of sera from a placebo-controlled birch SIT study demonstrated that 100- to 1,000-fold higher allergen doses are needed to activate birch allergen-specific T cells after SIT (van Neerven et al. 2004). Confirmation came from another study demonstrating that blocking of allergen-IgE binding to B cells was mediated through IgG antibodies that were induced through grass pollen SIT and more recently by SIT with recombinant hypoallergenic Bet v 1 derivatives (Wachholz et al. 2003; Pree et al. 2010).

Thirdly, it has been shown that the induction of allergen-specific IgG antibodies by SIT is associated with a suppression of the boost of secondary IgE production by allergen contact. It is well-established that allergic patients exhibit strong increases of allergen-specific IgE levels after seasonal allergen exposure (Henderson et al. 1975; Niederberger et al. 2007b). A suppression of the rise of allergen-specific IgE during the pollen season in patients after SIT was noted in several studies (Mothes et al. 2003; Niederberger et al. 2004; Creticos et al. 2006). Since allergen-specific IgG decline relatively quickly after discontinuation of SIT it has been speculated that the suppression of IgE boosts by several courses of SIT administered for two or more years may be responsible for the long-term effect of SIT after its discontinuation (Gadermaier et al. 2011).

## 3 Isolation and Characterization of Allergen-Specific IgG Antibodies for Therapy and Prevention

The availability of recombinant purified major allergens of high clinical relevance provides a solid basis for the application of technologies for the isolation, characterization and production of allergen-specific therapeutic antibodies (Valenta and Kraft 1995, 2002). Phage display, combinatorial libraries and hybridoma techniques have enhanced our ability to obtain monoclonal recombinant antibodies (Huse et al. 1989; McCafferty et al. 1990; Winter et al. 1994).

One strategy to obtain "blocking antibodies or antibody derivatives" is to isolate IgE antibody fragments–scFvs or IgE Fabs– from allergic patients (Steinberger et al. 1996; Flicker et al. 2002; Jylha et al. 2009). Due to the lack of the constant region, these fragments cannot bind to the FcεRs and hence do not elicit IgE-mediated effects. Another strategy is to generate IgE antibody fragments and convert them into complete human IgG antibodies by engineering the IgE variable region onto an IgG constant region. These antibodies combine two important characteristics that may be relevant. They may block IgE binding to the allergen and in addition may bind to FcγRs thus mediating co-cross-linking and silencing of effector cells or allergen uptake and clearance.

One example for a conversion of an allergen-specific IgE Fab into an $IgG_1$ antibody is an IgE Fab specific for grass pollen allergen Phl p 2 obtained by combinatorial cloning from an allergic patient. This antibody inhibited patients' IgE binding to Phl p 2 and Phl p 2-induced histamine release from basophils of grass pollen allergic patients (Flicker et al. 2002; Padavattan et al. 2009). Nevertheless, it has been observed that single IgG antibodies that were derived from allergic patients IgE did not block polyclonal IgE binding to the allergen (Flicker et al. 2000, 2006).

Another possibility for obtaining allergen-specific monoclonal antibodies is conventional cell cloning technology. For example, monoclonal allergen-specific IgG antibodies have been isolated by EBV-transformation of allergen-specific B cells of a SIT-treated patient (Visco et al. 1996; Lebecque et al. 1997; Denepoux

et al. 2000). However, as yet no human cell line producing allergen-specific IgE has been described.

Some essential features of allergen-specific IgG antibodies with protective potential are summarized in Table 1. The probably most important properties that make an IgG antibody a protective IgG antibody are epitope specificity and titer.

## 3.1 Epitope Specificity and Titer

It has been demonstrated that the blocking capacity of an allergen-specific antibody depends on its ability to occupy IgE binding sites or to bind in close vicinity so that there is steric hindrance of IgE binding (Flicker and Valenta 2003; Gieras et al. 2011).

In fact, SIT with allergen extracts can induce a large variety of IgG antibodies of which certain often do not even react with allergens, others recognize allergens but do not inhibit IgE binding and only some block IgE recognition (Flicker and Valenta 2003). Since SIT also induces "useless" IgG responses it has not been possible to associate the induction of IgG antibodies directly with clinical improvement (Djurup and Malling 1987; Birkner et al. 1990; Bodtger et al. 2005). Allergen-specific IgG antibodies which do not inhibit IgE binding to the allergen (Flicker et al. 2008) have been described and there is even evidence for IgG antibodies which may enhance IgE binding to allergens (Denepoux et al. 2000). Both reports clearly demonstrate that there are non-blocking IgG recognizing the wrong epitopes and it is not surprising that such antibodies have no therapeutic effect even when they are present in molar excess. The latter may also explain why certain allergic patients mount large amounts of allergen-specific IgG antibody responses but nevertheless suffer from allergy. Thus simple measurements of the allergen-specific IgG antibodies will not be sufficient surrogate markers for the clinical outcome of SIT.

However, if allergen-specific IgG antibodies have the correct epitope specificity and can inhibit IgE binding to the allergen, their protective effect will be greater if their concentration and titer is high.

It has been claimed that mainly allergen-specific $IgG_4$ antibodies are responsible for therapeutic effects during SIT (Aalberse et al. 1983). However, several studies demonstrate that the blocking activity of therapy-induced IgG antibodies does not reside exclusively in the $IgG_4$ fraction. Blocking antibodies of the $IgG_1$ subclass have been described (Visco et al. 1996; Flicker et al. 2002) and it has been shown that $IgG_4$-depleted sera retained their blocking activity (Ejrnaes et al. 2004).

Interestingly, it has turned out that the vast majority of allergens contain conformational IgE epitopes which are difficult to characterize because this requires sophisticated technologies e.g., three-dimensional structure analysis. With the application of structural biology methods first structures of complexes consisting of allergens and specific antibodies have been solved. The birch pollen allergen, Bet v 1, the bee venom allergen, Api m 2 (hyaluronidase) and the cockroach

allergen Bla g 2, were co-crystallized with recombinant mouse IgG Fabs (Mirza et al. 2000; Padavattan et al. 2007; Li et al. 2008). Furthermore, the major bovine milk allergen, $\beta$-lactoglobulin and the major grass pollen allergen Phl p 2 were crystallized in complexes with recombinant human IgE Fabs (Niemi et al. 2007; Padavattan et al. 2009). These allergen-antibody complexes gave us for the first time insight in the molecular interaction between allergen and antibodies and revealed the nature of conformational IgE epitopes (Niemi et al. 2007; Padavattan et al. 2009).

## 3.2 Cross-Reactivity

Another important feature of an allergen-specific protective antibody is its cross-reactivity to homologous allergens present in related species. This finding is of great importance because IgE antibodies from allergic patients often displayed cross-reactivity to allergens from other allergen sources (Valenta et al. 1996; Radauer et al. 2008). So far, several monoclonal human and murine IgG blocking antibodies were demonstrated to cross-react with homologous allergens from related species (Visco et al. 1996; Lebecque et al. 1997; Flicker et al. 2002; Gieras et al. 2011) and were even shown to inhibit patients' IgE to related allergens (Gieras et al. 2011).

## 3.3 Affinity

Aside from epitope specificity, the affinity of antibodies for the given epitopes is an important factor. Affinity is perceived as the strength of binding and can be calculated from the quotient of dissociation rate and association rate. Moreover, the kinetics of an antibody/allergen complex is of great importance because the kinetics determines the on and off rates of formed complexes. Nowadays, the determination of the affinity and the kinetics of such complexes can be assessed by surface plasmon resonance (SPR) (Padavattan et al. 2009). When targeting protective antibodies applicable for passive immunotherapy high-affinity and low-dissociation rates are preferable. Determinations by SPR revealed that Bet v 1-specific IgG antibodies gained from allergic patients who had undergone immunotherapy had high affinities recorded in the nM range and hence reside in the same range as Bet v 1-specific IgE gained from the same patient (Jakobsen et al. 2004, 2005).

Recently, a human monoclonal IgG antibody specific for the major grass pollen allergen Phl p 2 has been engineered by grafting the variable domain of the corresponding IgE onto the human $IgG_1$ constant region. This antibody showed an extremely high affinity to Phl p 2 and strongly inhibited allergic patients IgE binding to the allergen as well as allergen-induced basophil degranulation (Padavattan et al. 2009). Murine antibodies recognizing peptides derived from the

major IgE binding site of the major birch pollen allergen, Bet v 1 also showed high affinity to Bet v 1 (Gieras et al. 2011). Interestingly, these antibodies inhibited patients IgE binding but were less potent in inhibiting allergen-induced basophil degranulation.

The pairing of heavy and light chains also has an influence on the affinity and specificity of an antibody. Affinity of antibodies can be modified by pairing the original VH sequence with different VL sequences. Kim and Hong reported about an enhancement of affinity of a humanized monoclonal antibody after light chain shuffling (Kim and Hong 2007). For allergen-specific human IgE antibodies there is some evidence that the heavy chain determines allergen-specificity (Laffer et al. 2001; Rojas et al. 2004; Christensen et al. 2009; Gadermaier et al. 2010b). It is therefore quite possible, that the affinity of allergen-specific antibodies can be increased by shuffling of light chains.

## 3.4 Half-Life and Clearance

The affinity of a therapeutic allergen-specific antibody is not only important for the effective blocking of IgE but also affects other parameters. For example, high affinity of a therapeutic antibody would decrease dosing and frequency of administration, and thus reduce costs of treatment. Several reports describe different methods for improving affinity constants of antibodies toward their antigens. It could be shown that error-prone PCR using an excess of guanine and/ or thymine resulted in the retrieval of antibody fragments with significantly improved binding affinities for the corresponding antigen (Persson et al. 2008). The same authors demonstrated in a former study that the change of several residues situated in the paratope of an antibody was critical for the strength of the binding affinity (Persson et al. 2006). Aside from the random mutagenesis strategy another method called "codon-based mutagenesis" has been described. The principle of this targeted approach is that specifically the CDRs are mutated (Glaser et al. 1992). Utilizing this technique Wu et al. succeeded in improving the affinity of an anti-angiogenic antibody by greater than 90-fold (Wu et al. 1998). It turned out that targeted mutagenesis in the CDR 3 led to the greatest affinity improvements due to the diminished off rate of the antibodies (Finlay et al. 2009).

Besides the efforts to improve the affinity constants of antibodies for the given antigen, many studies focus on understanding the factors that affect the pharmacokinectics of monoclonal antibodies, a field that is summarized by the term Fc engineering. The goal is the protection of monoclonal IgG antibodies from rapid clearance from the body, a prerequisite for effective passive immunization. Much effort has been made to prolong the survival of applied IgG including protection of antibody degradation through binding to Fc-receptors, e.g., neonatal Fc receptor (FcRn) and Fc$\gamma$ receptors (Fc$\gamma$R). Several studies describe IgG stabilization by FcRn to their serum persistence by recycling internalized IgG to the cell surface (Ghetie et al. 1996; Akilesh et al. 2007; Roopenian and Akilesh 2007). In this

context it turned out that human IgG$_1$, IgG$_2$ and IgG$_4$ per se exhibit longer elimination half-lives than IgG$_3$ a fact that is attributed to the binding differences to FcRn, as result of a single amino acid difference in the FcRn binding domain (Ghetie and Ward 2002). In accordance, attempts to alter the human IgG$_1$ antibody sequences, i.e., a single mutation in the CH2–CH3 region of the Fc part, led to affinity improvement of the human FcRn which was shown by Biacore analysis but also by in vivo studies in cynomogulus monkeys (Yeung et al. 2009). Neverthe-less, the authors admitted that the increase of the affinity is not always associated with lower clearance (Yeung et al. 2009). Besides the cynomogulus monkey model which is well-known to be appropriate for investigating the half-lives of human IgG antibodies because the monkey FcRn binds human IgG, a human FcRn transgenic mouse model has been developed to evaluate the pre-clinical pharma-cokinetics of human therapeutic antibodies (Petkova et al. 2006).

Another emerging issue is that the kind of glycosylation, e.g., galactose, fucose, N-acetylglucosamine has a strong impact on the half-life of the antibody (Jefferis 2009a). Different glycosylation patterns were easily achieved by using different expression systems (mammalian cells, yeasts, plants, insect cells and prokaryotes). The most widely used production cell lines for human therapeutic IgG antibodies are currently mammalian cells because the glycosylation pattern of assembled anti-bodies almost resembles the naturally glycosylated antibodies. Nevertheless, recombinant antibodies produced in well-established hamster or mouse cell lines like CHO, NSO and Sp2/0 usually have small quantities of unnatural glycoforms bearing the risk to be immunogenic and thus be cleared quicker by forming immune complexes (Jefferis 2009b). Therefore first efforts have been made to create genetically modified CHO cells or as alternative, yeast lines that use human enzymes for glycosylation to produce recombinant glycosylated human IgG anti-bodies with high-structural fidelity with the natural IgG antibodies (Jefferis 2009b).

The route of antibody application e.g., topical administration or systemic treatment is also discussed to influence the in vivo half-life of antibodies. It turned out that antibody titers in the BAL fluid of naïve BALB/C mice 48 h after intranasal instillation are hardly detectable whereas antibodies administered by i.v. route are maintained for several months in the sera (Sehra et al. 2003).

It is further reported that complete IgG antibodies in general are beneficial compared to antibody fragments like scFvs or Fabs due to their extended half-lives (Presta 2008). However, if neutralizing antibodies without eliciting any Fc-mediated effects are in favor antibody fragments became again of interest despite their short half-life. Several attempts have been made to prolong serum half-life of antibody fragments including linkage to polyethylene glycol (PEGylation) (Chapman et al. 1999; Constantinou et al. 2010) or linkage to albumin (Constantinou et al. 2010).

## 3.5 Binding to Fc Receptors

An interesting question is whether allergen-specific IgG antibodies besides simply competing for epitopes on allergens may exert their beneficial roles also via binding to Fc$\gamma$R.

In this context, it has been shown in OVA-sensitized mice that complete OVA-specific IgG2a and not OVA-specific F(ab)$_2$ suppressed inflammation indicating that Fc$\gamma$R-mediated mechanisms may be responsible for the repression (Sehra et al. 2003). FACS analysis clearly showed that alveolar macrophages are the main population responsible for the OVA capture by binding to IgG and Fc$\gamma$R on these cells. In parallel, a two-fold increase in INF-$\gamma$-secreting T cells was observed in IgG-treated mice (Sehra et al. 2003). However, it is also possible that complete antibodies were more efficient simply due to their longer half-life.

Strait et al. obtained results in a murine model suggesting that besides allergen interception by IgG antibodies (i.e., Fc$\gamma$R-mediated cellular uptake of allergen) also cross-linking of Fc$\varepsilon$RI to the inhibitory Fc$\gamma$RIIb through the allergen/IgG/IgE complex contributes to the inhibitory effect of administered IgG antibodies. They concluded that Fc$\varepsilon$RI/Fc$\gamma$RIIb cross-linking makes an important contribution when blocking IgG levels are limited but is redundant when blocking IgG antibody concentrations are high relative to concentrations of antigen in mice (Strait et al. 2006). Another recent report indicated that co-administration of mouse IgE and mouse IgG prevented mast cell degranulation in BALB/c mice whereas no IgG-dependent inhibition of mast cell degranulation was observed in Fc$\gamma$RIIB$^{-/-}$ mice (Uermosi et al. 2010).

For the human system, there is relatively little evidence that cross-linking of the Fc$\varepsilon$RI to the inhibitory IgG receptor Fc$\gamma$RIIb indeed plays a role in inhibiting histamine release. There are data from cultured human mast cells and basophils (Zhu et al. 2002; Tam et al. 2004; Zhang et al. 2004) and from in vivo studies using transgenic mice expressing human Fc$\varepsilon$R$\alpha$ (Allen et al. 2007). By investigating the inhibitory role of Fc$\gamma$RIIb of cat allergic patients who have received SIT, Cady et al. found evidence that Fc$\gamma$RIIA may be involved in the inhibition of basophil activation (Cady et al. 2010).

However, the investigation of the potential role of Fc$\gamma$R co-cross-linking in reducing allergen sensitivity in patients receiving immunotherapy failed to provide any evidence for this mechanism in allergic patients (Ejrnaes et al. 2006).

The choice of the isotype/subclass of a therapeutic allergen-specific antibody may be driven by several considerations. IgG$_1$ antibodies are often used as therapeutic antibodies. They have a long in vivo half-life but have been proven to be effective in activation of ADCC and complement activation (Jefferis 2007). Both are certainly not desired features although allergic patients develop allergen-specific IgG$_1$ in the course of SIT without showing signs of inflammation. Most likely, allergen-specific IgG$_4$ antibodies that just activate Fc$\gamma$RI and Fc$\gamma$RIIIa depending on the allotype of the receptor and/or the glycoform of the IgG$_4$ antibody but do not activate complement represent the most desirable

subclass for passive immunization against allergens (Jefferis 2007, 2009b). In fact, allergen-specific IgG$_4$ is the prominent subclass associated with successful SIT, does not activate complement and can pass the placental barrier (Flicker et al. 2009) and thus may eventually protect children from becoming sensitized.

## 3.6 Safety of Recombinant Monoclonal Antibodies

Considerable efforts have been made in the last years to reduce side effects of therapeutic monoclonal antibodies. Among the side effects, immunogenicity is a major problem because it may lead to the development of antibodies against administered antibodies resulting in immune complexes, hypersensitivity or accelerated clearance of the applied antibodies. For decreasing immunogenicity of applied non-human antibodies different strategies like humanization, "resurfacing" (only surface-accessible framework amino acids are altered to human ones while the CDR and buried amino acids remain from parental rodent antibody) or SDR grafting (only a subset of CDR residues that are involved in binding of the antibody called "specificity-determining regions" is altered) have been suggested (Presta 2006). De-immunization, another technology, means the identification and alteration of potential T cell epitopes present in the antibody without reducing the binding affinity (Presta 2006). The availability of fully human monoclonal antibodies by phage display library or transgenic mice is another possibility to produce therapeutic antibodies with low immunogenicity. To date, just few examples of human therapeutic antibodies exist that have passed the safety requirements (FDA approval) and are already used in the field of oncology, psoriasis and asthma (Corren et al. 2010; Hansel et al. 2010). The lessons learned from the other fields may be translated to the field of type I allergy.

## 4 Proof of Concept Testing in Animal Models

In order to investigate the in vivo efficacy of passive administration of allergen-specific IgG antibodies a few murine models have been established so far. It has been shown that topical or systemic administration of allergen-specific IgG antibodies reduces allergic inflammation in sensitized mice (Sehra et al. 2003; Moerch et al. 2006; Strait et al. 2006) (Flicker unpublished data). Sera of OVA-sensitized mice that received anti-OVA specific IgG antibodies exhibited significantly reduced OVA-specific IgE levels, significantly reduced levels of leukocytes and eosinophil granulocytes in BALs and significantly reduced AHR to methacholine (Sehra et al. 2003; Moerch et al. 2006). The efficacy of a single administration of allergen-specific IgG antibodies was confirmed by data from mice sensitized to
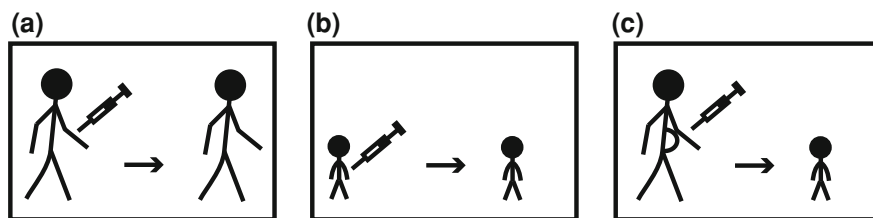
**Fig. 2** *Envisaged therapeutic and prophylactic immunization with allergen-specific antibodies.***a** Therapeutic application may protect allergic patients from symptoms in the course of allergen contact. **b** Prophylactic application shortly after birth to children may protect against allergic sensitization. **c** Application of allergen-specific IgG antibodies to pregnant mother may lead to diaplacental transfer to children and protect them against allergic sensitization after birth

three of the most important seasonal allergens, the major grass and birch pollen allergens, Phl p 1, Phl p 5 and Bet v 1 (Flicker unpublished data).

Very few data exist so far for prophylactic treatment with allergen-specific antibodies, i.e., administration of allergen-specific antibodies before sensitization to allergen takes place. It has already been demonstrated in experimental animal models and in clinical studies that prenatal induction of allergen-specific IgG antibodies protects against allergen-induced sensitization and allergic inflammation in the offspring (Glovsky et al. 1991; Jenmalm and Bjorksten 2000; Uthoff et al. 2003; Polte and Hansen 2008; Polte et al. 2008; Flicker et al. 2009; Victor et al. 2010).

It will thus be very interesting to explore if passive immunization of pregnant mothers or new born children with allergen-specific IgG antibodies can suppress allergic sensitization (Fig. 2).

# 5 Possible Application of Allergen-Specific IgG for Therapy and Prophylaxis

With the availability of defined reagents (i.e., clinically relevant allergens) and new technologies (e.g., combinatorial cloning technologies and large-scale production of fully human antibodies) it should be possible to produce allergen-specific human IgG antibodies for the clinically most relevant allergens. The following concrete applications for allergen-specific IgG antibodies can be envisaged. First, it should be possible to treat seasonal allergies by passive immunization of allergic patients shortly before the beginning of the pollen season (Fig. 2a). Since human allergen-specific $IgG_4$ antibodies have a half-life of approximately 21 days it is quite possible that allergic patients can be protected by a single passive immunization for a full pollen season. Perennial allergies are perhaps not easy to treat by passive immunization because repeated injections may be needed, but also Omalizumab, an injected therapeutic anti-IgE is successfully used to treat such patients (Holgate et al. 2009). It may thus well be envisioned that

passive immunization against allergens which elicit severe asthma attacks may be an alternative to heavy immunosuppressive treatment.

Another very interesting application of allergen-specific IgG antibodies may be prophylactic immunization (Fig. 2). There is some evidence that prenatal induction of allergen-specific IgG antibodies may protect against allergen-induced sensitization and allergic inflammation in the offspring (Glovsky et al. 1991; Jenmalm and Bjorksten 2000; Flicker et al. 2009). For prophylaxis, allergen-specific IgG may be either administered to pregnant women who transmit the protective IgG via their placenta to the children (Fig. 2c) or directly to the children shortly after birth in order to prevent allergic sensitization (Fig. 2b).

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# In Shape – The Art of Mapping Conformational Epitopes

Elisabeth Gadermaier

## Editorial

# In Shape – The Art of Mapping Conformational Epitopes

Elisabeth Gadermaier

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In type I allergy, IgE antibodies directed against specific epitopes on foreign proteins or glycoproteins are produced during the process of sensitization. These IgE antibodies are further bound to effector cells like mast cells and basophils, and their crosslink through the corresponding allergens is essential for the initiation of allergic symptoms. The vast majority of allergens contain conformational B cell epitopes, comprising amino acids that are in close vicinity only in the folded protein whereas they are noncontinuous in the linear sequence. Resulting from this three-dimensional architecture, conformational epitopes are difficult to characterize.

However, it is of great importance for the better understanding of immune responses and for the design of effective active and passive treatment strategies to gain also precise knowledge of the interacting surface between IgE antibodies and their corresponding epitopes.

The most direct way of identifying conformational epitopes is still the determination of the three-dimensional structure of the antibody-allergen complex by X-ray crystallography [1, 2]. But there are limitations in the performance as the procedure is very time consuming and needs a large amount of purified protein complexes. Another method is presented in the article of Gieras et al. [3] showing that peptide-specific monoclonal antibodies (mAb) can also map conformational IgE epitopes. An al-

ternative approach is the usage of filamentous phage libraries that display random sets of peptides. Panning of these libraries with the antibody of interest will select peptides that are involved in antibody-antigen interaction and that may mimic conformational B cell epitopes.

The article of Tiwari et al. [4] in this issue describes the mapping of a conformational epitope on the major cockroach allergen Bla g 2. This mapping was performed by the panning of a 12-mer phage display peptide library (i.e. Ph.D. library purchased from the New England Biolabs) with the mAb 7C11 [5] that is described as a surrogate for human IgE. For this reason, a modified version of the panning protocol was established to select in a more stringent way for high-affinity binding phages. Only two displayed peptides could be identified that share very high degrees of amino acid identities as they differed in only one amino acid. The isolated phages displayed peptides with similar specificity and binding strength. Peptides were further matched on the surface of the three-dimensional structure of Bla g 2 (PDB ID: 1YG9) with the help of the computational algorithm EpiSearch. In the present study, mapping of the two peptides led to the isolation of four overlapping surface patches that share four amino acids. The four patches identified 17 out of 18 experimentally determined residues; this corresponds to a discovery of 94%. The predicted conformational epitope

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in the current study was finally validated by comparison with the genuine epitope presented by the already published co-crystal of Bla g 2 and mAb 7C11 [5].

The strength of this article is the selection of two peptides that – via the identification of four high scoring patches – discovered in the end 94% of all experimentally determined residues. The selection of these peptides was performed by a modified phage display screening method that included an additional elution step to get rid of weakly bound phages as well as a 10-fold reduction step in mAb 7C11 concentration from one panning round to the next. These very stringent isolation conditions select for high-affinity binders, therefore accepting low panning output. The current approach uses EpiSearch for mapping isolated peptides, a program that is described to show the advantages of being quite flexible and time-saving in comparison to other computational algorithms. EpiSearch was already applied for epitope mapping in six unrelated experimental data sets; in these cases it correctly mapped the location of conformational epitopes and the identified highest scoring patches covered in most cases more than 50% of the experimentally determined residues [6]. The authors of the current paper could even report an improvement in prediction of participating amino acid residues compared to the described former studies although, quite surprisingly, it was shown that the EpiSearch program is able to map 17 out of 18 amino acids from the genuine epitope with the help of two 12-mer peptides.

Nevertheless, there are also some weak points within the paper. The isolated peptides that were described here for the very first time were characterized only in their phage-bound version. The production of soluble peptides would offer tools to precisely study their interaction with the specific antibody not only for binding specificity and inhibition potential but also for affinity in Biacore analysis. Additionally, it would have been beneficial to isolate and map more than the two described peptides. Therefore, more extensive screening or screening of a more diverse library would be essential.

The identification of conformational epitopes via screening of random peptide libraries and further their mapping with the use of the EpiSearch program as described in this article seem to be useful in terms of defining their correct localization. These days much effort is put on the development of new fast and precise strategies dealing with epitope mapping. An example for a recent tool is the software SPADE that is the first approach predicting IgE epitopes by combining structural and cross-reactivity data and that compared to other prediction tools yields highest specificities [7].

However, with currently available technologies, the only rational method of identifying all amino acid residues from a conformational – 'in shape' – epitope is measuring the crystal structure of an allergen in complex with the corresponding antibody.

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# Curriculum vitae

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**Personal Data:**

Date of Birth: March 18th, 1981

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**Education:**

| | |
|---|---|
| Since Sept. 2006: | PhD Thesis at the Department of Pathophysiology and Allergy Research, Centre for Pathophysiology, Infectiology and Immunology, Medical University of Vienna Title: Allergen-specific antibodies Under supervision of Prof. Valenta |
| August 2006: | Graduate degree with distinction |
| Nov. 2004-Aug. 2006: | Diploma Thesis at the Department of Pathophysiology, Centre of Physiology and Pathophysiology, Medical University of Vienna Title: Characterization of allergen-specific antibodies Under supervision of Prof. Valenta |
| 2001 – 2006: | Study of Genetics University of Vienna, Vienna Biocenter |
| 1999-2001: | Study of Biology/Genetics Paris Lodron University of Salzburg |
| June 1999: | Final grammar school examination with distinction |
| 1991-1999: | Grammar school, Ried/Innkreis, Austria |
| 1987-1991: | Primary school; Upper Austria |

**Memberships:**

Member of the Austrian Society for Allergology and Immunology (ÖGAI)

Junior member of the European Academy of Allergology and Clinical Immunology (EAACI)

**Scientific education:**

2009        Cold Spring Harbor laboratory course: Phage display of proteins and peptides. November 6 – 19th, Cold Spring Harbor, New York, USA.

2006        Biometry II, Human resource development, Medical University of Vienna, Vienna, Austria.

2006        Biometry I, Human resource development, Medical University of Vienna, Vienna, Austria.

**Awards and Grants:**

2010        Travel Grant from the Austrian Research Association for the 14th International Congress of Immunology (ICI 2010), Kobe, Japan.

2009        Scholarship support for the Cold Spring Harbor laboratory course "Phage display of proteins and peptides", New York, USA.

# List of publications

Elisabeth GADERMAIER

**Publications in peer-reviewed journals:**

Gadermaier E, Staikuniene J, Scheiblhofer S, Thalhamer J, Kundi M, Westritschnig K, Swoboda I, Flicker S, Valenta R. Recombinant allergen-based monitoring of antibody responses during injection grass pollen immunotherapy and after 5 years of discontinuation. **Allergy**. 2011;66:1174-1182. (PhD thesis)

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Gadermaier E, Staikuniene J, Flicker S, Westritschnig K, Swoboda I, Valenta R. Recombinant allergen-based monitoring of antibody responses during and after injection immunotherapy with a grass pollen vaccine. 6th EAACI-GA2LEN Davos Meeting, Pichl, Austria, 2008. (PhD thesis)

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### Poster presentations:

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Gadermaier E, Flicker S, Steinberger P, Valenta R. Importance of the heavy chain for the specificity of IgE antibodies in allergy. Annual Meeting of the Austrian Society for Allergology and Immunology (ÖGAI), Vienna, Austria, 2010. (PhD thesis)

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<u>Gadermaier E</u>, Staikuniene J, Scheiblhofer S, Thalhamer J, Kundi M, Westritschnig K, Swoboda I, Flicker S, Valenta R. Monitoring of antibody-responses during grass pollen extract immunotherapy and after five years of discontinuation with recombinant allergens. Allergen Vaccines 2009. Current Problems in Allergen Vaccines Manufacturing and Development. Varadero, Cuba, 2009. (PhD thesis)

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<u>Gadermaier E</u>, Staikuniene J, Scheiblhofer S, Thalhamer J, Westritschnig K, Swoboda I, Flicker, Valenta R. Recombinant allergens for following antibody responses during injection immunotherapy with a grass pollen vaccine. Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), Vienna, Austria, 2008. (PhD thesis)

<u>Gadermaier E</u>, Flicker S, Aberer W, Egger C, Reider N, Focke M, Vrtala S, Valenta R. The analysis of antibody responses of birch pollen allergic patients treated with injection immunotherapy suggests active vaccination as underlying mechanism. 25th Congress of the European Acadamy of Allergology and Clinical Immunology, Vienna, Austria, 2006. (Diploma thesis)